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The present program of research aims at determining the neuroprotective activity of novel estrogens in both male and female animal models for brain ischemic damage. We have proposed to achieve 4 technical aims over the course of 3 years. These aims relate to a description of the activity of several estrogens against cerebral damage related to middle cerebral artery occlusion in a rodent model, when the estrogens are administered prior to or following the ischemic event. We also are pursuing our observation that androgen reduction is neuroprotective in male subjects. We will assess (during years 2 and 3 of the grant) the potential mechanism(s) of the neuroprotective activity of estrogens (and of androgen reduction) in these models by determining the extent to which estrogens (or androgen reduction) reduce oxidative damage associated with stroke and then assess the involvement of signal transduction processes and anti-apoptotic proteins in the neuroprotective activity of estrogens. Collectively, these studies will provide the knowledge needed to determine if estrogen therapy and androgen reduction therapy are useful in protection of the brain tissue from damage related to the activities of U.S. Army male and female personnel.

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(4) Introduction: The present program of research aims at determining the neuroprotective activity of novel estrogens in both male and female animal models for brain ischemic damage. We have proposed to achieve 4 technical aims over the course of 3 years. These aims relate to a description of the activity of several estrogens against cerebral damage related to middle cerebral artery occlusion in a rodent model, when the estrogens are administered prior to or following the ischemic event. Also, we will assess (during years 2 and 3 of the grant) the potential mechanism(s) of the neuroprotective activity of estrogens in these models by determining the extent to which estrogens reduce oxidative damage associated with stroke and then assess the involvement of signal transduction processes and anti-apoptotic proteins in the neuroprotective activity of estrogens. Collectively, these studies will provide the knowledge needed to determine if estrogen therapy is useful in protection of the brain tissue from damage related to the activities of U.S. Army male and female personnel.

(5) Body:

Background: This report covers the period of August 1, 2000 to July 30, 2001 for research funded by the grant from the USAMRMC numbered DAMD 17-99-1-9473. During this period, I accepted the position of Chair, Department of Pharmacology and Neuroscience and Director, Institute for Aging and Alzheimer's Disease Research at the University of North Texas Health Science Center at Fort Worth (UNTHSCFW). This change necessitated the transfer of the funds remaining for the second and third years of the grant from the University of Florida to UNTHSCFW. This activity delayed the initiation of the 2nd year's funding of the present research until June 1, 2001.

Also, on September 6 to 7, 2001, Major Chessley Atchison, Ph.D., will conduct a site-visit at the UNTHSCFW. The progress reported in this document will also be reported to Dr. Atchison.

We have made substantial progress in achieving several of the technical aims of the grant and this progress is reported below.

Technical Aim 1: To assess the prophylactic and post-treatment neuroprotective effects of novel estratrienes in an animal model of cerebral ischemia.

New Compound Synthesis: (Although not specifically funded by this grant, the following program of drug synthesis provided new compounds to our stroke and brain trauma program.)

During Grant year 00-01 we synthesized and completed neuroprotective evaluations of more than three dozen novel estrogens to identify compound candidates for activity in stroke models.

Our synthetic strategy has been three-fold. First, we employed an enantiomer approach to produce more than a dozen novel estrogens and have completed *in vitro* assessment of their neuroprotective activity and their estrogen binding activity; we have

completed *in vivo* assessment of three of these compounds in an animals model for middle cerebral artery occlusion: 17α-estradiol (17 α-E2, Manuscript in preparation) and the complete enantiomer of 17 β-estradiol (17 β-E2, Appendix A), ent-E2 and the complete enantiomer of 17-desoxy estradiol (ZYC-13, Manuscript in preparation). All three of these compounds are as neuroprotective as 17 β-E2 in both *in vitro* and *in vivo* models for neuronal death and all lack activity at either the estrogen receptor α (ERα) and ERβ. Specific neuroprotection and estrogen receptor binding data are presented below.

A second synthetic strategy for production of novel estrogens is based upon the conjugation at the 17-oxygen of 17 β-E2 with alkyl groups. We synthesized 6 new 17-alkylestradiols and assessed their neuroprotective activity in an *in vitro* test (Prokai et al., 2001, Appendix B). One of these compounds will be tested in a MCA occlusion model during the next grant period.

A third strategy for production of novel estrogens was to do substitutions at one or more of the other carbons in the estradiol or estrone molecule. This later approach has produced several compounds that are at least as active in *in vitro* assays as is 17 β-E2 (Perez et al., 2000 abstract). During the next grant period, we will continue to synthesize and test additional compounds that may prove to be of interest in neuroprotection.

Pharmacokinetics of Estrogen Preparations: (Although not specifically funded by this grant, the following program of assessment of the kinetics of estrogen preparations is important to our preparation of compounds for clinical assessment.)

Having completed our assessment of the kinetics of 17 βE2 in rodents, we began a clinical assessment of the pharmacokinetics of 17 αE2 in post-menopausal women as an initial assessment of the potential for use of this non-feminizing estrogen for treatment and prevention of brain damage caused by stroke. We use oral dosing of 17 αE2 sodium sulfate in healthy post-menopausal women in an escalating dose paradigm. Each subject received doses of 50, 100 and 200 μg of 17 αE2 sodium sulfate and blood samples were obtained at ten times thereafter for assay of plasma 17 αE2 concentrations. We observed a dose-dependent increase in the maximum concentrations of plasma 17 αE2 and the expected other kinetic parameters of the compound. In brief, we have determined that 17 αE2 sodium sulfate can be administered orally to achieve neuroprotective concentration of plasma 17 αE2. These studies now allow us to begin Phase 2 clinical trials for the efficacy of 17 αE2 sodium sulfate as a non-feminizing estrogen for the protection of brain tissue from the effects of brain insult.

Studies in Female Rats:

Prophylactic Neuroprotection by Estrogens:

Middle Cerebral Artery (MCA) Occlusion: We have previously reported that 17 β-E2 is a potent neuroprotectant when administered prior to the onset of MCA occlusion. Initially, we demonstrated that administration of 17 β-E2 by either a sc or an iv formulation at 1 day prior to MCA occlusion protected animals from much of the cerebral damage related to the infarct (Simpkins et al., 1997). Subsequently, we demonstrated that a slow release preparation of 17 β-E2, administered beginning 1 week prior to the occlusion was potently effective in protecting brain tissue from ischemia

damage (Simpkins et al., Unpublished Observations). Similarly, administration of 17 β-E2 at 2 hours prior to the onset of the infarct was protective (Zhang et al., 1998). Finally, using nuclear magnetic resonance (NMR) imaging to perform real time imaging of the development of ischemic damage, we demonstrated that administration of 17 β-E2 at 2 hours prior to the onset of the occlusion can essentially protect much of the brain tissue from the damaging effects of occlusion (Shi et al., 2000, Appendix C).

Novel estrogens were also assessed for prophylactic neuroprotection. We completed an assessment of the protection against cerebral damage related to MCA occlusion is animals pre-treated with 17 α-estradiol (17 α-E2), through a subcutaneous Silastic® tube containing the steroid at two different doses. We observed that 17 α-E2 provided protection of both cortical and subcortical brain areas from damage induced by the occlusion. The extent of the protection provided by 17 α-E2 was similar to that afforded by 17 β-E2 (Stubley et al., 2000, Abstract and Manuscript in preparation).

We tested the complete enantiomer of 17 β-E2, ent-E2, for its neuroprotective activity in an MCA occlusion model of brain damage. We observed that ent-E2 was as effective as 17 β-E2 in protecting both cortical and subcortical tissue from damage (Green et al., 2001, Appendix A).

Additionally, two of our newly synthesized compounds, ZYC-3 and ZYC-13 has completed *in vivo* assessments in our MCA occlusion model. We have observed that both ZYC-3 and ZYC-13 are potently neuroprotective against ischemia induced brain damage (Abstract submitted and manuscripts in preparation). Collectively, these data indicate that non-feminizing estrogens, like 17 α-E2, ent-E2, ZYC-3 and ZYC-13 are potent neuroprotectant in at least one model for cerebral damage, the MCA occlusion model for stroke.

Subarachnoid Hemorrhage (SAH) Model

As we reported last years, we completed and have now published (Yang et al., 2001, Appendix D) an assessment of the prophylactic protective effects of 17 β-E2 in a model for SAH that we developed. Animals were administered 17 β-E2 at a dose of 100 μg/kg at 2 hours prior to the induction of an intraluminal hemorrhage using a thread that punctured the anterior cerebral artery. Pre-treatment with 17 β-E2 preserved blood flow during the bleeding episode, but did not effect the bleeding volume, and markedly reduced the extent of cerebral ischemic damage related to the bleeding episode (Yang et al., 2001, Appendix D). These data indicate that treatment with 17 β-E2 can provide protection from bleeding as well as occlusion type strokes.

Male Subjects

We assessed the possibility that estrogen could provide cerebral protection in male subjects as well as in females. In an initial study, published prior to the onset of this grant (Hawk et al, 1997) we reported that treatment for 1 week with 17 β-E2 by a sc Silastic® tube containing the steroid, resulted in protection from brain damage induced by MCA occlusion equivalent to that observed in females. Interestingly, however, this protection form brain damage was also observed when males were castrated. This raised the possibility that the effects of estrogens in males were related to their capacity to reduced serum androgens, such as testosterone. To test this possibility, we administered

estrogens, but maintained serum testosterone levels relatively constant through sc implants of Silastic® tubes containing the male sex steroid. When testosterone concentrations were maintained, estrogens lost most of their ability to protect brain tissue. These data indicated that serum androgen reduction was a key event in the neuroprotection afforded by estrogens.

In view of this surprising finding, we conducted a series of studies to determine the effects of androgen reduction, through a variety of means, on brain damage subsequent to an infarct. First, we administered the potent LHRH agonist, luprolide, which nearly completely suppresses secretion of LH and hence of testosterone, twice daily for one week. Luprolide reduced testosterone concentration to nearly undetectable levels after a week of administration. These animals were subjected to MCA occlusion and 24 hours later lesion size was evaluated. Luprolide reduced lesion size by 50%, the same extent of protection seen with estrogen pretreatment (Cartright et al., 2000).

Finasteride is a 5α-reductase inhibitor that prevents the conversion of testosterone to 5 α-dihydrotestosterone (HDT). We administered finasteride to male rats, at 6 hours prior to MCA occlusion and evaluated the size of the ischemic infarct 24 hours later. A single dose of finasteride reduced infarct size in male rats by about 40% (Cartright et al., 2000).

To determine if a non-pharmacological means of reducing testosterone would also protect brain from ischemic damage, we subjected male rats to the stress of administration of an anesthetic (or control animals were left undisturbed) at 6 hours prior to the MCA occlusion. Animals which were subjected to stress showed a major reduction in serum testosterone concentrations and a 50% reduction in infarct size (Yang et al., 2001).

Finally, to fully characterize the duration of testosterone reduction needed to protect animals from ischemic damage, we created a "testosterone clamp" by castrating male rats and implanting them with 2 testosterone filled Silastic® tube. These implants produced serum testosterone concentration which were equivalent to that seen in intact rats (Yang et al., 2001, Appendix E). Removal of these implants caused an 85% reduction in testosterone concentrations within 1 hour and undetectable levels of the hormone within 2 hours (Yang et al., 2001, Appendix E). MCA occlusion was initiated at 0 (no removal of the pellets), 1, 2, 4, or 6 hours after removal of the testosterone tubes. We observed a time-dependent reduction in the ischemic damage, with a 50% reduction seen at 6 hours (Yang et al., 2001, Appendix E). These data indicate that the removal of testosterone for as little as 6 hours can substantially protect the male brain from the damaging effects of ischemia.

To determine if androgens are directly toxic to neurons, we use an *in vitro* tissue culture model of glutamate toxicity in HT-22 mouse hippocampal cells. While estrogen treatment potently protected cells from this insults, testosterone exposure potentated the glutamate toxicity, suggesting that androgens are directly toxic to neurons (Yang et al., 2001, Appendix E).

Mechanism Studies:

Technical Aim 2: To determine the effects of novel estratrienes on oxidative damage during and following a cerebral ischemic event.

To determine the effects of estratrienes on oxidative damage, we undertook two major research projects. First, we developed an assay for a stable marker of lipid peroxidation, 8-isoprostan and applied it to the assessment of brain and plasma response to cerebral ischemic events. Second, we assessed the response of brain tissue NF-κB, a cellular oxidative response protein, to cerebral ischemia.

Plasma concentrations of 8-isoprostans were significantly increased within 30 min and were still elevated at 100 min. of reperfusion following a 20 min. 4 vessel occlusion in rats (Idris et al., 2001). The data suggest that brain ischemia causes a rapid and profound oxidative event, reflected in plasma increases in isoprostans. We are now evaluating the effects on brain isoprostans of 4-vessel occlusion.

We are also conducting studies to determine the effects of MCA occlusion on 8-iosprostan concentrations in the plasma and brain. To date, our data indicate the MCA occlusion increases plasma concentrations of isoprostans and elevates brain concentrations of this stable lipid peroxidation indicator. Collectively, these data indicate the cerebral ischemia causes a profound oxidative stress on the brain.

An indicator of cellular oxidation is production of NF-κB. Normally, NF-κB is maintained in an inactive state through the coupling to IκB. Reactive oxygen species (ROS) cause phosphorylation of IκB, which then dissociates from NF-κB, allowing it to translocate to the nucleus and there activate a variety of potentially harmful genes. We conducted studies to determine the effects of MCA occlusion on the production of both NF-κB (active form) and phosphorylated IκB (inactive form). We observed a marked increase in both NF-κB and the phosphorylated form of IκB in OVX rats subjected to MCA occlusion. We are now conducting studies to determine the effects of estratrienes on these markers of cellular oxidative stress.

Technical Aim 3: To determine the effects of novel estratrienes on CREB expression and phosphorylation following a cerebral ischemic event.

We conducted studies to determine the effects of MCA occlusion and reperfusion on levels of CREB and phosphorylated CREB (P-CREB). We observed that MCA occlusion and reperfusion decreased levels of both CREB (expression of the protein) and P-CREB (Activation of the protein) in the infarcted brain regions. Pretreatment with 17 βE2 prevented these changes in both CREB and P-CREB. These data indicate that CREB expression and CREB phosphorylation are responsive to the cerebral ischemia and that estrogens prevent this response. As CREB phosphorylation may be a neuroprotective signal, the ability of estrogens to prevent this response to ischemia may indicate a neuroprotective mechanism. During the next funding year, we will test the effects on CREB and P-CREB of non-feminizing estratrienes.

Technical Aim 4: To determine the effects of novel estratrienes on BCL-2 expression following a cerebral ischemic event.

Technical Aim 4 is schedule for completion during the 3rd grant year. However, we have begun studies to determine the effects of MCA occlusion on BCL-2

concentrations. Further, we will determine the effects of 17 βE2 and several non-feminizing estratrienes on the BCL-2 response to MCA occlusion.

(6) Key Research Accomplishments:

- Estradiol and non-feminizing estrogens like 17 α-estradiol, ent-estradiol, ZYC 3 and ZYC 13 are potent neuroprotectants against cerebral damage induced by brain ischemia.
- This neuroprotectant activity can be achieved even when the estrogen is administered after the ischemic event.
- The protective effects of estradiol can be observed in male subjects, and appears to have a strong component related to estrogen-induced androgen reduction.
- Acute androgen reduction is potently neuroprotective in male subjects.
- Transient cerebral ischemia causes a prompt increase in plasma levels of 8-isoprostan following a 4-vessle occlusion in rats.
- Transient middle cerebral artery occlusion causes a prompt increase in both plasma and brain 8-isoprostans.
- Brain levels of both CREB and phosphorylated CREB are decreased following cerebral ischemia and reperfusion.
- The in life phase of studies to determine brain levels of BCL-2 following MCA occlusion and the effects of estrogens has begun.

(7) Reportable Outcomes:

Papers Published as a direct result of the pursuing the technical aims of this grant:

1. Green, P. S., S.-H. Yang, K. R. Nilsson, A. S. Kumar, D. F. Covey, and J. W. Simpkins The Nonfeminizing Enantiomer of 17ß-Estradiol Exerts Protective Effects in Neuronal Cultures and a Rat Model of Cerebral Ischemia. *Endocrinology* 142: 400-406, 2001 (Appendix A).

2. Prokai, L, S-M Oon, K. Prokai-Tatrai, K. A. Khalil and J. W. Simpkins, Synthesis and biological evaluation of 17 β-alkoxyestra-1,3,5(10)-trienes: potential neuroprotectants against oxidative stress, *J. Med. Chemistry*, 44: 110-114, 2001(Appendix B).

3. Shi, J., J.D. Bui, S.H. Yang, T.H. Lucas, D. L. Buckley. S.P. Blackband, M.A. King, A.L. Day and J.W. Simpkins, Estrogens decrease reperfusion-associated cortical ischemic damage: a MRI analysis in a transient focal ischemia model. *Stroke* 32: 987-992, 2001 (Appendix C).

4. Yang, S.-H., Z He, S. S. Wu, Y.-J. He, J. Cutright, W.J. Millard, A. L .Day, and J.W. Simpkins, 17 β-estrdaiol can reduce secondary ischemic damage and mortality of subarachnoid hemorrhage, *J Cerebral Blood Flow and Metab.* 21: 174-181, 2001 (Appendix D).

5. Green, P. S., E. J. Perez, T. Calloway, and J. W. Simpkins, Estradiol attenuation of β-amyloid-induced toxicity: A comparison of MTT and Calcein assays, *J. Neurocytology* 29: 419-423, 2000 (Appendix F).

6. Yang, S.H., A. L. Day and J. W. Simpkins, Estradiol exerts neuroprotective effects when administrated after ischemic insult. *Stoke* 31: 745-749, 2000 (Appendix G).

7. Simpkins, J. W., P.S. Green, K.E. Gridley, J. Shi, E. M. Monck, Neuroprotective Effects of Estrogens, in "Biology of the Menopause", Ed. F. L. Bellino, Serono Symposium, Springer-Verlag, New York, pp. 103-111, 2000 (Appendix H).

8. He, Z., T. Yamawaki, S.-H. Yang, Q. Liu, M.A King, A.L. Day, J.W. Simpkins, and H. Naritomi, Definition of the anterior choroidal artery territory in rats using intraluminal technique, *J. Neurological Sciences* 182: 16-28, 2001 (Appendix I).

Papers Submitted:

1. Yang, S-H., E. Perez, J. Cutright, R. Liu, A. L. Day and J. W. Simpkins, Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model, *J. Applied Physiology, in Press* 2001 (Appendix E).

2. Wen, Y. E. Perez, P.S. Green and J.W. Simpkins, Nitric oxide may mediate estrogen neuroprotection through a non-genomic mechanism. *J. Neuroscience*, Submitted, August 2001 (Appendix J).

3. Zhen He, Yun-Ju He , Arthur L Day , and James W Simpkins, "Proestrus levels of estradiol during transient cerebral ischemia improve histological outcome of the hippocampal CA 1 region: perfusion-dependent and –independent mechanisms " *Journal of the Neurological Sciences*, Submitted May 2001 (Appendix K).

Abstracts Published:(Appended abstracts are attached but not individually identified)

1. Wen, Y., E. J. Perez, P. Green and J. W. Simpkins, Nitric Oxide may medicate estrogen's neuroprotection through a receptor-independent mechanism, Society for Neuroscience Abstracts Vol 26, pg 2045, 2000.

2. Perez, E. J., K. L. Eberst, S. M. Oon, Z. Y. Cai, L. Prokai, D. Covey and J. W. Simpkins, Structure-activity relationship of estratrienes against glutamate toxicity in a

mouse hippocampal cell line, Society for Neuroscience Abstracts Vol 26, pg 2045, 2000.

3. Green, P. S. C. T. Fulp and J. W. Simpkins, Estrogen modulation of Bcl-2 family protein expression, Society for Neuroscience Abstracts Vol 26, pg 1615, 2000.

4. Cutright, J. S. Yang, Z. He, A. L. Day, and J. W. Simpkins, Improvement in cerebral ischemia outcome with non-surgical methods of reducing testosterone in male rats, Society for Neuroscience Abstracts Vol 26, pg 780, 2000.

5. He. Z, S. H. Yang, Y. J. He, J. Cutright, A. L. Day, and J. W. Simpkins, Physiological estradiol delays neuronal death following transient forebrain ischemia in rats, Society for Neuroscience Abstracts Vol 26, pg 779, 2000.

6. Wang, J., E. J. Perez, P.S. Green and J. W. Simpkins, Estradiol protects against ATP depletion and mitochondrial membrane potential decline induced by 3-nitroproprionic acid in SK-N-SH neuroblastoma cells, Society for Neuroscience Abstracts Vol 26, pg 243, 2000.

7. Watson, D. G., C. Fiola and J. W. Simpkins, Inhibition or down-regulation of protein kinase C enhances estrogen-induced neuroprotection in an in vitro model, Society for Neuroscience Abstracts Vol 26, pg 2073, 2000.

8. Yang, S. H., Z. He, S. Wu, Y.J. He, J. Cutright, W. J. Millard, A. L. Day, and J. W. Simpkins, 17beta-estradiol can reduce secondary ischemic damage and mortality of subarachnoid hemorrhage, Society for Neuroscience Abstracts Vol 26, pg 780, 2000.

9. Watson, D. G. and J. W, Simpkins, Protein kinase C inhibition or down-regulation mimics and enhnces estrognes-induced neuroprotection, Southwestern and Rocky Mountain Division for the Advancement of Science, March, 2001.

10. Simpkins, J. W., Neuroprotection by estrogens and phennolic A ring compounds, International Busniness Communications 9th Annual Conference on Alzheimer's Disease, "Gene Discovery to Therpaeutic Applications", Atlanta, GA, Feb 8-9, 2001.

11. Simpkins J.W., S.-H. Yang and E.J. Perez Rapid effects of estrogen on neuroprotection after ischemic insult, Second International Meeting on Rapid Responses to Steroids, Denver, CO, June 16-18, 2001.

12. Simpkins, J.W., D. G. Watson, E. J. Perez, P.S. Pang, R. Liu, T. Fan and A. L. Day, Estrogen receptor-independent mechanisms of neuroprotection, First Annual Kronos Symposium, Bar Harbor, MI, 2001.

Abstracts Submitted:

1. D.G. Watson and J.W. Simpkins, Estradiol exposure inhibits PKC epsilon activation and ERK1/2 phosphorylation: potential mechanisms for its neuroprotective effects. 2001 Society for Neuroscience Annual Meeting Abstract, 2001.

2. E.J. Perez, S. Rothman, S. Xia, Z. Y. Cai, L. Prokai, D. F. Covey, and J.W. Simpkins, Neurotoxicity of ICI 182,780, tamoxifen, and 4-hydroxytamoxifen in primary cortical neurons, SK-N-SH neuroblastoma and HT-22 cells. Society for Neuroscience Annual Meeting Abstract, 2001.

3. He Z, He Y-J, Day AL, Simpkins JW, Proestrus Levels of Estradiol during Transient Global Cerebral Ischemia Improves Histological Outcome of the Hippocampal CA1 Region: Perfusion-Dependent and -Independent Mechanisms, Society for Neuroscience Annual Meeting Abstract, 2001.

4. Wen, Y., E.J. Perez, J.Chung, and J.W. Simpkins, Alzheimer's Disease is associated with local reduction of IGF-1 and Akt activity. Society for Neuroscience Annual Meeting Abstract, 2001.

5. Wang, J., K.D. Yi, S. Narayan, X. Zhang, J.W.Simpkins, Effect of 17β-estradiol on 3-NPA-induced increases in cytosolic calcium in SK-N-SH neuroblastoma cells and HT-22 murine hippocampal cells. Society for Neuroscience Annual Meeting Abstract, 2001.

6. Wang, X., K. D. Yi, E.J.Perez, J. Wang, Y. Wen, and J.W. Simpkins, A microplate assay for quantifying cellular oxidative stress using 2',7'-dichlorofluorescin-diacetate. Society for Neuroscience Annual Meeting Abstract, 2001.

7. Yang, S.H., E. Perez, J. Cutright, Z. He, A. L. Day, J.W. Simpkins, Testosterone increases neurotoxicity of glutamate *in vitro* and ischemia-reperfusion injury in an animal model. Society for Neuroscience Annual Meeting Abstract, 2001.

8. Fan, T., E.J. Perez, K.L. Eberst , D.F. Covey, and J.W. Simpkins, ZYC-13, The enantiomer of 1,3,5(10)-estratriene 3-ol, exerts neuroprotective effects *in vitro* and *in vivo*. Society for Neuroscience Annual Meeting Abstract, 2001.

9. Jung, M.E. and J. W. Simpkins, Protective Effects of estrogen on ethanol withdrawal-induced cerebellar damage and mortoric deficits in rats, Society for Neuroscience Annual Meeting Abstract, 2001.

10. Jung, M.E. and J. W. Simpkins, Protective Effects of estrogen on ethanol withdrawal syndrome, College of Problems of Drug Dependence 63rd Annual Meetings, June 16-21, 2001.

11. Idris, A. H., A. Gabrielli, Z. HE, J. W. Simpkins, A. L. Day, Oxidative injury occurs rapidly after global brain ischemia and reperfusion, 27th Annual American Heart Association International Stroke Conference, 2001.

12. Yang, S.H., R. Liu, E. J. Perez, S.Yo, Z. Y. Cai, D. F. Covey. A. L. Day, and J. W. Simpkins, Neuroprotective effects of a novel non-receptor binding estrogen analogue during stroke, 27th Annual American Heart Association International Stroke Conference, 2001.

Published Reports, Manuscripts and Abstracts Secondarily Funded by DAMD 17-99-1-9473 (While the grant DAMD 17-99-1-9473 was cited as contributing to these papers, their funding from this source was coincidental and related to the purchase of reagents. Also, the research papers listed below contribute heavily to our understanding of the mechanism of the neuroprotective effects of estrogens and estrogen-like compounds, knowledge that will be applied to our cerebral ischemia research during the 3rd years of the present grant period.)

1. Green, P.S. and J. W. Simpkins, Role of estrogens and estrogen-like non-feminizing compounds in the prevention and treatment of Alzheimer's disease, *Annals of the New York Academy of Sciences*, 924: 93-98, 2000.

2. Yang, J., P.S. Green and J. W. Simpkins, Estradiol protects against ATP depletion, mitochondrial membrane potential decline and the generation of reactive oxygen species by 3-nitropropionic acid in SK-N-SH human neuroblastoma cells. *J. Neurochem.* 77: 804-811, 2001.

3. Liang, Y., S. Belford, F. Tang, J.W. Simpkins and J.A. Hughes, Membrane fluidity effects of estratrienes. Brain Res. Bull. 54:661-668, 2001

4. Jung, M. E., S.H. Yang, A. M. Brun-Zinkernagel and J. W. Simpkins, Estradiol protects against ethanol withdrawal-induced cerebellar and motor deficit, *Alcohol*, Submitted, May 2001.

5. Zhang, H., H. Xu, S. N. Uljon, R. Gross, K. Hardy, J. Simpkins, L.M. Rofelo, S. Petanceska, R. Wang and K. Duff, Modulation of beta amyloid accumulation by estrogen in AD mouse model, *J. Neurochemistry*, Submitted, May 2001.

(8) Conclusions: The 2nd grant year has been very successful, resulting in numerous publications, manuscripts and abstracts. These papers demonstrate the following: (1) Estradiol and non-feminizing estrogens like 17 α-estradiol, ent-estradiol, ZYC-3 and ZYC-13 are potent neuroprotectants against cerebral damage induced by brain ischemia. (2) This neuroprotectant activity can be achieved even when the estrogen is administered after the ischemic event. (3) The protective effects of estradiol can be observed in male

subjects, and appears to have a strong component related to estrogen-induced androgen reduction. (4) Acute androgen reduction is potently neuroprotective in male subjects. (5) The mechanism of the effects of cerebral ischemia and the protective effects of estratrienes are now unfolding. We have observed that MCA occlusion caused a marked increase in NF-κB (active form) and phosphorylated IκB (inactive form), and decreases CREB and the phosphorylated form of CREB. Either or both of these changes are potential mechanisms of the neurodegenerative effects of cerebral ischemia and estrogens may block these responses.

As a result of these studies, we are now in a position to conduct detailed analyses of the mechanism of neuroprotective action of the compounds under study. Additionally, we believe that we have sufficient preclinical data with which to initiate a clinical trial (from other sources of funds) to assess the efficacy of estrogens in protection of the brain from damage.

(9) References:
The References in the text are cited by the author's name and are indicated in the Reportable Outcomes Section of this document.

(10) **Appendices:** Appended materials are identified in the text (Reportable Outcomes Section) and are attached.

Appendix A

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Endocrinology

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The Nonfeminizing Enantiomer of 17β-Estradiol Exerts Protective Effects in Neuronal Cultures and a Rat Model of Cerebral Ischemia*

P. S. GREEN, S.-H. YANG, K. R. NILSSON, A. S. KUMAR, D. F. COVEY, AND J. W. SIMPKINS

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ABSTRACT

Estrogens are potent neuroprotective compounds in a variety of animal and cell culture models, and data indicate that estrogen receptor (ER)-mediated gene transcription is not required for some of these effects. To further address the requirement for an ER in estrogen enhancement of neuronal survival, we assessed the enantiomer of 17β-estradiol (*Ent*-E_2), which has identical chemical properties but interacts only weakly with known ERs, for neuroprotective efficacy. *Ent*-E_2 was both as potent and efficacious as 17β-estradiol in attenuating oxidative stress-induced death in HT-22 cells, a murine hippocampal cell line. Further, *Ent*-E_2 completely attenuated H_2O_2 toxicity in human SK-N-SH neuroblastoma cells at a 10 nM concentration. In a rodent model of focal ischemia, 17β-estradiol (100 μg/kg) or *Ent*-E_2 (100 μg/kg), injected 2 h before middle cerebral artery occlusion, resulted in a 60 and 61% reduction in lesion volume, respectively. *Ent*-E_2, at the doses effective in this study, did not stimulate uterine growth or vaginal opening in juvenile female rats when administered daily for 3 days. These data indicate that the neuroprotective effects of estrogens, both *in vitro* and *in vivo*, can be disassociated from the peripheral estrogenic actions. (*Endocrinology* **142:** 400–406, 2001)

EPIDEMIOLOGICAL STUDIES ASSOCIATE postmenopausal estrogen replacement therapy with several beneficial neurological outcomes, including a reduction in incidence of Alzheimer's disease (1, 2), Parkinson's disease (3, 4), and death from stroke (5, 6). These effects may be mediated, in part, by estrogen-mediated enhancement of neuronal survival. The neuroprotective effects of estrogens, specifically the potent 17β-estradiol (βE_2), have been widely described in neuronal cultures against toxicities, including growth factor deprivation, glutamate toxicity, and oxidative stress (for review, see Ref. 7). Similarly, in rodents, βE_2 has been shown to attenuate neuronal loss after cerebral ischemia (8–10), kainic acid administration (11), and physical injury (12).

The role of estrogen receptor (ER)-dependent transcription in estrogen's neuroprotective activity is controversial (for review, see Ref. 7). The neuroprotective activity of βE_2 in culture models is attenuated by the ER antagonists tamoxifen or ICI 182,780 in some studies (13–15); however, others report no effect of these same ER antagonists on βE_2-mediated neuroprotection (16–20). Our laboratory (16, 21, 22) and others (17, 23) have reported equipotent (to βE_2) neuroprotective efficacy of 17α-estradiol, which only weakly activates ER-dependent gene transcription (24), implicating mechanisms other than ER-mediated transcription in estrogen-mediated protection of neuronal cultures. In mouse models of cerebral ischemia, the data are equally inconclusive. Sampei *et al.* (25) report no difference in total lesion size between wild-type and ERα-deficient mice. However, ICI 182,780 administration increases striatal lesion volume in the wild-type mouse (26). ICI 182,780 administration did not alter neocortical lesion volume in this study. It is important to note that, although protection of neocortical areas are consistently reported with βE_2 treatment (8–10), βE_2-mediated protection of striatal infarct is not consistently reported (9).

The present study addresses the requirement for ER-dependent transcription in the neuroprotective effects of estrogens, both *in vitro* and *in vivo*, using a novel enantiomer strategy. Ent-17β-estradiol (*Ent*-E_2), the enantiomer of the naturally occurring βE_2 (Fig. 1), has identical physiochemical properties as βE_2 except for interactions with other stereospecific molecules such as ERs. *Ent*-E is reported to interact only weakly with uterine-derived ERs (27, 28) and lacks estrogenic effects on reproductive tissues in rodents (29–31). Some reports indicate that *Ent*-E_2 exerts slight antiuterotrophic activity and can antagonize the uterotrophic effects of βE_2 (32, 33). In contrast, *Ent*-E_2 has been reported to elicit alterations in lipid profiles identical to βE_2, with similar potency (34). Further, *Ent*-E_2 is not enzymatically converted to βE_2 (35) and, therefore, is more adequately suited than 17α-estradiol for evaluating the role of ERs in estrogen-mediated neuroprotection. This study evaluates the neuroprotective effects of *Ent*-E_2, both in culture models of oxidative stress and in a rat transient focal ischemia model and, further,

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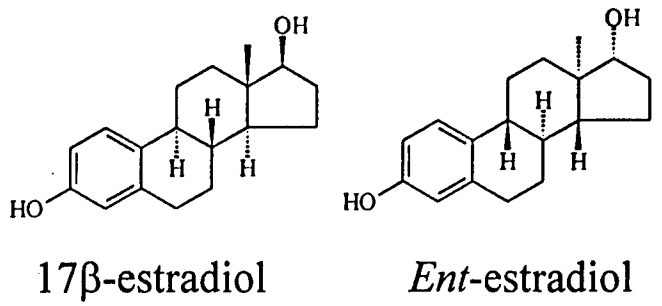


FIG. 1. Structure of the naturally occurring βE_2 and the nonnaturally occurring *Ent*-E_2.

determines whether *Ent*-E_2 can exert neuroprotective effects in the absence of stimulation of peripheral estrogen-responsive tissues.

Materials and Methods

Steroids

βE_2 was purchased from Steraloids, Inc. (Wilton, NH). *Ent*-E_2 was synthesized from the known starting material, [3*R*-(3α,3aα,9aα,9bβ)]-3-(1,1-dimethylethoxy)-1,2,3,3a,4,5,8,9,9a,9b-decahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxalan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one (Chemical Abstracts Registry Number 139973-49-2), which was prepared by a multistep synthetic pathway as described in the literature (36). This compound was then converted in either of two ways (method A or method B) to *Ent*-19-nortestosterone (Chemical Abstracts Registry Number 4091-86-5). In the first step of method A, the double bond is reduced, using lithium in liquid ammonia, and the resulting tricyclic compound is cyclized to *Ent*-19-nortestosterone in the second step. In the first step of method B, the double bond is reduced by catalytic hydrogenation, and the resulting tricyclic compound is again cyclized to *Ent*-19-nortestosterone in the second step. Method B has been previously used to prepare 19-nortestosterone (37). The hydroxy group of *Ent*-19-nortestosterone is then esterified, and the A-ring of the steroid is aromatized using $CuBr_2$ in acetonitrile. This reaction has been reported previously for the conversion of 19-nortestosterone, 17-acetate to 17β-estradiol, 17-acetate (38). Finally, the 17-acetate group is removed by saponification to give *Ent*-E_2 (Chemical Abstracts Registry Number 3736-22-9). The structure of *Ent*-E_2 was proven by experimental data, which showed that the compound had the same melting point (176-177 C), infrared absorption spectra (3449, 3246, 2936, 2864, 1611, 1587, 1500, 1450, 1283, 1250, 1057, 1012, 930, 874 cm^{-1}), ^1H NMR [(300 MHz, CD_3OD) δ 7.06 (1 H, d, J = 8.7 Hz), 6.54-6.46 (2 H, m), 3.64 (1 H, t, J = 8.4 Hz), 0.75 (3 H)] and ^{13}C NMR [(75 MHZ, CD_3OD) δ 156.07, 138.98, 132.80, 127.32, 116.18, 113.85, 82.57, 51.32, 45.34, 44.36, 40.50, 38.01, 30.67 (2 × C). 28.48, 27.56, 23.99, 11.62) spectra but opposite optical rotation ($[\alpha]_D^{28}$ = −71.2 (c = 0.99, CH3OH)) as βE_2.

Steroids were initially dissolved in ethanol at a 10 mM concentration and then diluted to the appropriate concentration in culture media or assay buffer for cell culture or *ex vivo* assays, respectively. Steroids were dissolved in corn oil at the concentration necessary to yield the indicated dose in 1 ml/kg injection volume for rodent studies.

Cell culture

SK-N-SH human neuroblastoma cells were obtained from ATCC-(Manassas, VA), and HT-22 cells (immortalized hippocampal neurons of murine origin) were a generous gift of Dr. David Schubert (The Salk Institute, San Diego, CA). Cells were maintained in RMPI-1640 and DMEM media (Life Technologies, Inc., Gaithersburg, MD), respectively, supplemented with 10% charcoal/dextran-stripped FBS (HyClone Laboratories, Inc., Logan, UT) and 200 μg/ml gentamycin, according to standard culture conditions.

Cells were plated, 24 h before initiation of experiment, at a density of 20,000 cells/well (SK-N-SH cells) or 5,000 cells/well (HT-22 cells), in both clear- and white-bottomed Nunc 96-well plates (Fisher Scientific, Orlando, FL). Steroids were added at concentrations ranging from 0.1 nM to 10 μM, either 2 or 24 h before exposure to either glutamate (5 mM) or H_2O_2 (3-60 μM). Ethanol was used at concentrations of 0.001-0.1% vol/vol as a vehicle control. These concentrations of ethanol had no discernible effect on cell viability. After 24 h of toxin exposure, cells were rinsed with PBS, pH 7.4, and viability was assessed by the addition of 1 μM calcein AM (Molecular Probes, Inc., Eugene, OR) and 1 μg/ml propidium iodide (Sigma, St. Louis, MO) in PBS for 15 min. Calcein AM fluorescence was determined at an excitation of 485 nm and an emission of 538 nm. Percent viability was calculated by normalization of all values to the toxin-free control group (= 100%). Percent protection was calculated as the difference between each experimental value and the average of the toxin-only group normalized to the difference between the toxin-free control and toxin-only groups (= 100% protection). Cells that had been lysed by addition of 1% SDS were used for blank readings. Staining was visualized using a fluorescent Nikon (Melville, NY) microscope, and cells were photographed for qualitative documentation.

Animals

Female Sprague Dawley rats (Harlan, Indianapolis, IN) were housed in pairs in hanging, stainless steel cages in a temperature-controlled room (25 ± 1 C) with a daily light cycle (on from 0700 to 1900 h daily). All rats had free access to laboratory chow and tap water. All procedures performed on animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida before the initiation of the study.

Ovariectomy

Female Sprague Dawley rats (220-225 g BW) were given 3-5 days to acclimate, then were bilaterally ovariectomized using a dorsal approach. Animals were anesthetized with methoxyflurane (Pitman Moore, Inc., Crossing, NJ) inhalant anesthesia. A small (1 cm) cut was made through the skin, facia, and muscle. The ovaries were externalized, clipped, and removed; then the muscle, facia, and skin were sutured closed. Ovariectomy was performed 2 weeks before experiments.

Middle cerebral artery (MCA) occlusion

Either oil vehicle or 100 μg/kg βE_2 or *Ent*-E_2 was administered by sc injection, 2 h before the onset of MCA occlusion. Animals were anesthetized by ip injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). MCA occlusion was performed as previously described (8). Briefly, the left common carotid artery, external carotid artery, and internal carotid artery were exposed through a midline cervical incision. A 3-0 monofilament suture was introduced into the internal carotid artery lumen and gently advanced until resistance was felt. The suture was kept in place for 60 min and then withdrawn to allow MCA reperfusion. The procedure was performed within 20 min, with minimal bleeding. Rectal temperature was maintained between 36.5 and 37.0 C during the entire procedure.

Animals were decapitated and the brain removed 24 h after onset of MCA occlusion. The brain was then dissected coronally into 2-mm sections using a metallic brain matrix (ASI Instruments, Inc., Warren, MI). The sections located 3, 5, 7, 9, and 11 mm posterior to the tip of the olfactory bulb were stained by incubation in a 2% solution of 2,3,5-triphenyltetrazolium chloride in a 0.9% saline solution at 37 C for 30 min. Slices were then fixed in 10% formalin and photographed, and the ischemic lesion area was determined for each slice using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Percent ischemic lesion area was calculated as the sum of the ischemic lesion area for the five slices divided by the total cross-sectional area of these five slices.

Plasma levels of βE_2

Ovariectomized female Sprague Dawley rats were injected sc with either oil vehicle or 100 μg/ml βE_2 or *Ent*-E_2. Blood samples were obtained by cardiac puncture, 5 min before injection or 1 h, 4 h, or 24 h post injection. Plasma was stored at −20 C, until assayed using the ultrasensitive βE_2 RIA kit from Diagnostic Systems Laboratories, Inc. (Los Angeles, CA) according to the manufacturer's instructions. *Ent*-E_2 showed no cross-reactivity with the RIA at concentrations up to 10 μM.

Uterotrophic assay

Juvenile (25 days old) female Sprague Dawley rats were injected sc with oil, βE_2 (0.01–1 μg/rat), or *Ent*-E_2 (1–100 μg/rat) daily (at 0830 h) for 3 days. On the fourth day, the rats were killed using methoxyflurane, and the uteri were excised. Extraneous tissue was gently removed from the uteri before wet weight was determined. Vaginal opening was assessed before uterine removal.

Ligand competition of ER binding

5 nM [2,4,6,7-^{3}H]-βE_2 (specific activity, 84.1 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ) and 400 pM recombinant human ERα or ERβ (Affinity BioReagents, Inc., Golden, CO) were incubated in ER binding buffer (20 mM Tris, 1 mM EDTA, 400 mM KCl, 1 mM dithiothreitol, 10% glycerol, 0.1% BSA, pH 7.8) for 1 h at 25 C either with no added steroid (total binding), 1.2 μM diethylstilbestrol (nonspecific binding), or 0.1 nM–10 μM βE_2 or *Ent*-E_2. Bound and unbound radioligand were separated using Sephadex G-25 (Amersham Pharmacia Biotech) columns (1.5 ml bed volume) with a 1-ml elution volume. Ten milliliters of scintillation fluid was added, and counts were determined. This method resulted in greater than 90% receptor recovery and less than 15% nonspecific binding.

Brain membrane oxidation

The brain was removed from ovariectomized female Sprague Dawley rat, and the neocortex was homogenized in ice-cold Tris buffer (100 mM, pH 7.4) with 1% Triton X-100 using a Teflon/glass tissue homogenizer. The homogenate was centrifuged at 2,000 rpm for 10 min. The resulting supernatant was incubated with βE_2 or *Ent*-E_2 at concentrations ranging from 0.1–100 μM for 30 min at 37 C. $FeSO_4$ was then added to a final concentration of 200 μM and incubated for an additional 30 min at 37 C. Butylated hydroxytoluene (100 μM) and diethylenetriaminepentaacetic acid (100 μM) were then added. 2-thiobarbituric acid reactive products (TBARs) were immediately determined by addition of 0.5% 2-thiobarbituric acid, 3.125% trichloroacetic acid, and 0.2 N HCl; and incubation was performed, at 95 C for 1 h. Samples were centrifuged at 10,000 rpm for 10 min, and the absorbance of the supernatant at 532 nm was determined.

Statistical analysis

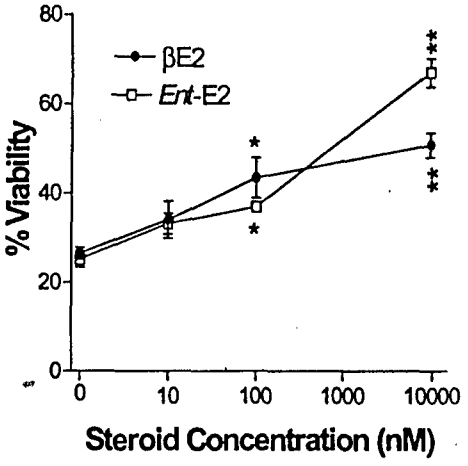
All data are presented as mean ± SEM. Comparison of ischemic lesion volume was performed using a one-way ANOVA with a Kruskal-Wallis test for planned comparisons between groups. For all other experiments, the significance of differences among groups was determined by one-way ANOVA with a Tukey's multiple-comparisons test for planned comparisons between groups when a significant difference was detected. For all tests, $P < 0.05$ was considered significant.

Results

Ent-E_2 attenuates oxidative stress-induced death in neuronal cultures

HT-22 cells, transformed hippocampal neurons, are sensitive to glutamate toxicity via a mechanism that involves glutathione depletion and the resulting oxidative stress (39). Exposure of HT-22 cells to 10 mM glutamate caused a 70–75% reduction in neuronal viability, by 24 h of exposure (Fig. 2). As previously reported (40), βE_2 treatment, commencing 2 h before glutamate exposure, conferred significant protection in this model, with a 10,000 nM concentration protecting 35 ± 4% of the cells. *Ent*-E_2, performed similarly in this model of neuroprotection, with 100 nM and 10,000 nM *Ent*-E_2 protecting 16 ± 2% and 56 ± 4% of HT-22 cells, respectively.

In another model of oxidative stress, both βE_2 and *Ent*-E_2 significantly attenuated H_2O_2-induced toxicity in HT-22 cells (Fig. 3). H_2O_2 exposure resulted in a concentration-dependent toxicity in HT-22 cells with a 30-μM concentration resulting in 21 ± 5% reduction in viability (Fig. 3) and a 60-μM concentration resulting in a 97 ± 8% reduction (data not shown);10 nM of either βE_2 or *Ent*-E_2 completely attenuated the toxicity of 30 μM H_2O_2 and protected 48 ± 14% or 63 ± 8% of the cells from 40 μM H_2O_2 toxicity, respectively (Fig. 3). No protection was seen with the 10 nM concentration of either steroid at H_2O_2 concentrations greater than 40 μM



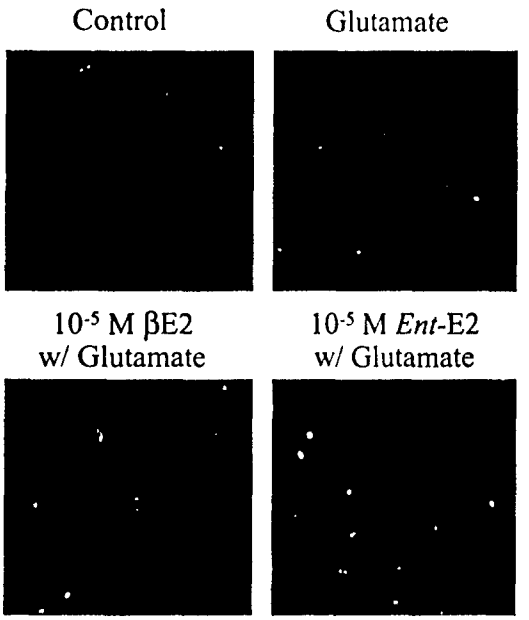


FIG. 2. Effects of βE_2 and *Ent*-E_2 on glutamate toxicity in HT-22 cells. The indicated concentration of steroid was added 2 h before the addition of glutamate (5 mM), and viability was assessed 24 h later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective toxin-free group as 100% viability. Shown is mean ± SEM for four to eight wells, representative of at least two individual experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* toxin only group. Pictured are representative fields stained with calcein AM (*green*) and propidium iodide (*red*).

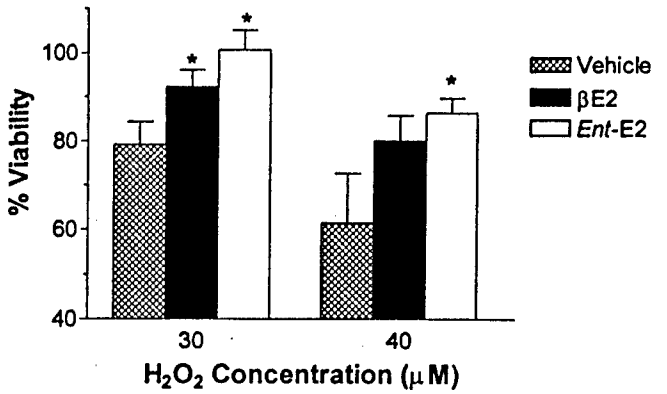


FIG. 3. Effects of βE_2 and *Ent*-E_2 on H_2O_2 toxicity in HT-22 cells. The steroid (10 nM) was added to HT-22 cells 2 h before the addition of the indicated concentration of H_2O_2. Viability was assessed 24 h later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective toxin-free group as 0% reduction in viability. Shown is mean ± SEM for four wells, representative of at least two individual experiments. *, $P < 0.05$ *vs.* toxin-only group.

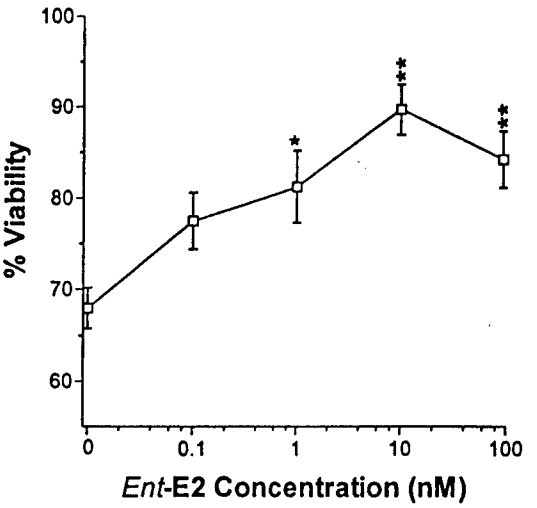


FIG. 4. Effect of *Ent*-E_2 on H_2O_2 toxicity in SK-N-SH cells. The indicated concentration of *Ent*-E_2 was added 24 h before the addition of 3 μM H_2O_2. Viability was assessed 24 h later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective toxin-free group as 100% viability. Shown is mean ± SEM for 3–4 wells, representative of at least two individual experiments. *, $P < 0.05$; **, $P < 0.001$ *vs.* the toxin-only group.

(data not shown). SK-N-SH cells were more sensitive than HT-22 cells to the toxic effects of H_2O_2 exposure, with 3 μM H_2O_2 reducing SK-N-SH cell viability by 32 ± 2% (Fig. 4). This kill was significantly attenuated by *Ent*-E_2, with a 1 nM concentration conferring 30 ± 9% protection (Fig. 4). In other studies, 1 nM βE_2 prevented 40 ± 5% of H_2O_2-induced toxicity in SK-N-SH cells (data not shown). Neither steroid exerted protective effects in these low nM concentration ranges with higher concentrations of H_2O_2 (data not shown).

Ent-E_2 reduces ischemic lesion size after transient MCA occlusion

Transient (1 h) occlusion of the MCA resulted in an average lesion area of 13 ± 2%, with the lesion localized primarily in the parietal cortex and basal ganglia (Fig. 5). Comparable with our previous reports (8), sc injection of βE_2, 2 h before onset of ischemia, reduced total lesion area by 60 ± 13%. Administration of *Ent*-E_2 similarly reduced total ischemic area by 60 ± 12%. This estrogen-mediated protection was observed in both neocortical and subcortical/allocortical regions of the ischemic infarct. βE_2 and *Ent*-E_2 reduced neocortical lesion size by 77 ± 11% and 59 ± 12%, respectively. Similarly, subcortical/allocortical lesion volume was reduced by 48 ± 8% and 47 ± 7% by βE_2 and *Ent*-E_2 administration, respectively. The subcortical/allocortical ischemic area includes regions of the caudate/putamen, hypothalamus, and hippocampus.

The protective effects of *Ent*-E_2 in this model are not attributable to conversion of *Ent*-E_2 to the more estrogenically potent βE_2. Plasma βE_2 levels after *Ent*-E_2 administration did not change from the preinjection baseline of 0.05 ± 0.01 nM (Fig. 6). In contrast, sc injection of βE_2 resulted in a rapid rise in plasma βE_2 levels, with values of 5.16 ± 0.94 nM within 1 h, and returned to near baseline (0.24 ± 0.08 nM) by 24 h.

Ent-E_2 is a weak ER agonist/antagonist

Daily administration of βE_2 for 3 days caused a dose-dependent increase in uterine wet weight, with a 1-μg/rat dose (average dose of 13.8 μg/kg) increasing wet uterine weight by 2-fold (Fig. 7). By contrast, *Ent*-E_2, at doses of 1–10 μg/rat, had no effect on uterine wet weight. At a dose of 100 μg/rat (average dose of 1400 μg/kg), *Ent*-E_2 exerted a slight antiuterotrophic effect, decreasing uterine wet weight by 23 ± 3%. *Ent*-E_2 also slightly antagonized the uterotrophic effects of 1 μg/rat βE_2, with a 100 μg/rat dose reducing the uterotrophic effect of βE_2 by 27 ± 8%. These results are comparable with previous reports in immature mice, where *Ent*-E_2 (doses of about 1200 μg/kg) exerted antiuterotrophic effects (29) and *Ent*-E_2 antagonized the uterotrophic effects of βE_2 when *Ent*-E_2 was present in a 100-fold excess (32).

Daily injections of βE_2 (1 μg/rat) induced vaginal opening in 100% of the animals examined (Table 1). *Ent*-E_2 exerted mixed agonist/antagonist effects on vaginal opening, with a 100-μg/rat dose causing vaginal opening in 50% of the juvenile rats. This dose of *Ent*-E_2 prevented βE_2-induced vaginal opening in 40% of the rats. No change in body weight was observed with administration of βE_2, *Ent*-E_2, or combinations thereof. BW of the juvenile rats averaged 72 ± 1 g.

In competition binding experiments, *Ent*-E_2 showed weak binding to both known ERs, with 4.2% and 6.3% of the relative binding affinity of βE_2 to ERα and ERβ, respectively. *Ent*-E_2 has been previously reported to have 0.9–6% of βE_2's relative binding affinity to cytosolic uterine ERs (27, 28).

Ent-E_2 can attenuate brain lipid oxidation ex vivo

Because estrogens have been previously reported to reduce oxidative damage to brain lipids (41–43), we examined the potency of both βE_2 and *Ent*-E_2 in an *ex vivo* assay of brain membrane oxidation. Thirty-minute incubation of the neocortical homogenate resulted in a 16-fold increase in TBAR formation. βE_2 and *Ent*-E_2 were equipotent in the attenuation of $FeSO_4$-induced lipid oxidation, as determined by TBAR formation (Fig. 8), with a 50-μM concentration of either steroid significantly attenuating $FeSO_4$-induced TBAR formation.

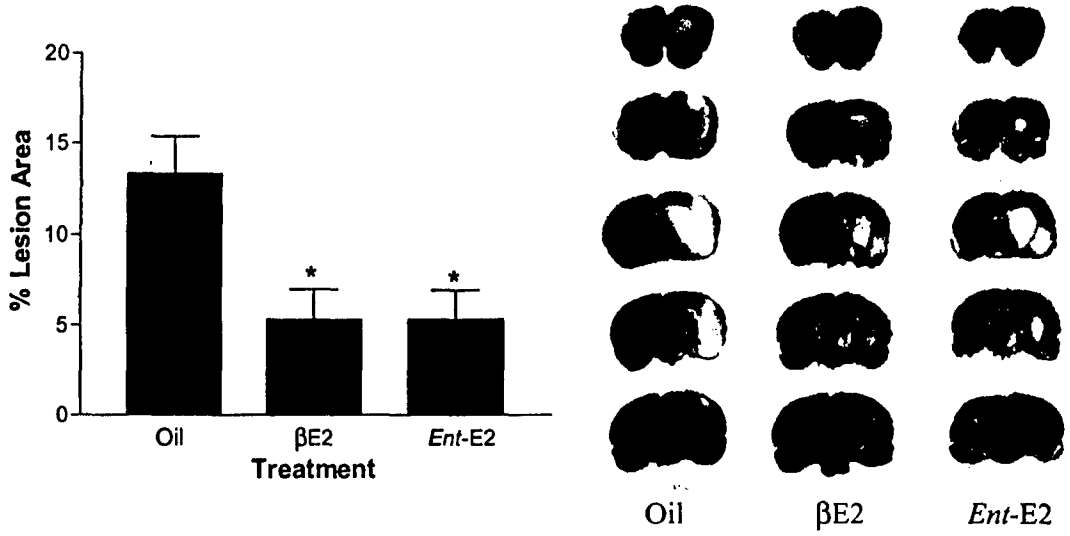


FIG. 5. Effects of βE_2 and *Ent*-E_2 on MCA occlusion-induced lesion volume in ovariectomized female rats. Rats were ovariectomized 2 weeks before occlusion, and steroids were administered, by sc injection, 2 h before onset of focal ischemia. After 1 h of MCA occlusion and 23 h of reperfusion, the brains were removed, and 2-mm slices were prepared at 3, 5, 7, 9, and 11 mm posterior to the olfactory bulb. Lesion area was determined by 2,3,5-triphenyltetrazolium chloride staining. Graphed is mean ± SEM for six rats per group. *, $P < 0.05$ *vs.* vehicle-treated rats. Pictured are representative slices for each treatment group.

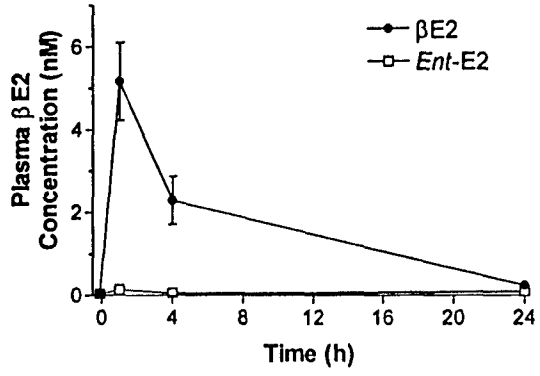


FIG. 6. Plasma βE_2 levels after βE_2 and *Ent*-E_2 administration. Ovariectomized female Sprague Dawley rats were injected sc with 100 μg/kg of either βE_2 or *Ent*-E_2. Blood was drawn by cardiac puncture, either 5 min before injection, 2 h post injection, 4 h post injection, or 24 h post injection. Plasma was collected, and βE_2 concentration was determined by RIA. Plasma βE_2 concentration is given in nM units: 1 nM = 272 pg/ml. Shown are mean ± SEM for three rats per group.

Discussion

Ent-E_2 was both as potent and efficacious as βE_2 in culture models of neuroprotection; and further, *Ent*-E_2 reduced ischemic lesion area after MCA occlusion to the same degree as βE_2. In contrast, *Ent*-E_2 showed only minimal binding affinity for either known ER, was more than 100-fold less potent than βE_2 in exerting effects on uterine growth or vaginal opening, and had weak antiuterotrophic effects. These data indicate that the neuroprotective effects of estrogens can occur without stimulation of peripheral estrogen-responsive tissues.

The neuroprotective effects of *Ent*-E_2 are not likely caused by conversion to the more estrogenically potent βE_2, because the conversion requires isomerization of five individual chiral carbons. Isomerization of the 17-hydroxy group could be facilitated by 17β-hydroxysteroid dehydrogenase; however, *Ent*-E_2 is not a substrate for this enzyme (35). Further, there was no detectable increase in plasma βE_2 levels during 24 h after sc injection of *Ent*-E_2 in female rats, indicating that *Ent*-E_2 is itself neuroprotective.

The minimal neuroprotective concentration of both βE_2 and *Ent*-E_2 varied with the cell culture model used. High physiological concentrations (low nM) were sufficient to attenuate H_2O_2-induced toxicity in SK-N-SH cells, but significantly higher supraphysiological concentrations (low μM) were required to lessen glutamate toxicity in HT-22 cells. This difference in the neuroprotective potency of estrogens between these models may be attributable to a number of factors, including differences in culture media and differences in toxicity. Further, the concentration of steroid required for protection may depend on the degree of insult, because low concentrations of *Ent*-E_2 or βE_2 did not protect SK-N-SH or HT-22 cells from H_2O_2 exposure if viability was reduced by more than 70% (P. S. Green and J. W. Simpkins, unpublished observations).

The high doses of *Ent*-E_2 used in some, but not all, of the experiments in the present report could show appreciable ER binding; however, this does not adequately explain the equipotent neuroprotection conferred by the enantiomer. The 16- to 100-fold-lower affinity of *Ent*-E_2 for the known ERs (this report, and Refs. 27 and 28) would be apparent as a similar 16- to 100-fold-lower potency in effects mediated by either ERα or ERβ. This potency difference was seen in uterotrophic and vaginal opening responses but not in assays of neuroprotection. Regardless of the minimum dose required for neuroprotection in each model, *Ent*-E_2 attenuated neuronal death with a potency equivalent to that of βE_2. This result indicates that enantiospecific interactions between estrogens and other cellular molecules are not required for the neuroprotective actions of estrogens.

Several lines of evidence connote that the neuroprotective effects of estrogens do not require ER-dependent gene tran-

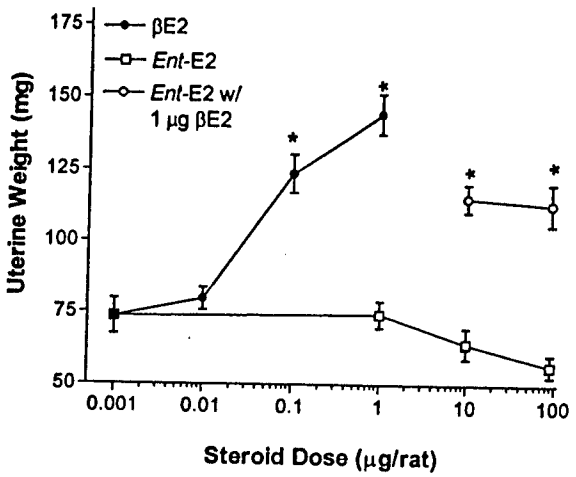


FIG. 7. Effects of βE_2 and *Ent*-E_2 on uterine wet weight in juvenile rats. Twenty-five-day-old female rats were injected sc with the indicated dose of βE_2 or *Ent*-E_2, or concurrent administration of the indicated dose of *Ent*-E_2 with 1 μg/rat βE_2 daily for 3 days. On day 4, the uteri were resected and weighed. Shown are mean ± SEM for three to nine rats per group. *, $P < 0.05$ *vs.* oil injection.

TABLE 1. Effects of βE_2 and Ent-E_2 on vaginal opening in juvenile female rats

Ent-E_2 Dose (μg/rat)	Number with vaginal opening	
	Without βE_2	With 1 μg/rat βE_2
0	0 of 4	5 of 5
10	1 of 4	3 of 4
100	2 of 4	3 of 5

Twenty-five-day-old female Sprague-Dawley rats were injected sc with the indicated dose of Ent-E_2 with or without concurrent administration of 1 μg/kg βE_2, daily for 3 days. On day 4, vaginal opening was assessed.

scription, including potent neuroprotective efficacy of several nonfeminizing estrogens including *Ent*-E_2 (Figs. 2–5) and 17α-estradiol (16, 21–23). Further, functional ERs have not been found in either HT-22 cells (40, 44) or SK-N-SH cells (22), although this study (Figs. 2–4) and others (16, 21–23, 40, 44) demonstrate estrogen-mediated protection of these neuronal cell lines. Similarly, βE_2-mediated protection can occur in the presence of ER antagonists (16–20). Together, these findings, though not excluding a role for ERs in neuroprotection, implicate cellular mechanisms other than classical ER activity in the neuroprotective effects of estrogens.

Antioxidant effects have been proposed as one mechanism for the neuroprotective effects of estrogens (19). Interestingly, the structure-activity relationship for the antioxidant effects of estrogens (42) is identical to the structure-activity relationship for the neuroprotective effects (22, 23). Further, it has been reported that the concentrations of βE_2 that are capable of exerting *ex vivo* antioxidant effects were required for neuroprotective effects (19). βE_2 attenuation of lipid peroxidation has been shown to require μM concentrations (19, 41–43). The neuroprotective concentration for βE_2 in culture models ranges from 0.1 nM (21, 45) to 50 μM (18). In this study, *ex vivo* antioxidant effects of βE_2 and *Ent*-E_2 required a minimum concentration of 50 μM, whereas neuroprotective effects were seen at much lower concentrations.

Neuronal effects of estrogens with weak ER agonist ac-

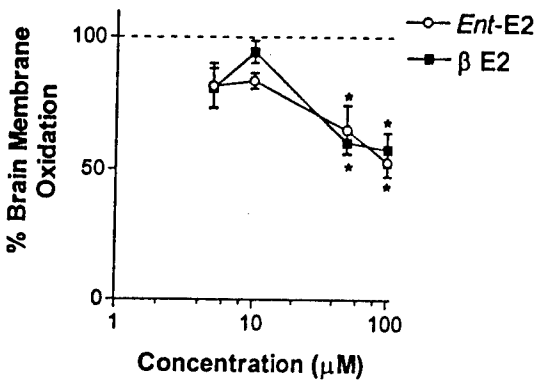


FIG. 8. βE_2 and *Ent*-E_2 inhibit $FeSO_4$-induced lipid oxidation in a rat brain homogenate. Homogenate was prepared from the neocortical tissue of an ovariectomized female Sprague Dawley rat. Homogenate was incubated with the indicated concentration of steroid for 30 min, and then oxidized by a 30-min incubation with 200 μM $FeSO_4$ at 37 C. The extent of lipid oxidation was determined by TBAR formation. Data were normalized to $FeSO_4$-only group as 100% oxidation. Shown are mean ± SEM for three samples per group. *, $P < 0.05$ *vs.* $FeSO_4$-only group.

tivity are being increasingly described. The classically inactive estrogen, 17α-estradiol, has been shown to be neuroprotective in both culture (16, 21–23) and MCA occlusion models (8). Similarly, the weak ER agonist dihydroequilin has been shown to exert neurotrophic effects in cultured neurons (46). The cellular mechanisms for these effects of weak ER agonists is not known; however, several cellular effects of 17α-estradiol have been described. Exposure to αE_2 can activate the MAP kinase pathway (47), and this pathway is implicated in βE_2-mediated neuroprotection (14). In addition, αE_2 has also been shown to have several other direct effects on neurons, including modulation of the mitochondrial $Na+/K^+$-ATPase activity (48), alteration of membrane fluidity (49), and inhibition of toxin-induced activation of NFκB (P. S. Green and J. W. Simpkins, unpublished observations). It is unknown whether *Ent*-E_2 can also interact with any of these cellular pathways.

A profusion of data indicates that estrogens enhance the survival of neurons both *in vitro* and *in vivo*, suggesting that estrogens may be useful in the treatment of neurodegenerative disease or acute neuronal death. Estrogens, such as *Ent*-E_2, may offer the beneficial neuroprotective effects of estrogens without the complicating peripheral estrogenic actions and could be useful in both men and women for whom estrogen therapy is contraindicated.

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Synthesis and Biological Evaluation of 17β-Alkoxyestra-1,3,5(10)-trienes as Potential Neuroprotectants Against Oxidative Stress

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17β-*O*-Alkyl ethers (methyl, ethyl, propyl, butyl, hexyl, and octyl) of estradiol were obtained from 3-*O*-benzyl-17β-estradiol with sodium hydride/alkyl halide, followed by the removal of the *O*-benzyl protecting group via catalytic transfer hydrogenation. An increase compared to estradiol in the protection of neural (HT-22) cells against oxidative stress due to exposure of glutamate was furnished by higher (C-3 to C-8) alkyl ethers, while methyl and ethyl ethers decreased the neuroprotective effect significantly. Lipophilic (butyl and octyl) ethers blocking the phenolic hydroxyl (3-OH) of A-ring were inactive.

Introduction

Estrogens have long been recognized as antioxidants in a variety of in vitro and in vivo models. The antioxidant action is believed to be due to their ability to scavenge free radicals that cause neuronal cell death. Oxidative stress has been linked to neuronal cell death resulting from either acute insults due to ischemia, trauma, or chronic neurodegenerative diseases such as Alzheimer's disease (AD)[2] characterized by a progressive loss of memory and cognitive function. Although the histopathogenesis of AD is yet to be fully understood, one theory hypothesizes that the oxidative microenvironment surrounding the accumulated amyloid-β-peptide (Aβ) plaques is responsible for peroxidation of cell membrane lipids leading to cell lysis and death.[3] Another oxidative stressor suggested in AD's pathogenesis is the amino acid glutamate, the major excitatory neurotransmitter in the central nervous system.[4]

Population studies have shown that estrogen replacement therapy in postmenopausal women can decrease the incidence of AD or delay its onset.[4] It has been demonstrated[5] that the most biologically active estrogen, 17β-estradiol (**1**), is a potent antioxidant and has neuroprotective activity; however, the mechanism of action is still unclear. The direct neuroprotective effects of **1** on SK-N-SH human neuroblastoma cells under serum deprivation were first reported in 1994.[6] Numerous recent studies have demonstrated similar effects of **1** against a variety of toxicities, including oxidative stress,[7] in different types of neuronal cells. Interestingly, an enantiomer of estradiol ("*ent*-estradiol"), its epimer (17α-estradiol), and estratrien-3-ol are equipotent to **1** in protection of neural cells against oxidative damage, although they have significantly reduced estrogenic activities.[6,8] All neuroprotective derivatives or analogues of **1** possess, however, a common structural element which is an intact, unsubstituted phenolic A-ring with its free 3-hydroxyl group.[9] Since lipophilic phenols also protect neural cells against glutamate and peroxide-mediated oxidative damage and cell death,[10] we decided to functionalize the 17-hydroxyl function of **1** by preparing alkyl ethers having a wide range of lipophilicity. We hypothesized that these ethers not only retain but also eventually increase neuroprotection compared to **1**. (In the meantime, these 17β-*O*-ethers also are expected to have a significantly less "feminizing" effect than **1**.) As controls, lipophilic 3-*O*-alkyl ethers have also been prepared and evaluated to further confirm the structural requirements of a phenolic A-ring for neuroprotection.

Chemistry

The previously reported preparation of 17β-methylestradiol[11] could have offered an attractive and convenient entry to a series of 17β-alkylestradiols. Unfortunately, we were unable to reproduce the procedure. Since alkylation on the phenolic 3-hydroxyl group proceeds under much milder conditions than that at the 17-position, we decided to protect the 3-OH selectively (and reversibly) before alkylating on the 17-position under strong basic conditions with the relevant alkyl halide. However, the initially prepared 3-*tert*-butyldimethylsilylated estradiol proved to be too unstable in both very mild acidic or basic conditions against alkyl halides, which resulted in a rapid desilylation followed by alkylation of the 3-OH. Similarly, the commercially available 3-benzoylestradiol was also unsuitable for the preparation of 17-alkylated ethers, since the phenolic ester group also hydrolyzed rapidly under the condition of our attempted alkylations. Therefore, the protection of the 3-OH of **1** as benzyl (Bz) ether[12] (**2**) was employed, followed by elaboration of the 17β-OH to the corresponding 17β-alkoxyl congeners **3a**−**f**. The 17-OH group was successfully alkylated with the corresponding alkyl halide in the presence of sodium hydride in DMF. However, the subsequent removal of the 3-benzyl pro-

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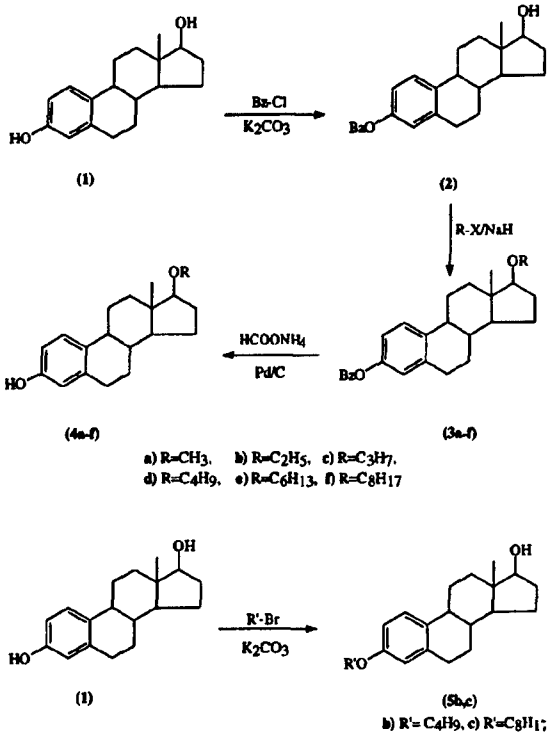
Scheme 1. Synthesis of 17- and 3-Alkyl Ethers of Estradiol



tecting group was extremely slow in the usual fashion by using a Parr hydrogenator with Pd/C as the catalyst in glacial acetic acid. On the other hand, the 3-Bz protecting group was removed rapidly under ambient conditions by catalytic transfer hydrogenation using ammonium formate resulting in the desired products **4a**–**f**.[13,14] 3-*O*-Butyl and -octyl ethers of **1** (**5b,c**; Scheme 1) as controls were prepared directly from **1** by using alkyl halide in the presence of potassium carbonate.

In addition to NMR, mass spectrometry, chromatographic, and combustion analyses to characterize the compounds prepared, crystallography data were obtained for two representative 17β-ethers (**4a,d**). The solid-state conformation (ORTEP-type plot) of **4d** is shown in Figure 1. The crystals were monoclinic and belong to the *P*2(1) space group, and this confirmed that the 17-methoxy and -butoxy groups assumed a β-orientation in the D-ring.

Results and Discussion

Cytotoxicity studies on the compounds involved were done on mouse clonal hippocampal HT-22 cells, and the Calcein AM assay was used to quantify cell viability because it is robust, reproducible, has high capacity, and reliably determines cell viability against a variety of insults.[15] We have previously found this assay to be the most reliable and reproducible of all of the viability assays used. [For example, in an exhaustive assessment of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) versus Calcein AM in measuring toxicity induced by Aβ, we observed that Calcien AM, but not MTT, accurately measured viability – an observation similar to that reported by others;[16] both assays were equally effective in detecting H_2O_2-induced toxicity on the other hand.[17]]

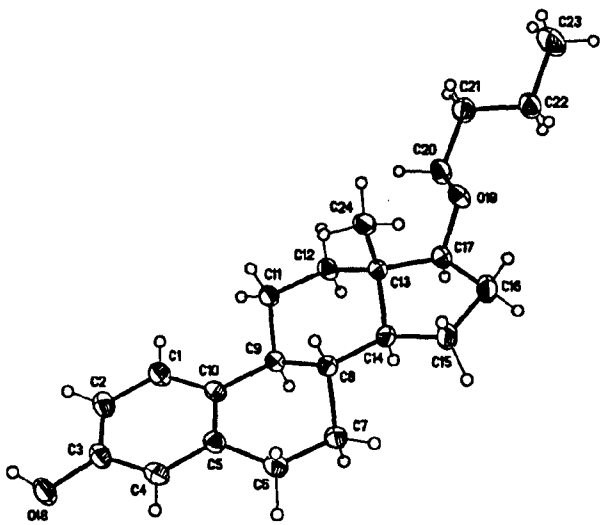


Figure 1. ORTEP plot of the X-ray crystal structure of 17-*O*-butylated 17β-estradiol **4d**. Thermal ellipsoids are shown at the 30% probability level.

When testing the neuroprotective potency of estrogens, the ED_{50} for estrogen neuroprotection is in the nanomolar range by dye exclusion techniques,[17] morphological criteria/cell counting,[18] and vital dyes (Calcein AM),[19] whereas ED_{50}s in the micromolar range were observed when MTT reduction is used as an endpoint. We have abandoned the frequently used trypan blue exclusion assay, as it requires the application of an additional stress – the harvesting of cells to conduct counts. This additional stress confounds the data. On the other hands, lactic acid dehydrogenase (LDH) release from dead or dying cells was highly variable in our hands and was too cumbersome to be used for screening in this study.

Compared to **1**, only **4c**–**f** of the six 17β-*O*-alkylestradiols tested improved neuroprotection in a dose-dependent manner against the glutamate-induced oxidative damage in murine HT-22 cells at concentrations of 0.1 μM and higher (Figure 2). These compounds were essentially equipotent at 1 μM (approximately twice as many cells were viable compared to the control) and showed no apparent relationship with a single molecular property such as lipophilicity (based on the calculated log P^{20}) or water solubility (according to our solubility studies all of the tested compounds are essentially water-insoluble). The butyl (**4d**) and octyl (**4f**) ethers were, for example, no more neuroprotective at a concentration of 10 μM than at 1 μM. The parent compound (**1**) was effective only at 10 μM but was clearly superseded by **4c,e** at this concentration. 17β-Methylestradiol (**4a**) was also ineffective below 10 μM and decreased cell viability compared to **1** at 10 μM, while 17β-ethylestradiol (**4b**) were ineffective even at 10 μM. Similarly to earlier observations regarding the inability of 3-*O*-methylestradiol to exert neuroprotection,[11] **5b,c** ethers blocking the phenolic hydroxyl in the A-ring also were inactive.

The complex relationship of neuroprotection and 17-alkoxy chain length was surprising. A comparison of the solid-state conformation of **4a,d** revealed no apparent differences in the preferred geometry of the steroid backbone between a representative "active" (**4d**) and an

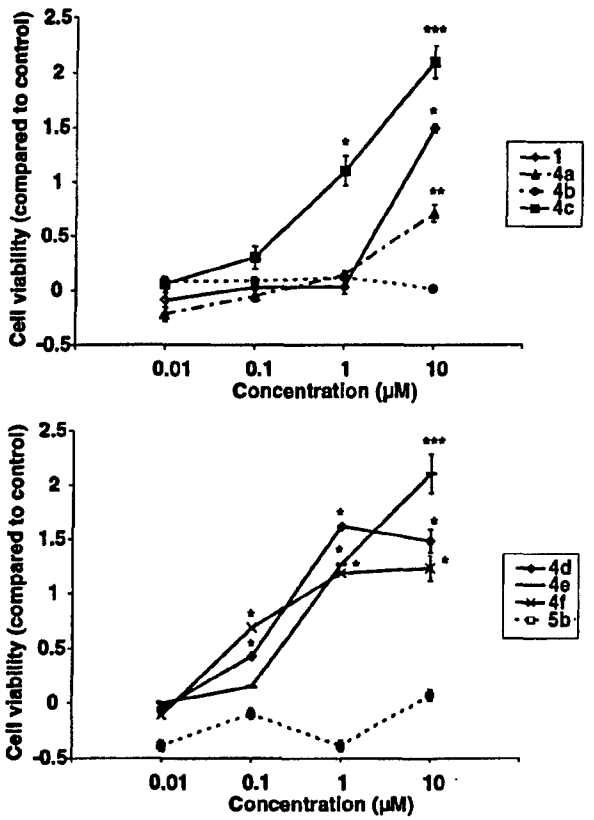


Figure 2. HT-22 cell viability in vitro after glutamate exposure (20 mM) following treatment with estradiol (**1**), its 17β-alkyl ethers (**4a–f**), and 3-butylestradiol (**5b**, as a typical representative of the 3-alkyl ethers). Statistically significant differences between groups were tested by analysis of variance (ANOVA) followed by post hoc Tukey test: *significant increase ($p < 0.05$) vs vehicle control, **significant increase ($p < 0.05$) vs vehicle control but decrease compared to 10 μM estradiol (**1**), ***significant increase ($p < 0.05$) vs vehicle control and statistically significant increase compared to 10 μM estradiol (**1**).

"inactive" (**4a**) ether derivative of **1**. One possible explanation is that the interaction of the alkyl chain of the 17β-substituent with the target site or the lipoidal cell membrane plays an important role in the efficacy of the derivative as a neuroprotectant. Thus, **4a,b** having a compact alkyl group may not have the flexibility (i.e., sufficient degrees of freedom for bond rotation) to embed into a cell membrane effectively; however, a longer alkyl chain (C $\geq$ 3) may provide this property. We also noted that the two "inactive" 17-ethers (**4a,b**) were compounds with high melting points, while the active ones have significantly lower melting points. Further studies correlating neuroprotection by phenolic A-ring steroids (including **4a–f**) with their effect on membrane fluidity are underway.

In summary, our results indicate that higher 17β-alkyl ethers of estradiol (**4c–f**) show a dose-dependent neuroprotection in vitro against oxidative stress in HT-22 cells. Moreover, this effect is manifested at lower concentration (<1 μM) than that of the parent compound (**1**).

Experimental Section

Instruments and Materials. All solvents and materials were obtained from Fisher Scientific (Atlanta, GA) or Aldrich (Milwaukee, WI). Estradiol (**1**) and 3-*O*-methyl-17β-estradiol (**5a**) were purchased from Sigma (St. Louis, MO). Sodium hydride was used as a 60% dispersion in mineral oil. Melting points were determined on a Fisher-Johns melting point apparatus and uncorrected. Thin-layer chromatography (TLC) was done on Whatman silica gel plates (on aluminum backing) containing UV fluorescence indicator by using ethyl acetate:hexane (1:4, v/v) eluent. All chromatographic purifications were done on gravity columns with 230–435 mesh neutral silica gel with ethyl acetate:hexane (1:4, v/v) as an eluent. Elemental analyses were performed by the Atlantic Microlab, Inc. (Norcross, GA). NMR spectral data were recorded for all compounds using a Varian XL-300 spectrometer and TMS as an internal standard. Mass spectral data were obtained by using atmospheric-pressure chemical ionization (APCI) on a quadrupole ion trap instrument (LCQ, Finnigan MAT, San Jose, CA). Analytical reversed-phase high-performance liquid chromatography was performed on a ThermoSeparation/SpectraPhysics (Fremont, CA) system consisting of an SP8810 isocratic pump, a Rheodyne (Cotati, CA) model 7125 injector valve equipped with a 20-μL sample loop, an SP8450 variable wavelength UV/VIS detector operated at 280 nm, and an SP4290 computing integrator. A 15-cm x 4.6-mm i.d. octadecylsilica column (Phase Sep S5 ODS2, Queensferry, Clwyd, U.K.) and a mobile phase of acetonitrile containing 1% acetic acid at a flow rate of 1.0 mL/min were used for the analyses.

X-ray crystallography data were collected at 173 K on a Siemens SMART PLATFORM equipped with A CCD area detector and a graphite monochromator utilizing Mo Kα radiation (λ = 0.71073 Å). Cell parameters for each structure were refined using up to 8192 reflections and a hemisphere of data (1381 frames) was collected using the ω-scan method (0.3° frame width). The first 50 frames were remeasured at the end of data collection to monitor instrument and crystal stability (maximum correction on I was <1%). Absorption corrections by integration were applied based on measured indexed crystal faces. Both structures were solved by the Direct Methods in SHELXTL5[21] and refined using full-matrix least-squares. The non-H atoms were treated anisotropically, whereas the hydrogen atoms were calculated in ideal positions and were riding on their respective carbon atoms, except the hydroxyl protons H_{18} in **4a** and H_{18} and H_{26} in **4d**. These protons were obtained from a difference Fourier map and refined without any constraints. While no solvent crystallized with **4a**, a methanol molecule was found in the general position in the lattice of **4d**. A total of 196 parameters of **4a** were refined in the final cycle of refinement using 2961 reflections with $I > 2\sigma(I)$ to yield R_1 and wR_2 of 5.03% and 12.66%, respectively. For **4d**, a total of 247 parameters were refined in the final cycle of refinement using 3294 reflections with $I > 2\sigma(I)$ to yield R_1 and wR_2 of 3.71% and 8.90%, respectively. Refinement was done using F^2. Tables of geometric data, indicating H-bonding interactions, are available as Supporting Information.

3-Benzyloxyestra-1,3,5(10)-trien-17β-ol (2).[12] To 5 g (18 mmol) of **1** and 10 g (72 mmol) of potassium carbonate in 100 mL of dry acetone was added 5.7 g (4.0 mL, 34 mmol) of benzyl bromide. The mixture was refluxed overnight under nitrogen atmosphere. Upon cooling the solid was removed by filtration. The filtrate was collected and acetone was removed in vacuo leaving behind clear yellowish oil, which solidified on standing. Recrystallization from ethyl acetate/hexane gave 6.1 g (93% yield) of a white fluffy solid: mp 61–63 °C; TLC R_f 0.23; ^{1}H NMR ($CDCl_3$) δ 7.44–7.19 (m, 5H, C_6H_5 of benzyl), 7.12 (d, J = 8.6 Hz, 1H, 1-CH), 6.78 (dd, J = 8.7 and 2.7 Hz, 1H, 2-CH), 6.72 (d, J = 2.4 Hz, 1H, 4-CH), 5.05 (s, 3H, OCH_2 of benzyl), 3.37 (tr, J = 8.4 Hz, 1H, 17α-CH), 2.87–2.82 (m, 2H, 6-CH_2), 2.34–1.18 (m, 13H), 0.78 (s, 3H, 13-CH_3); MS m/z 363 $[M + H]^+$.

General Procedure for Preparation of 3-Benzyloxy-17β-alkoxyestra-1,3,5(10)-trienes 3a–f. Compound **2** (0.8 g, 2.2 mmol) was dissolved in 5 mL of anhydrous DMF and, then, sodium hydride (0.3 g) was added. The mixture was stirred at room temperature for 30 min before the addition of 20 mmol

of alkyl halide. The stirring was continued overnight. The reaction mixture was quenched by pouring it into 20 mL of diluted hydrochloric acid and extracted with methylene chloride. The organic phase was dried over Na_2SO_4 and the solvent removed in vacuo leaving behind a clear, yellowish oil which solidified on standing. The crude products were purified by either recrystallization or column chromatography. Synthesis information and analytical data of a representative compound (**3a**) in the series are given below; see Supporting Information for those of **3b–f**.

3-Benzyloxy-17β-methoxyestra-1,3,5(10)-triene (3a): recrystallization from methanol, 63% yield, yellowish solid; mp 92–94 °C; TLC R_f 0.83; ^{1}H NMR ($CDCl_3$) δ 7.48–7.32 (m, 5H, C_6H_5 of benzyl), 7.27 (d, J = 8.7 Hz, 1H, 1-CH), 6.81 (dd, J = 8.7 and 2.1 Hz, 1H, 2-CH), 6.73 (d, J = 2.4 Hz, 1H, 4-CH), 5.05 (s, 2H, OCH_2 of benzyl)), 3.39 (s, 3H, 17β-OCH_3), 3.33 (t, 1H, J = 8.7 Hz, 17α-CH), 2.83 (m, 2H, 6-CH_2), 1.22–2.34 (m, 13H), 0.80 (s, 3H, 13-CH_3); MS m/z 377 $[M + H]^+$.

General Procedure for Preparation of 17β-Alkoxyestra-1,3,5(10)-trienes 4a–f. To a solution of 2.0 mmol of **3a–f** in 10 mL of methanol were added 0.2 g of Pd/C (10%) and ammonium formate (1.00 g, 16 mmol). The reaction mixture was stirred at room temperature for 1 h. Pd/C was then removed by filtration and solvent was evaporated in vacuo. To the oily residue water was added and the resulting solid was collected by filtration. Either recrystallization or column chromatography was used for purification. Synthesis information and analytical data of a representative compound (**4a**) in the series are given below; see Supporting Information for those of **4b–f**.

17β-Methoxyestra-1,3,5(10)-trien-3-ol (4a): recrystallization from methanol, 50% yield, white solid; mp 242–244 °C; TLC R_f 0.48; ^{1}H NMR (DMSO) δ 7.05 (d, J = 8.40 Hz, 1H, 1-CH), 6.51 (dd, J = 8.40 and 2.10 Hz, 1H, 2-CH), 6.45 (d, J = 2.40 Hz, 1H, 4-CH), 3.30 (s, 3H, 17β-OCH_3), 3.28 (t, J = 8.25 Hz, 1H, 17α-CH), 2.73–2.72 (m, 2H, 6-CH_2), 2.56–1.22 (m, 13H), 0.74 (s, 3H, 13-CH_3); ^{13}C NMR (DMSO) δ 156.7(C-3) 139.3 (C-5 or C-10), 132.7 (C-10 or C-5), 128.0 (C-1), 116.8 (C-2 or C-4), 114.5 (C-4 or C-2), 92.2, 58.7 (OCH_3), 51.7 (C-17), 45.6, 44.6, 40.2, 39.8, 31.1, 29.2, 28.8, 28.1, 24.4, 13.6 (13-CH_3); MS m/z 287 $[M + H]^+$, 255 $[M - OCH_3]^+$. Anal. C, H.

General Procedure for Preparation of 3-Alkoxyestra-1,3,5(10)-trienes 5b,c. To a suspension of compound **1** (0.5 g, 1.8 mmol) and potassium carbonate (1.00 g, 7.2 mmol) in 5 mL of dry acetone was added 10 mmol of 1-bromoalkane. The mixture was refluxed overnight then allowed to cool and it was filtered. The acetone was removed in vacuo and the oily residue was purified. Synthesis information and analytical data of a representative compound (**5b**) in the series are given below; see Supporting Information for those of **5c**.

3-Butoxyestra-1,3,5(10)-trien-17β-ol (5b): recrystallization from methanol:water (1:1, v/v), 68% yield, white solid; mp 86–88 °C; TLC R_f 0.62; ^{1}H NMR ($CDCl_3$) δ 7.17 (d, J = 8.7 Hz, 1H, 1-CH), 6.70 (dd, J = 8.4 and 2.40 Hz, 1H, 2-CH), 6.62 (d, J = 2.4 Hz, 1H, 4-CH), 3.93 (t, J = 6.30 Hz, 2H, OCH_2 of 3-OC_4H_9), 3.71 (t, J = 8.1 Hz, 1H, 17α-CH), 2.86–2.80 (m, 2H, 6-CH_2), 2.20–1.10 (m, 17H, including $(CH_2)_2$ of 3-OC_4H_9), 0.96 (t, J = 7.4 Hz, 3H, CH_3 of 3-OC_4H_9); ^{13}C NMR ($CHCl_3$) δ 156.9 (C-3), 137.7 (C-5 or C-10), 132.3 (C-10 or C-5), 126.1 (C-1), 114.4, 111.9, 81.7, 67.5 (OCH_2 of 3-OC_4H_9), 49.9, 43.8, 43.1, 38.7, 36.6, 31.3, 30.4, 29.7, 27.2, 26.3, 23.0, 19.2, 13.7 (CH_3 of 3-OC_4H_9), 10.9 (13-CH_3); MS m/z 311 $[M - OH]^+$.

Cytotoxicity Studies. HT-22 cells (generously provided by Dr. David Schulbert, Salk Institute, La Jolla, CA) were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum under the usual conditions. All wells in the 96-well culture plate contained approximately 5000 cells as determined by a Neubauer hemacytometer and the cells were incubated for 24 h before the compounds were added. The estradiol derivatives purified by recrystallization or column chromatography were free from **1** as determined by HPLC. All agents were dissolved in absolute ethanol and diluted, with the culture media, to a final concentration of 0.01, 0.1, 1.0, and 10 μM in their respective wells. The cells were further incubated for 24 h before sodium glutamate in a solution of phosphate buffer was added. Cell viability was quantified 2 h later by the Calcein AM assay in a phosphate buffer solution.

Statistical Analysis. ANOVA was used to determine the significance of differences among groups. Comparison between groups were done using the Tukey test. A $p < 0.05$ was considered significant.

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Supporting Information Available: Detailed information on the synthesis and characterization of **3b–f**, **4b–f**, and **5c**; X-ray crystallographic data of **4d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Appendix C

Estrogens Decrease Reperfusion-Associated Cortical Ischemic Damage

An MRI Analysis in a Transient Focal Ischemia Model

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Background and Purpose—Early identification of irreversible cerebral ischemia is critical in defining strategies that influence neuronal survival after stroke. We used MRI to investigate the effects of 17β-estradiol (E2) on the temporal evolution of focal ischemia.

Methods—Female rats were ovariectomized and divided into 1 of 2 groups: ovariectomy alone (OVX; n=4) or ovariectomy with estrogen replacement (OVX+E2; n=3). Both groups were then subjected to 1-hour middle cerebral artery occlusion (MCAO), with the use of a standardized endovascular monofilament model, followed by reperfusion. Sequential diffusion-weighted (DWI) and T2-weighted (T2WI) MRI were obtained during and after the MCAO. In separate groups of animals (n=5 for OVX and OVX+E2), cerebral blood flow (CBF) was measured by laser-Doppler methods before, during, and after occlusion.

Results—DWI detected similar lesion characteristics during MCAO in both groups. In the OVX group, lesion size did not change during reperfusion, but the signal intensity ratio increased early and stabilized during the latter stages. In contrast, DWI lesion size decreased during reperfusion in OVX+E2 rats by 50% to 60% ($P<0.05$), a size reduction almost exclusively limited to cortical regions. During MCAO, the signal intensity ratio in OVX+E2 rats was reduced compared with OVX rats. Reperfusion further attenuated the signal intensity ratio in cortical but not subcortical regions ($P<0.05$ versus OVX). T2WI revealed no lesions in either group during MCAO, but it detected lesion sizes similar to that of DWI during reperfusion. Furthermore, similar patterns and magnitudes of estrogen treatment–related decrease in lesion size were noted after reperfusion. T2WI demonstrated less intense signal intensity ratio changes in both groups compared with DWI. There were no differences in CBF between groups either during occlusion, early reperfusion, or 1 day after reperfusion.

Conclusions—This study strongly suggests that estrogens selectively protect cortical tissue from ischemic damage during MCAO and that this protection is exerted during both the occlusion and reperfusion phases of ischemia and does not involve an estrogen-related change in CBF. (*Stroke*. 2001;32:987-992.)

Key Words: cerebral ischemia, focal ■ estrogens ■ magnetic resonance imaging ■ neuroprotection ■ reperfusion injury

Stroke ranks as the third leading cause of death and the leading cause of disability in the United States.[1] Stroke patients must not only survive the acute stages of infarction but must then cope with significant mental, physical, and economic stresses associated with neurological impairment. When one considers the cost in both loss of life and loss of self-esteem and productivity, the need for effective therapeutic interventions is obvious. Most strokes occur when perfusion to the middle cerebral artery (MCA) is reduced by a clot within the major cerebral arteries, producing a region of focal cerebral ischemia and a subsequent cascade of neuronal and microvascular changes ultimately leading to infarction.[2] Experimental ultrastructural evidence suggests that some of the damage occurs in the interval of reduced or absent perfusion (the occlusion phase), but most arises during the reperfusion stage, after flow has been restored by clot lysis or opening of collateral channels.[3] Since new thrombolytic therapy can now dissolve clots and restore arterial patency, the search for neuroprotective agents that can blunt the reperfusion-

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Stroke is available at http://www.strokeaha.org

TABLE 1. Physiological Parameters in Rats Subjected to Transient MCAO (n=4 for Each Group)

Group	MABP, mm Hg	pH	Pco_2	Po_2	Hematocrit, %
Before MCAO					
OVX	101±8	7.29±0.03	55±3	67±4	42±1
OVX+E2	90±3	7.27±0.02	61±2	69±4	43±2
Ischemia 30 min					
OVX	124±4	7.33±0.02	50±4	70±13	41±1
OVX+E2	116±7	7.31±0.05	51±5	70±8	43±3
Reperfusion 30 min					
OVX	99±11	7.35±0.01	45±1	74±5	44±1
OVX+E2	92±10	7.28±0.06	56±7	76±9	43±3

associated injury and elongate the tissue interval for safe intervention assumes critical strategic importance.[4]

Observations from our and other laboratories indicate that estrogens are potent neuroprotective agents and decrease focal and global ischemia-induced lesion size by as much as 50%.[5–15] An understanding of the events affected by estrogen during occlusion and reperfusion will allow us to define the therapeutic window for application of estrogens in stroke. The histological methods used in these previous studies limited our ability to dynamically assess the protective effects of estrogen during occlusion and reperfusion.

MRI can provide a wealth of critical information about the initiation, progression, and localization of cerebral ischemic events during their occurrence. Diffusion-weighted MRI (DWI), a sensitive indicator of random movement of water molecules, is thought to reveal the early changes associated with stroke-induced cytotoxic edema.[16] On the other hand, conventional T2-weighted MRI (T2WI), a sensitive indicator of vasogenic edema that occurs later in the pathophysiology of stroke, can detect subacute ischemic damages, even though it fails to show acute ischemic changes.[16] As such, MRI can quantify the progression of 2 major pathological consequences of cerebral infarction. In the present study we applied MRI techniques to noninvasively analyze, for the first time, the temporal and spatial effects of 17β-estradiol (E2) in focal cerebral ischemic events.

Materials and Methods

Animals

Sprague-Dawley female rats (250 g body wt) purchased from Charles Rivers Laboratories, Inc (Wilmington, Mass), were housed in pairs in hanging, stainless steel cages in a temperature-controlled room (25±1°C) with daily light cycle (light on 7 AM to 7 PM daily) for a minimum of 3 days before surgery. All rats had free access to Purina Rat Chow and tap water. All procedures performed on animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida before initiation of the study. Two weeks before the focal ischemia was induced, all rats were ovariectomized to eliminate endogenous estrogens. Rats in the ovariectomy with estrogen replacement (OVX+E2) group were administrated a single dose of E2 (100 μg/kg) 2 hours before focal ischemia surgery, while those in the ovariectomy alone (OVX) group received an oil vehicle injection. The sample size needed for the MRI study was calculated on the basis of published observations by us and others that E2 treatment caused a 50% reduction in MCA occlusion (MCAO)–induced lesion size.[5–15] A small number of animals sufficient to show statistical significance were assigned to each group.

Focal Ischemic Model

MCAO was achieved according to the methods described previously.[5] Briefly, after administration of anesthetics, the left common carotid artery, external carotid artery, and internal carotid artery on the left side were exposed and dissected through a midline cervical incision. A 3-0 monofilament suture was introduced into the left internal carotid artery lumen and gently advanced until resistance was felt, indicating MCAO and compromised blood flow. The suture

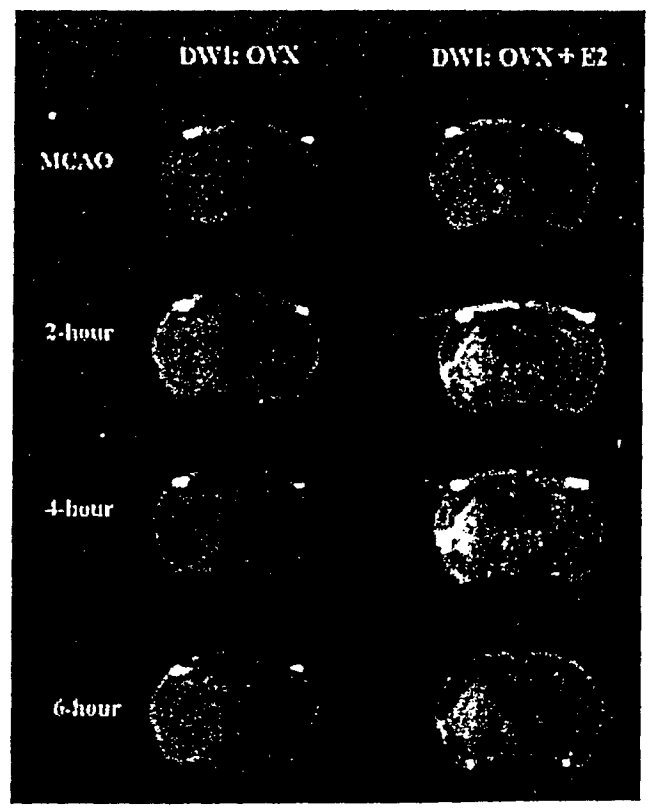

Figure 1. Sequential DWI from representative OVX and OVX+E2 rats during MCAO and after reperfusion. Two weeks after ovariectomy, female rats were divided into OVX (n=4) and OVX+E2 (n=3) groups. Both groups were then subjected to 1-hour MCAO. Sequential DWI were then obtained for each animal beginning at 30 minutes of the MCAO and at 2, 4, and 6 hours after monofilament removal (reperfusion interval). The imaging sections shown were captured at 9 mm caudal to the end of olfactory bulb.

was kept in place for 60 minutes and then withdrawn to allow MCA reperfusion. The operating procedure was performed within 20 minutes with little bleeding. Rectal temperature was monitored and maintained between 36.5°C and 37.0°C during the entire stroke procedure.

Magnetic Resonance Imaging

Imaging was performed in a 4.7-T, 33-cm magnet with a Brucket console with an actively shielded gradient set capable of 220 mT/m. The animals were supported on a cradle, and their heads were placed in a home-built birdcage coil with a 5-cm outer diameter (operating in quadrature transmit/receive mode). After the acquisition of scout images, 6 coronal plane images were prescribed beginning 3 mm behind the olfactory bulb. The slices were each 1.5 mm thick and were separated by 2 mm. All images were acquired over a 5-cm field of view with a 128×128 matrix (0.39×0.39-mm in-plane resolution), with a repetition time of 1.75 seconds and 2 signals averaged. Each set of 6 images was acquired in 7.5 minutes. DWI was performed with a standard pulsed gradient, spin-echo technique with an echo time of 33 ms. The gradient pulses were each applied for 9 ms and were separated by 13 ms around the 180° refocusing pulse. The gradient amplitude used was 152 mT/m, resulting in a b value of 1400 s/mm^2. T2WI was performed with a standard spin-echo technique with an echo time of 75 ms. Both DWI and T2WI were captured sequentially for each animal (n=4 for OVX and n=3 for OVX+E2 group) at 30 minutes during MCAO (the occlusion interval) and 2, 4, and 6 hours after withdrawal of the monofilament (the reperfusion period).

Quantification of Ischemic Lesion Sizes and Intensity

The ischemic lesion sizes and lesion intensity of the MR images were anatomically measured with Image-Pro Plus software (Media Cybernetics). The lesion area was subdivided into cortical and subcortical areas according to neuroanatomic landmarks. The percentage of the lesion size over the whole brain coronal section was calculated. The lesion intensity ratio was calculated, with the intensity of the nonlesioned hemisphere assigned a value of 1.

Cerebral Blood Flow Measurement

Cerebral blood flow (CBF) was measured in a separate group of rats (n=5 in each group for OVX and OVX+E2) that underwent focal ischemia surgery by methods that we have previously described in detail.[8] A middle line section exposed the small area around bregma. Two symmetrical holes were drilled through the skull and adjacent to the dura. These 2 holes were located at 1.5 mm posterior to and 3.5 mm left/right of bregma. Two probes of a digital laser perfusion monitor (MICROFLO DSP, Oxford Optronix Ltd) were placed on the dura to record the second-to-second change in CBF before, during, and after MCAO. Recordings were made from each animal for at least 10 minutes before occlusion, during the entire hour of occlusion, and beginning at 10 minutes of reperfusion and again at 24 hours after the occlusion. For each recording period, data representing a 10-minute period of stable CBF measurements were used to determine CBF for that sample period for each animal.

Physiological Parameters

Physiological parameters were monitored in a separate group. The left femoral artery was catheterized for mean arterial blood pressure monitoring and arterial blood sampling in the OVX and OVX+E2 animals (n=4 in each group). Physiology parameters were measured with a portable clinical analyzer (i-STAT).

Statistical Analysis

The Mann-Whitney U test was applied to determine the significance of the difference between OVX and OVX+E2 groups. R^2 was calculated to analyze the coherence of the 2 measurements. A value of $P<0.05$ was considered significant.

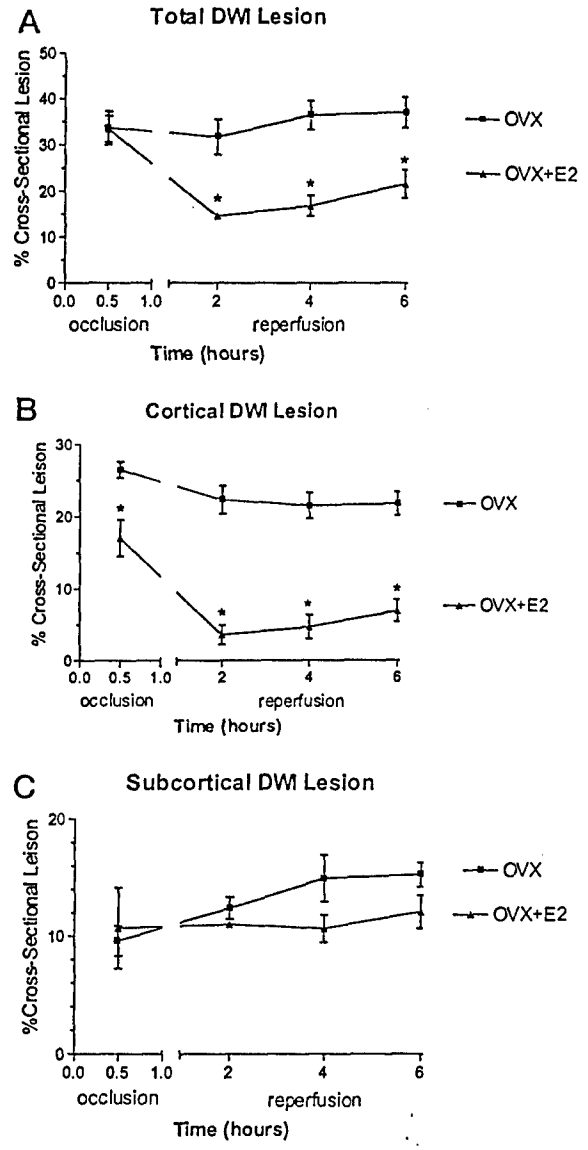

Figure 2. Effects of estrogen on MCAO-induced lesion sizes in female rats assessed by DWI. Serial DWI was applied to assess the total (A), cortical (B), and subcortical (C) lesion sizes in both OVX (n=4) and OVX+E2 (n=3) groups. Average measurements of lesion sizes at 7 and 9 mm were used for statistical analysis because they represented the widest extent of the MCAO lesions. The Mann-Whitney U test was applied to determine the significance of the difference between OVX and OVX+E2 groups. Mean±SEM values are depicted. When SEM is not shown, it is too small to be depicted. *$P<0.05$ vs OVX group.

Results

The mean arterial blood pressure was kept in the normal range during the experiment. The relatively lower Po_2 and relatively higher Pco_2 were due to the anesthesia. There were no significant differences in the determined parameters between OVX and OVX+E2 groups (Table 1).

DWI detected early changes in lesion sizes at 30 minutes into the MCAO. The total MCAO-induced lesion size was similar in both groups (33.7% and 33.5% of the whole hemisphere in the OVX and OVX+E2 groups, respectively) (Figures 1 and 2A) but was larger in cortical regions in the OVX group (26.5% versus 17.1% of OVX+E2) (Figures 1 and 2B). During reper-

TABLE 2. Effects of Estrogen in SIR of Ischemic Lesions Assessed by MRI

	SIR							
	DWI				T2WI			
	Occlusion	2 h	4 h	6 h	Occlusion	2 h	4 h	6 h
Cortex								
OVX	2.35±0.15	2.43±0.25	2.34±0.27	1.81±0.09	0	1.45±0.05	1.67±0.06	1.84±0.05
OVX+E2	1.63±0.12*	1.15±0.38*	1.23±0.40*	1.28±0.26	0	1.26±0.08	1.36±0.08*	1.40±0.11*
Subcortex								
OVX	2.20±0.21	2.38±0.15	2.27±0.22	1.87±0.06	0	1.40±0.04	1.59±0.06	1.76±0.06
OVX+E2	1.51±0.32	1.86±0.05*	2.11±0.11	1.94±0.09	0	1.28±0.01	1.35±0.04	1.52±0.06

Values are mean±SEM. Changes in ischemic lesion intensity were measured by SIR. The Mann-Whitney *U* test was applied to determine the significance of the difference between OVX (n=4) and OVX+E2 (n=3) groups.

*$P<0.05$ vs OVX group.

fusion, the lesion size remained constant in the OVX group but decreased in the OVX+E2 group by 50% to 60% ($P<0.05$) (Figures 1, 2A, and 2B). The size reduction was primarily located in cortical regions (Figures 1, 2A, and 2B).

The intensity of the ischemic lesion, as measured by the signal intensity ratio (SIR), increased at 30 minutes during MCAO in the OVX group (lesion side versus nonlesion side, 2.35 and 2.20 in cortical and subcortical regions, respectively) and reached a plateau during the latter stages of reperfusion until 6 hours of reperfusion, when SIR dropped to 1.81 and 1.87 in cortical and subcortical regions, respectively (Table 2). By comparison, the SIR during MCAO in OVX+E2 animals was reduced (1.63 and 1.51 in cortical and subcortical regions, respectively; $P<0.05$). Reperfusion further attenuated the SIR in cortical but not subcortical regions to 1.28 ($P<0.05$ versus OVX) in the OVX+E2 group (Table 2).

T2WI failed to detect the early vasogenic edema induced by MCAO in either group. During reperfusion, the OVX group demonstrated a continuous increase in lesion sizes in cortical (19.7%, 22.1%, and 24.7% at 2, 4, and 6 hours during reperfusion, respectively) and subcortical (8.3%, 10.1%, and 12.5% at 2, 4, and 6 hours during reperfusion, respectively) regions (Figures 3, 4A, and 4B). In contrast, the OVX+E2 group showed a 70% to 80% decrease in lesion size in cortical regions ($P<0.05$) (Figures 3 and 4B) and a 10% to 25% decrease in subcortical regions (Figures 3 and 4C). T2WI showed less attenuation of SIR by E2 treatment compared with DWI. E2 treatment did not significantly decrease SIR in subcortical regions compared with OVX groups (Table 2) but did cause a 20% decrease ($P<0.05$) in SIR in cortical regions during later reperfusion.

In separate groups of animals, CBF was measured before and during MCAO and 2 times during reperfusion. In both groups, CBF was reduced to approximately 20% of baseline during MCAO. Consistent with our previous observations,[8] CBF gradually recovered during the reperfusion phase, reaching 81% and 97% of baseline in OVX and OVX+E2 groups, respectively, by 24 hours of reperfusion. There were no significant differences between groups at any of the sampling times assessed (Figure 5).

Discussion

Early detection and localization of potentially reversible ischemic damage are crucial for designing and investigating clinical therapeutic interventions against stroke. The present study demonstrates that MRI can provide a wealth of critical information about the initiation, progression, and localization of cerebral ischemic events and herein is used to define the location and the component of the developing ischemic lesion as affected by estrogens. DWI, which is sensitive to the random movement of water molecules, is thought to reveal the early changes associated with stroke-induced cytotoxic edema. On the other hand, conventional T2WI is sensitive to vasogenic edema that occurs later in the pathophysiology of

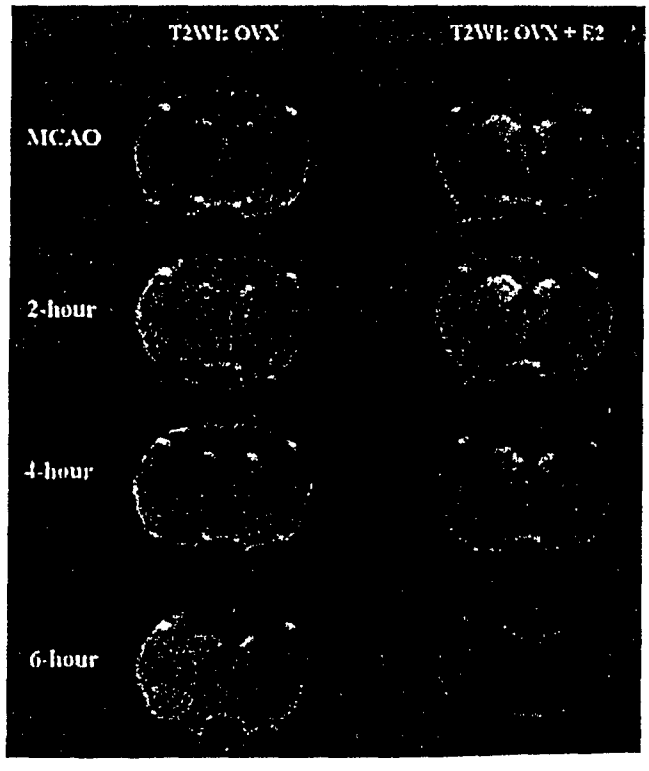

Figure 3. Sequential T2WI from representative OVX and OVX+E2 rats during MCAO and after reperfusion. Two weeks after ovariectomy, female rats were divided into OVX (n=4) and OVX+E2 (n=3) groups. Both groups were then subjected to 1-hour MCAO. Sequential T2WI were then obtained for each animal halfway through the MCAO and at 2, 4, and 6 hours after monofilament removal (reperfusion interval). The imaging sections shown were captured at 9 mm caudal to the end of olfactory bulb.

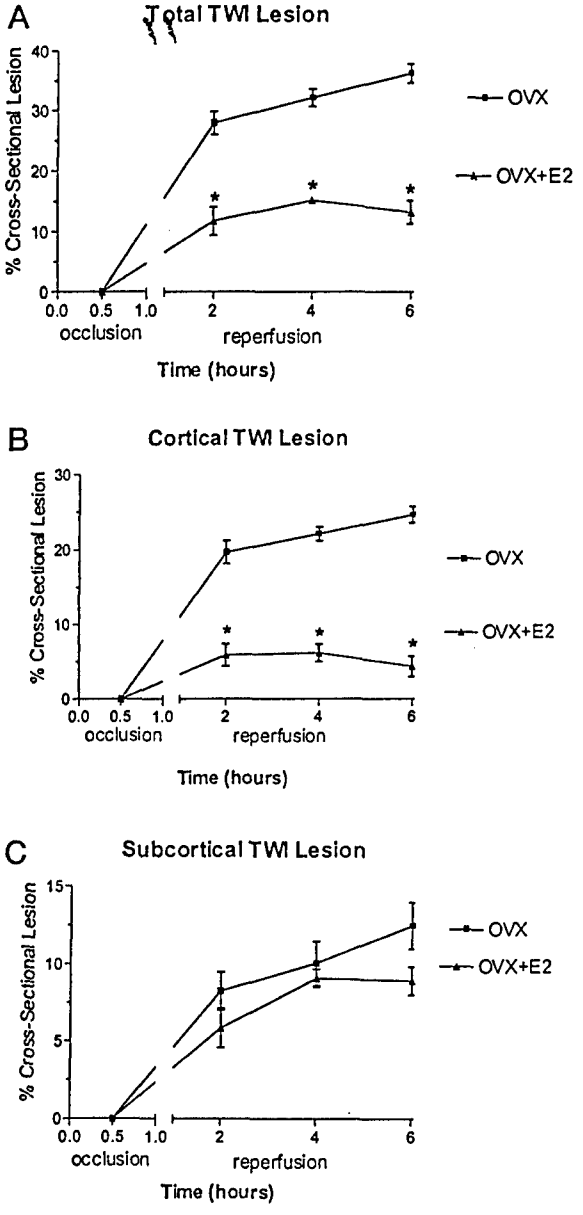

Figure 4. Effects of estrogen on MCAO-induced lesion sizes in female rats assessed by T2WI. Serial T2WI was applied to assess the total (A), cortical (B), and subcortical (C) lesion sizes in both OVX (n=4) and OVX+E2 (n=3) groups. Average measurements of lesion sizes at 7 and 9 mm were used for statistical analysis because they represented the widest extent of the MCAO lesions. The Mann-Whitney *U* test was applied to determine the significance of the difference between OVX and OVX+E2 groups. Mean±SEM values are depicted. When SEM is not shown, it is too small to be depicted. *$P<0.05$ vs OVX group.

stroke.[16] It can detect subacute ischemic damages, although it fails to show acute ischemic changes.

The early detection of ischemic lesion volumes by DWI is predictive of the clinical severity and outcome of stroke patients.[17,18] In our study DWI provided the earliest detected evidence of cerebral ischemia and was the parameter most affected by E2 treatment. This reduction in DWI changes by E2 treatment can account for most of the observed beneficial effects of estrogen pretreatment.[5–14]

The preferential protection provided by estrogen to cortical versus subcortical tissue could reflect the differential severity

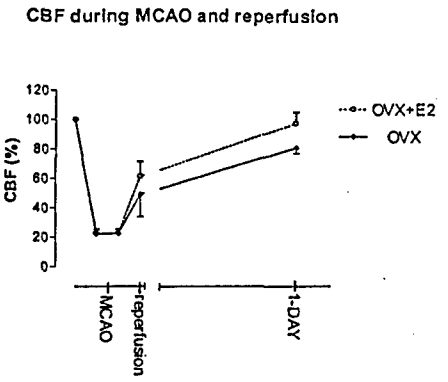

Figure 5. Effects of estrogen on CBF during MCAO and reperfusion in OVX and OVX+E2 rats. Mean±SEM values for OVX (n=5) and OVX+E2 (n=4) rats are shown. There were no differences between the groups at any of the sampling times.

of the ischemic damage of these 2 brain regions. The differential severity could result from their different blood supplies. The penumbra of the cortical ischemic region receives collaterals from leptomeningeal anastomosis, as well as from the watersheds between the anterior cerebral artery and the MCA and between the posterior cerebral artery and the MCA, while the cores of the cortical ischemic region and the subcortical region are supplied by terminal arteries of the MCA only.[19] During MCAO, the penumbra may continue to receive limited blood flow from the anterior cerebral artery, while the core and basal ganglion are believed to be more severely occluded. Alternatively, subcortical white matter is more vulnerable to the effects of focal ischemia than cortex.[20] These differences in the vulnerability and blood supply to the core and penumbra area could lead to the difference in neuroprotective effects of estrogen between these 2 areas.

The protective effects of E2 treatment appear to CBF independent. We have previously reported the neuroprotective effects of estradiol in the absence of changes in CBF.[8,15,21] In addition, others have reported a blood flow–independent neuroprotective effect of estrogens in both transient[13] and permanent[10] occlusion models. Consistently in this study, we saw no estrogen-related changes in the extent of decline in CBF during the occlusion and no significant difference in CBF during reperfusion.

E2 treatment appears to exert part of its protective effects by preventing permanent damage associated with reperfusion. Reperfusion causes structural alteration of the Golgi apparatus and compromises the energy supply to brain cells. Hoehn-Berlage et al[22] applied bioluminescence and fluorescence techniques to correlate DWI and energy disturbance during MCAO and found a depletion of ATP in the ischemic core, while the area of tissue acidosis spread beyond the ATP-depleted core region. These findings are consistent with our triphenyltetrazolium chloride staining observation (data not shown) that the white core infarct region is surrounded by the pink ischemic penumbra.[5–14] Additionally, since part of the E2-related improvement in DWI outcome occurs during the reperfusion phase, this delayed effect of the steroid may help to explain the observation that E2 treatment can be delayed until up to 3 hours after the onset of ischemia and lesion size reductions are still observed.[5,15]

Reperfusion of ischemic tissue can produce an influx of oxygen followed by an accumulation of oxygen-derived free radicals.[2] The oxidative stress may damage unsaturated fatty acids in the plasma membrane, which in turn could increase calcium influx into the cell and worsen ischemia-initiated neuronal injuries. We and others have shown that estrogens can attenuate free radical–induced peroxidative damage,[23,24] modulate calcium homeostasis in neurons,[25] and interact with neurotrophins, their receptors, and signaling pathways.[26] All of these effects of estrogen may contribute to its protective effects during reperfusion.

The suggestion that estrogens may have significant protective properties during reperfusion could have profound impact in stroke therapies. Many centers in the United States are now treating stroke acutely, using thrombolytic agents to dissolve the offending clot. Clinical trials have demonstrated a significant clinical improvement in such patients, especially when the treatment is delivered within 3 hours of stroke onset. Reopening an occluded intracranial vessel, however, is not without serious risks, which may include acute or delayed intracerebral hemorrhage, reperfusion hyperemia, and progression to infarction despite a patent lumen. The identification of an agent that can protect against such mechanisms, if delivered before or early after the vessel is reopened, could minimize an otherwise preprogrammed infarction and perhaps also positively influence hemorrhagic risks by stabilizing energy metabolism in vascular endothelium.

The present study suggests that estrogens are good candidates for producing such effects. During reperfusion, E2 treatment dramatically decreases ischemic lesion sizes and intensities, as demonstrated by both DWI and T2WI, and these decreases are almost exclusively located in cortical regions. In our previous study we found that a single dose (100 μg/kg) of E2 could increase the serum E2 concentration to physiological level in rats, producing levels sufficient for neuroprotection.[6] These findings are also consistent with a study showing that OVX can increase the ischemic lesion volume in a focal stroke model.[5]

In summary, we applied MRI techniques to demonstrate the temporal and spatial ischemic changes in a focal ischemic animal model. We have demonstrated that estrogens selectively protect cortical tissue from ischemic damage and that this protection is exerted during both the occlusion and reperfusion phases of ischemia. This study suggests that estrogen could have direct clinical applications by protecting against thrombolytic-induced reperfusion injury.

Acknowledgments

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Appendix D

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17-β Estradiol Can Reduce Secondary Ischemic Damage and Mortality of Subarachnoid Hemorrhage

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Summary: Subarachnoid hemorrhage (SAH) is a unique disorder commonly occurring when an aneurysm ruptures, leading to bleeding and clot formation, with a higher incidence in females. To evaluate the influence of 17-β estradiol (E2) in the outcome of subarachnoid hemorrhage, SAH was induced by endovascular puncture of the intracranial segment of internal carotid artery in 15 intact females (INT), 19 ovariectomized females (OVX), and 13 ovariectomized female rats with E2 replacement (OVX + E2). Cerebral blood flow was recorded before and after SAH. All animals were decapitated immediately after death or 24 hours after SAH for clot area analysis. Brains were sliced and stained with 2,3,5-triphenyltetrazolium chloride (TTC) for secondary ischemic lesion analysis. The cortical cerebral blood flow (CBF), which was measured by a laser–Doppler flowmeter, decreased to 29.6% ± 17.7%, 22.8% ± 8.3%, and 43.5% ± 22.9% on the ipsilateral side ($P = 0.01$), and decreased to 63.4% ± 14.1%, 57.4% ± 11.0%, and 66.6% ± 17.9% on the contralateral side ($P = 0.26$) in INT, OVX, and OVX + E2, respectively. The subcortical CBF, which were measured by the H_2 clearance method, were 7.77 ± 12.03, 7.80 ± 8.65, and 20.58 ± 8.96 mL 100 g^{-1} min^{-1} on the ipsilateral side ($P < 0.01$), and 21.53 ± 2.94, 25.13 ± 3.01, and 25.30 ± 3.23 mL 100 g^{-1} min^{-1} on the contralateral side in INT, OVX, and OVX + E2, respectively. The mortality was 53.3%, 68.4%, and 15.4% in INT, OVX, and OVX + E2, respectively ($P = 0.01$), whereas no significant difference in clot area was noted among the groups. The secondary ischemic lesion volume was 9.3% ± 8.4%, 24.3% ± 16.3%, and 7.0% ± 6.4% in INT, OVX, and OVX + E2, respectively ($P < 0.01$). This study demonstrated that E2 can reduce the mortality and secondary ischemic damage in a SAH model without affecting the clot volume. **Key Words:** Estrogens—Subarachnoid hemorrhage—Ischemia—Neuroprotection.

Stroke is the third most common cause of death in the adult population in the United States, after ischemic heart disease and all forms of cancer (Camarata et al., 1994). Subarachnoid hemorrhage (SAH) accounts for approximately 10% of all strokes (Selman et al., 1999). However, SAH affects a younger population and results in death in more than 50% of subjects, most of whom die within the first 24 hours. Subarachnoid hemorrhage accounts for more premature mortality than ischemic stroke (Broderick et al., 1994; Zhang et al., 1998). Subarachnoid hemorrhage can result in vascular changes such as acute vasospasm and intracranial hypertension, which lead to decrease of cerebral perfusion pressure and cerebral blood flow (CBF). All of these can contribute to secondary ischemic damage after SAH. Histologic studies of brains of patients who died shortly after SAH show extensive ischemic damage, and secondary ischemia has been reported to be one of the major causes of death shortly after SAH (Adams et al., 1981).

Unlike other kinds of strokes, aneurysmal SAH occurs more frequently in women than in men (Davis, 1994). Gender differences in the outcome of SAH are controversial, and the influence of the female sex hormone is unclear (Kongable et al., 1996; Simpson et al., 1991; Johnston et al., 1998). However, estrogens have been found to exert neuroprotective effects in models of ischemic stroke both *in vitro* and *in vivo* (Simpkins et al., 1997; Alkayed et al., 1998; Dubal et al., 1998; Zaulyanov et al., 1999; Yang et al., 2000). Whether estrogens exert similar protective effects in SAH as in ischemia is currently unknown. The purpose of this study was to

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determine whether 17β-estradiol (E2) influences the outcome of SAH and, if so, whether the influence relates to the ischemia associated with SAH.

MATERIALS AND METHODS

Preparation of animals

Female Charles River Sprague–Dawley rats (250 g, Wilmington, MA, U.S.A.) were maintained in laboratory acclimatization for 3 days before ovariectomy. Bilateral ovariectomy was performed two weeks before SAH under methoxyflurane inhalant anesthesia. All animal procedures were approved by the University of Florida Animal Care and Use Committee.

E2 administration and serum concentration

To obtain a sustained stable elevation in serum E2 concentration, implantation of a Silastic® pellet containing the steroid was used. To assess serum concentrations of E2 after this treatment regimen, a group of OVX animals (n = 5) was anesthetized with methoxyflurane inhalant and a control blood sample was taken through the jugular vein. Then a 30-mm-long Silastic® tube (1.57 mm inner diameter, 3.18 mm outer diameter) containing E2 (4 mg/mL in corn oil) was implanted subcutaneously in 5 OVX animals. Animals were returned to their cages and blood samples then were taken through the jugular vein at 24 and 48 hours after steroid administration, under methoxyflurane inhalant anesthesia. Serum was separated from blood cells by centrifugation and stored frozen (−20°C). Serum E2 concentrations were determined using duplicate serum aliquots in an ultrasensitive estradiol radioimmunoassay kit (Diagnostic Systems Lab, Webster, TX, U.S.A.).

Endovascular subarachnoid hemorrhage model

Animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal temperature was monitored and maintained between 36.5°C and 37.5°C during the procedure. With the aid of an operating microscope, the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a midline cervical skin incision. A 3–0 monofilament suture with a blunt tip was introduced into the ICA through the ECA lumen, and advanced until resistance was encountered. The distance between the CCA bifurcation and the resistive point was approximately 1.8 cm. The suture was advanced another 5 mm and then withdrawn immediately. The CCA and ICA were coagulated and the skin incision was closed.

Measurement of regional cerebral blood flow

A laser–Doppler flowmeter (LDF) was used for cortical CBF measurements. The scalp was incised on the midline, and bilateral 2-mm burr holes were drilled 1.5 mm posterior and 4.0 mm lateral to the bregma. The dura was left intact to prevent cerebral spinal fluid leakage. Laser–Doppler flowmeter probes held in place by a micromanipulator were stereotaxically advanced to gently touch the intact dura mater. The lower stable readings were obtained and recorded for at least 10 minutes from both sides (baselines measurement) (Dubal et al., 1998; Cholet et al., 1997). For each animal, the lower CBF reading was recorded at the same sites within 30 minutes after SAH. The CBF values were calculated and expressed as a percentage of the baseline values. Cerebral blood flow values reported represent the mean ± SD for the average of the CBF recordings obtained.

Hydrogen clearance method was used for subcortical CBF measurement: two Teflon-coated platinum electrodes held in place by a micromanipulator were stereotaxically advanced to 3 mm posterior, 0.5 mm lateral to the bregma, and 4.0 mm deep into the subcortical region on both sides (He et al., 1995). For each animal, CBF were recorded 30 minutes after SAH.

Measurement of clot and lesion volume

Each group of animals was decapitated immediately after death or 24 hours after SAH, the brain was removed, and the base of the brain was photographed by a digital camera (Sony MVC-FD5, Tokyo, Japan) for measurement of the clot area. Then the brain was placed in a metallic brain matrix (Harvard, Holliston, MA, U.S.A.) for tissue slicing. Two-millimeter sections were made beginning at 3, 5, 7, 9, 11, and 13 mm posterior to the olfactory bulb. Each slice was incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in physiologic saline at 37°C and then fixed in 10% formalin. The stained slices were photographed and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 3.0.1, Silver Springs, MD, U.S.A.). Clot area was calculated as the percentage of the base of the brain covered by clot to represent the clot size. Ischemic lesion volume was calculated as the sum of the areas of the ischemic lesion across the six slices divided by the total cross-sectional area of these six brain slices.

Experimental protocol

Forty-seven animals were placed into 3 groups: 15 intact females (INT), 19 ovariectomized females (OVX), and 13 ovariectomized females with estrogen replacement (OVX + E2), respectively. In OVX + E2 animals, a 30-mm-long Silastic® tube containing E2 (4 mg/mL oil) was implanted subcutaneously 24 hours before SAH under methoxyflurane inhalant anesthesia. Intact and OVX animals received a Silastic® tube containing oil as a control.

Twenty-four hours later, after a baseline CBF reading was obtained, SAH was induced in each animal. Cerebral blood flow was recorded bilaterally at the same sites within 30 minutes after SAH. Then the animals were returned to their home cages under careful observation. Each animal was decapitated for clot and ischemic volume analysis immediately after death or 24 hours after SAH.

Subcortical CBF was measured in 15 animals 30 minutes after SAH—5 each for INT, OVX, and OVX + E2—using the hydrogen clearance method. The right femoral artery was catheterized for blood pressure monitor in each animal. Mean arterial pressure was recorded before, immediately after, and 30 minutes after SAH.

Statistic study

Statistical analyses were performed using SAS Software (SAS Institute, Cary, NC, U.S.A.). Paired *t*-tests were performed to assay serum concentration of E2 treatment after implantation. The authors compared the clot areas, lesion volumes, and CBF from the three groups. For each comparison, *P* values from one-way analysis of variance were provided. Chi-squared test was used to compare the mortality rate among the three groups.

RESULTS

E2 administration and serum E2 concentration

Implantation of a 30-mm E2 (4 mg/mL corn oil) pellet maintained stable serum E2 concentrations for at least 48 hours after administration. E2 concentration was 24.9 ± 6.6 pg/mL and 31.3 ± 11.5 pg/mL at 24 and 48 hours after administration, respectively, compared with 3.5 ± 1.2 pg/mL in OVX animals ($P < 0.01$) (Fig. 1).

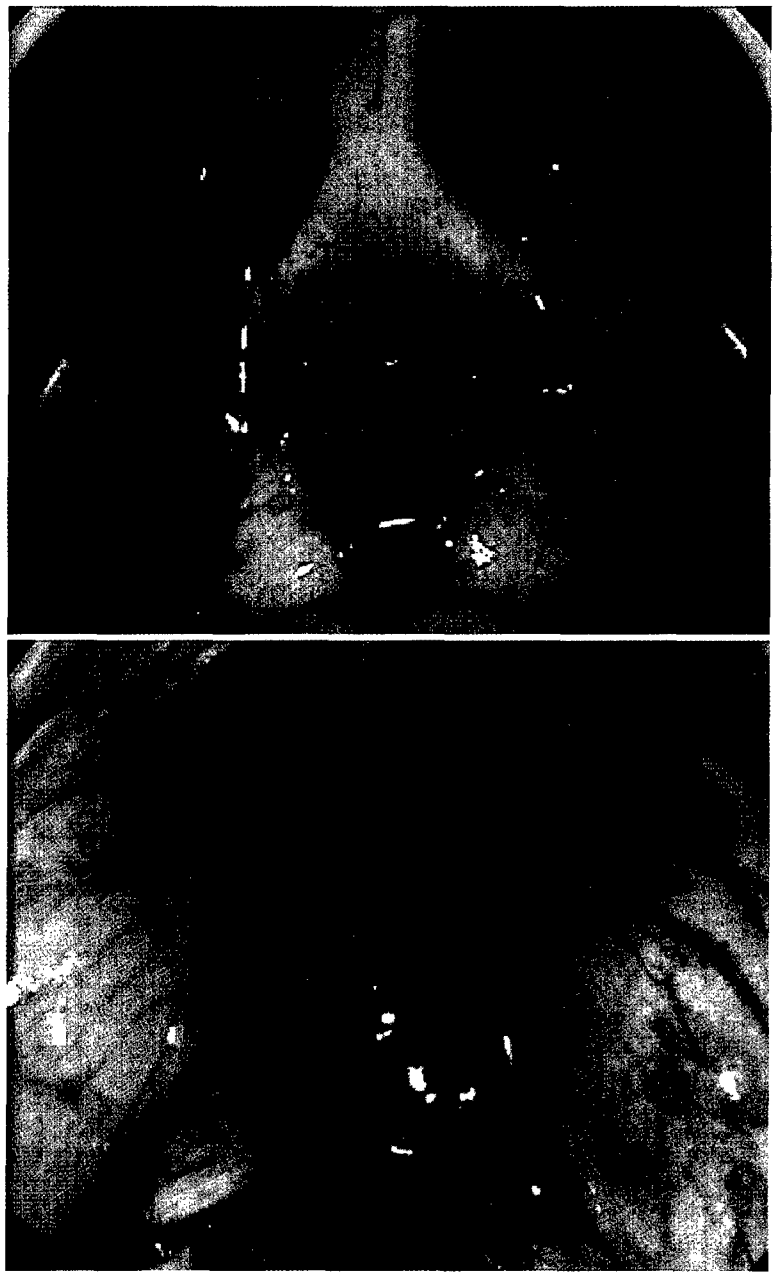

FIG. 2. Figure depicts sham **(top)** and blood distribution in subarachnoid hemorrhage **(bottom)**. Clotted and unclotted blood distributes to both sides of the brain with the majority on the ipsilateral side. Blood was also found as a thin layer overlaying both sides of the cortex.

distribution of blood throughout the subarachnoid space. This blood distribution is similar to that observed with SAH in human subjects and makes the studies of acute pathophysiologic changes of SAH, such as secondary ischemic damage, more clinically relevant (Veelken et al., 1995; Bederson et al., 1995; Matz et al., 1996). Diffusion magnetic resonance imaging study in this SAH model has shown that the acute secondary ischemic damage is confined primarily to the ipsilateral somatosensory cortex and basal ganglia and is involved in the contralateral somatosensory cortex in some of the cases, which was consistent with the authors' secondary ischemia result (Busch et al., 1998). Asymmetric ischemic damage could have resulted from the asymmetric clot distribution in this model, because there is a direct relation between the location of the thickest blood clots and the most severe vasospasm (Camarata et al., 1994).

Deprivation of ovarian steroids, which result from ovariectomy, increased secondary ischemic damage in the current study. In contrast, replacement of E2 in OVX animals decreased the secondary ischemic damage to the level below that of normal females. This study demonstrated that E2 exerted the similar neuroprotective effects in secondary ischemic damage of SAH as previously reported for ischemic stroke (Simpkins et al., 1997; Shi et al., 1997; Dubal et al., 1998; Toung et al., 1998; Chen et al., 1998; Hawk et al., 1998; Wang et al., 1999; Rusa et al., 1999; Yang et al., 2000). The neuroprotective ef-

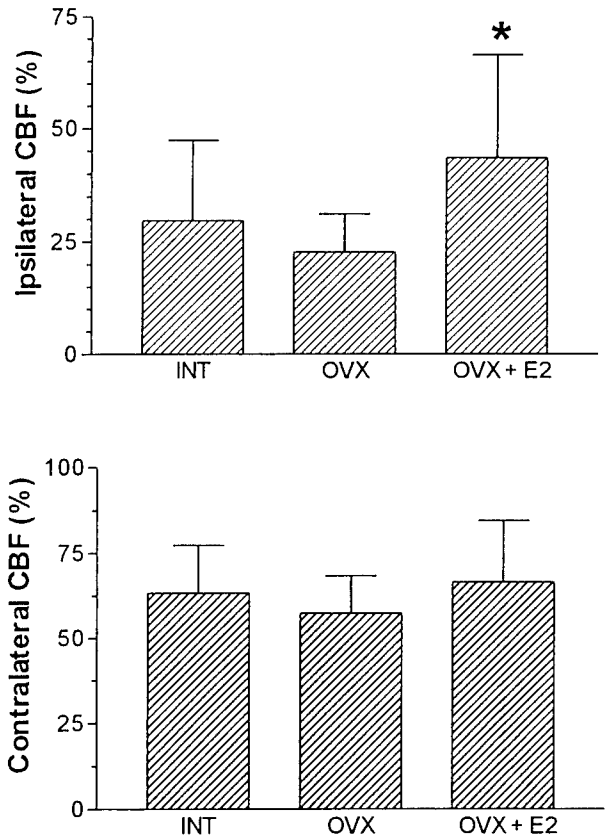

FIG. 5. Cortical cerebral blood flow (CBF) in both sides of INT, OVX, and OVX + E2. In the left side (ipsilateral), cortical CBF of OVX + E2 was significantly greater than that of OVX and INT (P = 0.0119). In the right side (contralateral), no significant differences were noted among the groups. Graph shows mean ± SD.

by stimulating endothelial nitric oxide synthase (Shaul, 1999). The current results suggest that the blood flow-preserving effect could only be induced by exogenous estrogen replacement. Because the exogenous estrogen level in the current study was 24 to 31 pg/mL, which is within the physiologic range (Butcher et al., 1974; Nequin et al., 1979), the lack of blood flow preservation in INT rats was not because of circulating estradiol levels. One explanation for the lack of this effect in endogenous steroids is the presence of progestin in intact female, which varied from 5 to 82 ng/mL, depending on the stage of the estrous cycle, in INT females and less than 5 ng/mL in OVX females (Butcher et al., 1974; Nequin et al., 1979; Murphy et al., 2000). Sarrel (1999) has shown that the vasodilation response to estrogen in women is blunted by progestin. However, the observation that secondary ischemia is similarly reduced in both INT and OVX + E2 rats suggests that progestin is not influencing the neuroprotective effects of estrogen and that the observed blood flow-preserving effects of exogenous estrogens are not the primary neuroprotective mechanism. In other words, in the SAH model, estrogen's neuroprotective effects are flow-independent.

Although both endogenous female steroids and exogenous E2 reduce secondary ischemic damage in SAH, endogenous female steroids (INT group) were not associated with a significant reduction of mortality, whereas E2 replacement markedly reduced mortality. The observation of effects of endogenous ovarian steroids on mortality is consistent with the clinical studies of the outcome of SAH. Kongable et al. (1996) showed that the SAH outcome of women and men is the same even though women were older and harbored more aneurysms. Simpson et al. (1991) found that men have a high risk of unfavorable outcome after SAH. In contrast to the above studies, Johnston et al. (1998) found the mortality of SAH was 62% greater in females than in males. Overall, sex differences in the clinical outcome of SAH are unresolved. The different effects of exogenous estrogen and endogenous female steroids on CBF could attribute to the different mortality between the INT and the OVX + E2 group. The vasodilation effect of estrogens can be reduced by progestin (Sarrel, 1999), suggesting that a progestin blockade of this important action of estrogens could account, in part, for the increased mortality of

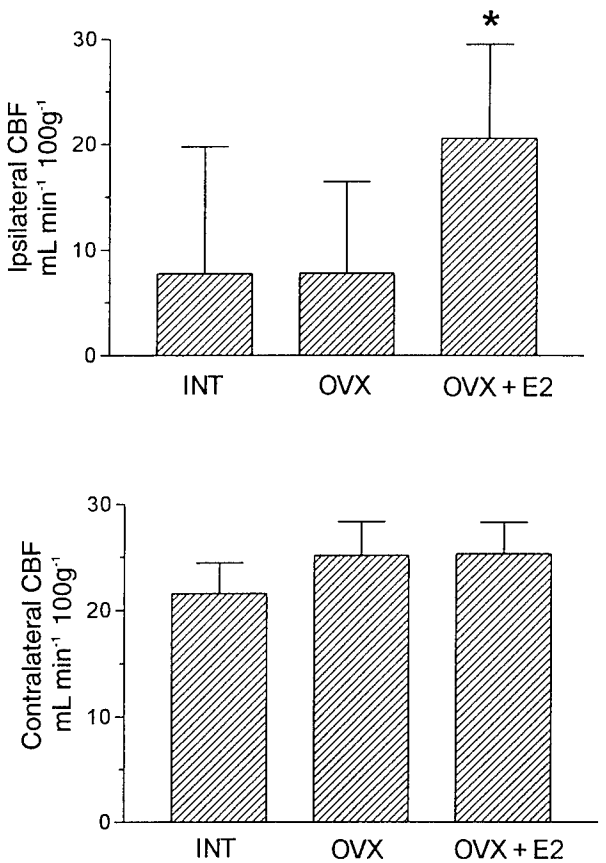

FIG. 6. Subcortical cerebral blood flow (CBF) in both sides of INT, OVX, and OVX + E2. In the left side (ipsilateral), subcortical CBF of OVX + E2 was significant greater than that of OVX and INT (P < 0.01). In the right side (contralateral), no significant differences were noted among the groups. Graph shows mean ± SD.

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Appendix E

TESTOSTERONE INCREASES NEUROTOXICITY OF GLUTAMATE *IN VITRO* AND ISCHEMIA-REPERFUSION INJURY IN AN ANIMAL MODEL.

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Abstract

Increasing evidence has demonstrated striking sex differences in the outcome of neurological injury. While estrogens contribute to these differences by attenuating neurotoxicity and ischemia-reperfusion injury, the effects of testosterone are unclear. The present study was undertaken to determine the effects of testosterone on neuronal injury in both a cell culture model and a rodent ischemia-reperfusion model. Glutamate-induced HT-22 cell death model was used to evaluate the effects of testosterone on cell survival. Testosterone was shown to significantly increase the toxicity of glutamate at a 10 μM concentration, while 17β-estradiol significantly attenuated the toxicity at the same concentration. In a rodent stroke model, ischemia-reperfusion injury was induced by temporal middle cerebral artery occlusion (MCAO) for 1 hour and reperfusion for 24 hours. To avoid the stress-related testosterone reduction, male rats were castrated and testosterone was replaced by testosterone pellets implantation. Testosterone pellets were removed at 1, 2, 4 or 6 hours prior to MCAO to determine the duration of acute testosterone depletion effects on infarct volume. The ischemic lesion volume was significantly decreased from 239.6 ± 25.9 mm^3 in control to 122.5 ± 28.6 mm^3 when testosterone pellets were removed at 6 hours before MCAO. The reduction of lesion volume was associated with the amelioration of the hyperemia during reperfusion. Our *in vitro* and *in vivo* studies suggest that sex differences in response to brain injury are partly due to the consequence of a damaging effects of testosterone.

Keyword: androgen, stroke, neuroprotection

Running title: deleterious effects of testosterone on stroke.

Introduction

Gonadal steroid hormones such as androgens and estrogens may affect various target tissues throughout the body including central nervous system. Clinical evidence has demonstrated striking sex differences in the incidence and outcome of stroke (26), which precipitated the studies of the potential impact of gonadal steroid hormones in disturbances of the central nervous system. A major focus in basic and clinical research in the last decade has been related to the activities of estrogens. Although the impact of postmenopausal estrogen replacement therapy on stroke prevention and stroke severity remains inconsistent (7,27), data from experimental studies in laboratory animals suggest that estrogens may have neuroprotective properties (3,12,33,42), which have led to a growing appreciation of the positive impact of estrogens on the central nervous system. In contrast, the effects of androgens on central nervous system are much less studied.

Testosterone has been shown to be a survival factor for axotomized motoneurons and promotes motor axon regeneration (21,22). Recently, several *in vitro* studies suggested that testosterone possessed neuroprotective effects on cerebellar granule neuron (1,2). In view of the proposed neuroprotective effects both of estrogens and androgens, sex difference of the outcome of stroke (3,26,44), could not be explained by the sex hormones. We have previously reported that chronic testosterone replacement increased while chronic castration and chronic 17β-estradiol treatment decreased ischemic damage related to middle cerebral artery occlusion (MCAO) in male rats (19). And the decrease of ischemic lesion volume with chronic 17β-estradiol treatment was associated with a marked reduction of testosterone level in intact males (19). In the present study, effects of acute testosterone depletion on ischemic stroke were evaluated.

Our objective was twofold. First, the direct effects of testosterone on neuronal survival were evaluated in a HT-22 cell culture model using glutamate insult. Second, the effects of acute testosterone depletion on ischemic lesion volume from MCAO were assessed in male rats. Our strategy was to compare the ischemic lesion volume from MCAO between testosterone depletion animals and animals with physiological level testosterone. Sustained physiological testosterone levels were obtained by castration and steroid pellets replacement technique, which we have previously reported (19). Acute testosterone depletion was achieved by pellets withdraw two days after castration and testosterone pellets implantation, while sham withdraw was used to maintain physiological testosterone levels in control. Using this strategy, the effects of timed depletion of testosterone before ischemic insult on the lesion volume and regional cerebral blood flow (CBF) from temporary MCAO were assessed in male rats.

Materials and Methods

CELL CULTURE AND TREATMENT

HT-22 cells (A gift from David Schubert, Salk Institute, San Diego, CA), which are a murine hippocampal cell line, were maintained in DMEM media (GIBCO, Gaithersburg, PA) supplemented with 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and 20 μg/ml gentamycin under standard cell culture conditions (5% CO2, 95% air, 37 °C). HT-22 cells (passages 18-25) were seeded into Nunc 96-well plates at a density of 5,000 cells / well.

Testosterone and 17β-estradiol were initially dissolved in absolute ethanol and diluted in DMEM media to the final concentration 0.01 to 10 μM. Exposure to testosterone and 17β-estradiol was initiated immediately before the addition of glutamate.

Ethanol was used at a final concentration of 0.1% as vehicle control. Glutamate was diluted to final concentration 10 mM in culture media and cells were exposed to glutamate for about 24 hours. All the cell culture experiments are repeated at least three times.

CELL VIABILITY ASSAY

Cells were exposed to steroids and glutamate for about 24 hours then cell viability was determined by calcein AM (Molecular Probes, Eugene, OR), an assay that measures cellular esterase activity and plasma membrane integrity. Wells were rinsed with PBS and after which a 25 μM solution of calcein AM in PBS was added. After incubation at room temperature for 15 minutes, fluorescence was determined (excitation 485, emission 530). Raw data were obtained as RFU. All data were then normalized to % cells killed, as calculated by treatment value / control value times 100.

EXPERIMENTAL ANIMALS

Male Charles Rivers Sprague-Dawley rats (250g, Wilmington, MA) were maintained in laboratory acclimatization for three days prior to surgery. All animal procedures were approved by the University of North Texas Health Science Center Animal Care and Use Committee and University of Florida Animal Care and Use Committee.

TESTOSTERONE CONCENTRATION IN THE TESTOSTERONE REPLACEMENT AND WITHDRAW ANIMALS:

To determine the effect of testosterone pellets implantation on serum testosterone concentration and the time course of testosterone reduction after pellets withdraw, bilateral castration was performed under methoxyflurane inhalant anesthesia, and two

15mm long testosterone Silastic pellets containing crystalline steroid were implanted subcutaneously immediately thereafter. Blood samples (0.5ml each time) were taken via jugular vein at 24 (n=4) and 48 (n=4) hours following the implantation of steroid pellets under methoxyflurane inhalant anesthesia. Then the pellets were removed and blood samples were taken via jugular vein at 1, 2, 4, 8, 12 and 24 hours (n=4 each time point) following steroid pellets removal. Serum was separated from blood cells by centrifugation and stored frozen (-20°C). Serum testosterone concentrations were determined using duplicate serum aliquots in a radioimmunoassay (Diagnostic Systems Laboratories, Los Angeles, CA). Animals used for testosterone assessment were not used for ischemia outcome studies.

EXPERIMENT PROTOCOL

Two days after castration and testosterone pellets implantation, ischemic stroke was induced in animals after testosterone pellets removal or sham removal. Pellets were removed in the testosterone depletion groups at 1 (n=7), 2 (n=5), 4 (n= 5) or 6 (n= 5) hours before ischemia under the methoxyflurane inhalant anesthesia, respectively. Sham pellets removal was performed in the physiological testosterone level group as control in the same condition as the pellets removal at 1 (n=5), 4 (n=5) and 6 (n=5) hours before MCAO, respectively. Ischemic stroke was induced by MCAO described as before (18,31). Briefly, animals were anesthetized by intraperitoneal injection of ketamine (60mg/kg) and xylazine (10mg/kg). Rectal temperature was monitored and maintained between 36.5 and 37.5°C during the procedure. With the aid of an operating microscope, the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed through a midline cervical skin incision. The CCA and ECA

were permanently cauterized. A 3-0 monofilament suture was introduced into the ICA via ECA lumen, and advanced until resistance was encountered. The distance between the CCA bifurcation and the resistive point was about 1.9 cm. The middle cerebral artery was occluded for one hour and then suture was withdrawn for reperfusion. The ICA was coagulated and the skin incision was closed.

Animals in each group were decapitated 24 hours after reperfusion. Then the brain was harvested and placed in a metallic brain matrix for tissue slicing (Harvard Apparatus, Holliston, MA). Seven slices were made at 3, 5, 7, 9, 11 13 and 15mm posterior to the olfactory bulb. Each slice was incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in physiological saline at 37 ° C, and then fixed in 10% formalin. The stained slices were photographed by a digital camera (Sony MVC-FD5, Tokyo, Japan) and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD).

REGIONAL CEREBRAL BLOOD FLOW MEASUREMENT AND PHYSIOLOGICAL PARAMETERS MONITOR.

In a separate study, MCAO was induced 6 hours after the pellets removal (n=6) or sham removal (n=6). Left femoral artery was canalized and connected to a blood pressure monitor. Arterial blood samples (150 μl each time) were taken before, 30 minutes during and 30 minutes after MCAO, respectively. Physiological parameters were measured by an ISTAT portable clinical analyzer (East Windsor, NJ).

Hydrogen clearance blood flowmeter (Digital UH meters, Unique Medical Co., Tokyo, Japan) was used for regional CBF measurement. Two Teflon coated platinum probes were stereotaxically insert into core area of ischemia (posterior bregma 0.5mm,

4mm lateral and 5mm depth). Regional CBF was monitored bilaterally during occlusion and within 30 minutes after reperfusion in testosterone pellets removal and sham removal groups.

STATISTIC ANALYSIS

All data are present as mean ± S.E.Ms. Cell death, CBF, ischemic volumes and physiological parameters in each group were compared by one-way analysis variance (ANOVA) followed by Tukey tests. A probability of 0.05 or less was considered significant.

Results

EFFECT OF TESTOSTERONE AND 17β-ESTRADIOL ON GLUTAMATE TOXICITY

10 μM testosterone significantly increase glutamate toxicity to 87.5 ± 3.7% of cells killed, comparing with 71.9 ± 6.9% at 0 μM testosterone. Opposite to the deleterious effect of testosterone, 10μM 17β-estradiol ameliorated glutamate toxicity to 40.3 ± 3.1% of cells killed, comparing with 78.3 ± 3.3% at 0 μM 17β-estradiol (Figure 1).

TESTOSTERONE CONCENTRATIONS IN TESTOSTERONE REPLACEMENT AND WITHDRAW ANIMALS:

Subcutaneous implantation of testosterone pellets increased serum testosterone concentrations to 2.58 ± 0.47 and 1.83 ± 0.13 ng/ml at one and two days after implantation, respectively, both of which are within reported physiological range of testosterone in male rats (Figure 2). Serum testosterone concentrations decreased to 0.24 ± 0.01 ng/ml at 1 hours after removal of the pellets. Thereafter testosterone

concentrations decreased to less than 0.08 ng/ml, the limits of sensitivity of the assay (Figure 2).

EFFECT OF TESTOSTERONE ON ISCHEMIC LESION VOLUME

Ischemic lesion volume was significantly decreased when testosterone pellets were removed at 6 hours before MCAO. The lesion volume was 217.8 ± 24.69, 192.6 ± 13.90, 151.3 ± 45.54 and 122.5 ± 28.62 mm^3 at 1, 2, 4 and 6 hours after pellets removal, respectively, compared to 239.6 ± 25.89 mm^3 in control animals in which physiological testosterone concentrations were maintained (Figure 3). As no differences in ischemic lesion volume were found between sham pellets removal animals at 1, 4 or 6 hours before MCAO, all the sham pellets removal animals were pooled together as controls.

EFFECT OF TESTOSTERONE ON BLOOD PRESSURE, GASES, pH, IONS AND REGIONAL CEREBRAL BLOOD FLOW

Physiological parameters are shown in table 1. .There were no significant differences between testosterone and testosterone depletion groups for any parameters measured.

Regional CBF decreased to 8.7 ± 2.1 and 7.5 ± 1.9 ml/min/100g tissue during MCAO in the testosterone and testosterone depletion group, respectively. Hyperemia was observed during reperfusion in the testosterone group, which showed a CBF of 82.2 ± 12.2 ml/min/100g tissue, compared to the non-ischemic side in testosterone group and testosterone depletion group ($p<0.05$), which had CBF of 32.0 ± 1.7 and 46.0 ± 3.6 ml/min/100g tissue, respectively. In the testosterone depletion group, no significant hyperemia was observed (Fig 4).

Discussion

Brain injury by transient global brain ischemia (cardiac arrest) and focal brain ischemia (ischemia stroke) is the leading cause of serious and long-term disability in the US (40). The striking differences in the incidence and outcome of stroke between the males and females have been suggested to be resulted from the neuroprotective effects of estrogens (3,12,27,33,42). In the present study, testosterone was shown to posses deleterious effects on ischemic stroke in a focal ischemia model, while acute testosterone depletion exert neuroprotective effects, which suggests that effects of testosterone could also contribute to these gender differences of stroke.

Experimental focal brain ischemia is one of the models most widely used to test the neuroprotective effects of estrogens *in vivo*. The protective effects of estrogens have been documented using MCAO model (3,12,33,42). In male rats, castration has also been reported to decrease ischemia-reperfusion injury in this model (19), while another report showed that castration did not affect the ischemia-reperfusion injury using a similar model (36). Two reasons could attribute to the different result between these studies. First, difference in the duration of MCAO, which was 1 hour in the former study compared to 2 hours in the latter study. Second, wide range of testosterone concentrations in noncastrated animals in the latter study, which ranged from 0.05 to 1.62 ng/ml. The wide range of testosterone concentrations in the intact male animals could be resulted from the different kinds of stress and daily rhythms of testosterone. Testosterone had a daily rhythm in young male rats, with daily troughs as low as around 0.5 ng/ml and peaks as high as 2.0 ng/ml (32). In the present study, castration and testosterone replacement techniques were used to evaluate the effects of acute testosterone depletion on ischemia-

reperfusion injury. This technique produces a sustained physiologically relevant testosterone level and avoids the influence of stress and daily rhythms in testosterone levels. Testosterone levels decline rapidly in respond to both physical and psychological stress (14), and testosterone levels are reduced in stroke patients (10,16). Testosterone levels have been shown to be inversely associated with stroke severity and 6-month mortality, while estradiol levels were not reduced in stroke patients (20). We have also shown that testosterone levels decrease significantly after MCAO (Figure 5). The physiological consequences of this response are still unclear. It has been shown that adrenomedullary activation may be influenced by the stress-induced decline in testosterone (15). Testosterone receptor blockade using flutamide appeared to ameliorate the depressed adrenal function in males after trauma and sever hemorrhagic shock (5). The stress-induced testosterone reduction could positively influence stroke outcome through adrenomedullary activation. In the present study, acute depletion of testosterone significantly decrease the ischemic lesion volume suggests that stress-related testosterone reduction could be a protective response.

Interestingly, acute depletion of testosterone before ischemic insult caused a time-dependent improvement in MCAO outcome. One of the reasons for the time-dependent effects of testosterone depletion could be resulted from the delayed degradation of testosterone in the brain. Our previous study suggested that plasma testosterone was a primary determinant of the size of ischemic lesions following MCAO in male rats (19). The half-life of serum testosterone is very short and serum testosterone decrease to undetectable level within two hours after pellets removal. Testosterone is highly hydrophobic and is cleared much more slowly from lipid rich tissue, such as brain, than

from blood. So the central nervous system effects of testosterone can persist after androgen depletion (13). The delayed effects of testosterone depletion also suggest that the effects could be mediated through a transcriptional mechanism, which could take 4 to 6 hours terminate.

Our data shown that there was a similar CBF reduction in both of the testosterone and testosterone depletion groups during MCAO. Hyperemia was shown clearly in the ischemic side within 30 minutes after reperfusion compared to the contralateral side in the testosterone group, but not in the testosterone depletion group. This suggests that the deleterious effects of testosterone could be CBF related. Reactive hyperemia and delayed hyporemia have been found during reperfusion and both are thought to be harmful to the ischemic tissue (34,37). Ischemic edema and blood-brain barrier disruption have been found to be exacerbated after acute reperfusion, which are related to the sudden surge reperfusion with hyperemia (23,41). Gradual blood flow restoration could significantly reduce the exacerbation of ischemic edema and blood-brain barrier opening (17). As such, the damaging effects of testosterone could be partially resulted from the reactive hyperemia during reperfusion.

The mechanism of the testosterone's effect on CBF is unclear. Testosterone has been shown to be vasoactive in the peripheral artery system. Treatment with testosterone causes a vasorelaxant response in rabbit coronary arteries (43). Other studies also indicated that testosterone infusion into coronary arteries in men with coronary artery disease induced vasodilation, and intravenous administration of testosterone reduced exercise-induced ischemic response in men with coronary artery disease (29,39). Testosterone's effect on vascular tone could be due to aromatization of testosterone to

estradiol, as aromatase has been identified in the arterial wall (11). However, E2 inhibits Ca^{2+} entry, whereas testosterone causes coronary relaxation by inhibiting other mechanisms in addition to Ca^{2+} entry (8). Further, testosterone has been shown to exacerbate while estrogen decrease the vulnerability of lateral striatal artery to chemical hypoxia (25). The direct mechanism of testosterone action on artery should be also taken into account.

Consistence with our *in vivo* study, testosterone was shown to exacerbate glutamate toxicity in an *in vitro* model. Toxic insults by glutamate in neuronal cell culture mimic a key component of ischemic brain injury. Microdialysis studies have shown that there are severalfold increase in extracellular glutamate during global ischemia, beginning within 1-2 minutes (6,24). There is a similar rise during focal ischemia, beginning within 2 minutes of MCA occlusion (38). Furthermore glutamate can cause both apoptosis and necrosis (28). In HT-22 cells, glutamate competes with cystine for uptake, leading to a reduction in glutathione, accumulation of reactive oxygen species and, ultimately cell death (35). The present study show that testosterone treatment exacerbates the glutamate toxicity to HT-22 cells, while 17β-estradiol treatment decreases the cell's susceptibility to glutamate toxicity, which provides us *in vitro* evidence to support our *in vivo* study. Although the deleterious effects of testosterone only present at the micromolar level *in vitro*, which is thousands times higher than the peak physiological levels in reproductive males, physiological levels of testosterone exert damaging effects on ischemia-reperfusion injury *in vivo*.

It has been shown that *in vivo* treatment of postnatal rats with testosterone rendered cerebellar granule neurons less vulnerable to oxidative stress-induced apoptosis

in vitro, which was associated with increase in catalase activity as well as in the activity of superoxide dismutase (1). However the decreased susceptibility to oxidative stress induced by the postnatal treatment with testosterone was more likely due to an accelerated maturation with a consequent developmental age-dependent increase in the antioxidant defence (30). The effects of testosterone could be different in the mature animals, as was shown with cerebral ischemia in our study. Testosterone treatment *in vitro* has also been shown to be neuroprotective for cerebellar granule neurons (2). As 17β-estradiol is also neuroprotective in cerebellar granule neurons (9), the neuroprotective effects of testosterone could be due to the conversion of testosterone into 17β-estradiol by aromatase. Further, testosterone has been reported to attenuate neuronal death in mice in response to excitotoxins, which was blocked by aromatase (4).

In summary, the present data show that testosterone can increase neuronal toxicity and exacerbate ischemia–reperfusion injury. These results suggest that sex differences in outcome after stroke may resulted from both the protective effects of estrogens and the damaging effects of testosterone. Further, acute depletion of testosterone provides neuroprotective effects on ischemia-reperfusion injury, which could be partially related to the amelioration of the hyperemia during reperfusion.

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Table 1. Physiological Parameters in Rats Subjected to Transient MCAO:

	Testoterone			Testosterone depletion		
	before MCAO	during MCAO	after MCAO	before MCAO	during MCAO	after MCAO
MABP(mmHg)	87.0 +/- 2.5	77.8 +/- 2.5	87.0 +/- 5.4	84.3 +/- 1.9	82.0 +/- 5.6	80.4 +/- 2.7
PCO_2 (mmHg)	48.8 +/- 2.9	46.9 +/- 1.3	45.3 +/- 1.3	56.1 +/- 2.3	47.6 +/- 4.8	42.1 +/- 3.9
PO_2 (mmHg)	78.2 +/- 7.2	73.0 +/- 3.1	86.0 +/- 4.3	67.2 +/- 4.1	71.0 +/- 7.2	80.6 +/- 8.3
HCO_3 (mmol/L)	26.6 +/- 0.4	25.8 +/- 0.2	25.0 +/- 0.3	27.0 +/- 0.7	26.2 +/- 1.1	25.2 +/- 0.9
SO_2 (%)	93.2 +/- 2.3	93.2 +/- 0.9	95.7 +/- 0.8	88.8 +/- 2.9	90.8 +/- 3.7	94.2 +/- 1.8
pH	7.35 +/- 0.02	7.35 +/- 0.01	7.35 +/- 0.01	7.29 +/- 0.02	7.36 +/- 0.03	7.39 +/- 0.03
Hb (g/dL)	14.4 +/- 0.2	14.2 +/- 0.5	14.6 +/- 0.5	14.5 +/- 0.2	14.0 +/- 0.0	14.4 +/- 0.2
Na^+ (mmol/L)	141.4 +/- 0.9	140.0 +/- 0.9	140.2 +/- 1.6	141.2 +/- 1.4	138.0 +/- 1.1	139.8 +/- 1.5
K^+ (mmol/L)	4.5 +/- 0.1	5.3 +/- 0.2	5.0 +/- 0.3	4.8 +/- 0.1	5.8 +/- 0.2	5.1 +/- 0.2

Values are mean ± SEM. N=6 for each group. There were no significant differences between treatment groups at any sampling time.

Figure 1: Effect of testosterone and 17β-estradiol on glutamate toxicity. HT-22 cells were exposed to 10mM glutamate for about 24 hours. Cell viability was determined by calcein assay. All data were normalized of % cells killed as calculated by treatment value / control value X 100. Values are mean ± SEM. * $p<0.05$ vs 0 μM testosterone, ** $p<0.05$ vs 0 μM 17β-estradiol.

Figure 2: Testosterone concentrations in testosterone replacement and depletion animals. Rats (n=4) were castrated and 2 testosterone pellets were implanted subcutaneously. Testosterone concentrations were maintained at 2.58 ± 0.47 and 1.83 ± 0.13 ng/ml at one and two days after implantation, respectively. * $p<0.05$ vs pellets in. (upper panel). Testosterone concentration decreases to 0.24 ± 0.01 ng/ml at 1 hours after removal of the pellets. Thereafter, testosterone concentrations decreased to less than 0.08 ng/ml. (lower panel). All values are mean ± SEM.

Figure 3: Effect of testosterone depletion on ischemic lesion volume. The lesion volume was 217.8 ± 24.7, 192.6 ± 13.9, 151.3 ± 45.5 and 122.5 ± 28.6 mm^3 at 1 (n=7), 2 (n=5), 4 (n=5) and 6 (n=5) hours after pellets removal, respectively, compared to 239.6 ± 25.9 mm^3 in control animals with testosterone (n=15). All values are mean ± SEM. *$p<0.05$ vs sham.

Figure 4: Effect of testosterone on regional CBF. T-ipsilateral: ischemic side in testosterone group. T-contralateral: contralateral side in testosterone group. T-depletion ipsilateral: ipsilateral side in testosterone depletion group. T-depletion contralateral: contralateral side in testosterone depletion group. All values are mean ± SEM. ## $p<0.01$ vs T-contralateral during MCAO. ** $p<0.01$ vs T-depletion contralateral. * $p<0.05$ vs T-contralateral and T-depletion contralateral.

Figure 5: Effect of ischemia-reperfusion injury on serum testosterone level in normal male rats (n=7). Blood samples were taken before MCAO and 24 hours after reperfusion. All blood samples were taken in the morning. Testosterone concentration was reduced to 0.73 ± 0.19 ng/ml at 24 hours after reperfusion, comparing with 2.46 ± 0.31 ng/ml before MCAO ($p<0.01$). All values are mean $\pm$ SEM.

Figure 1: Effect of testosterone and 17β-estradiol on glutamate toxicity.

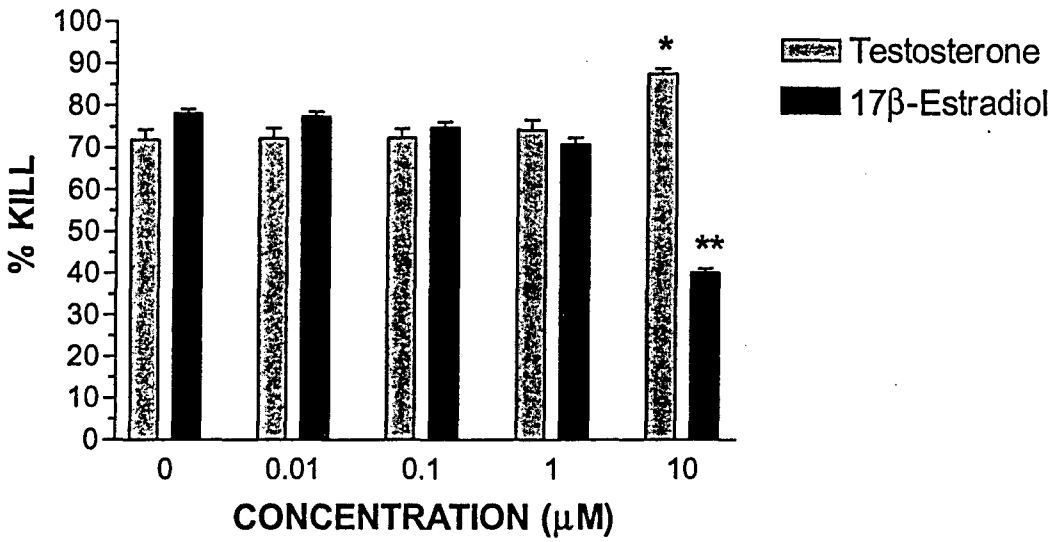
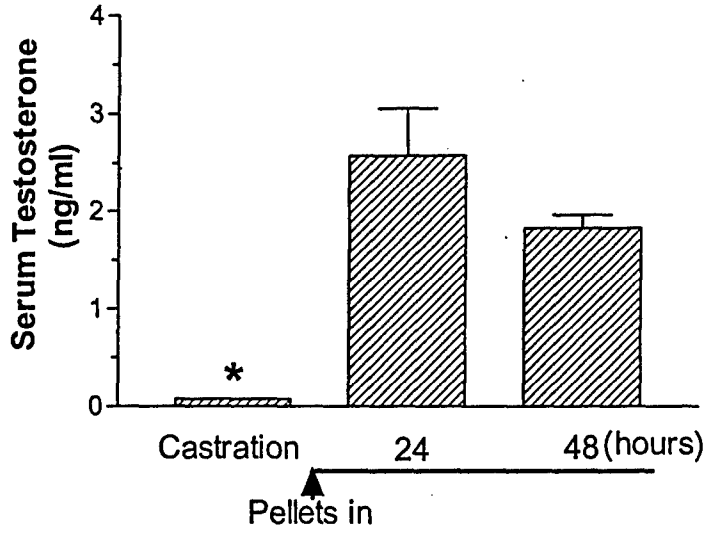


Figure 2: Serum testosterone concentration after testosterone pellets implantation and pellets removal.



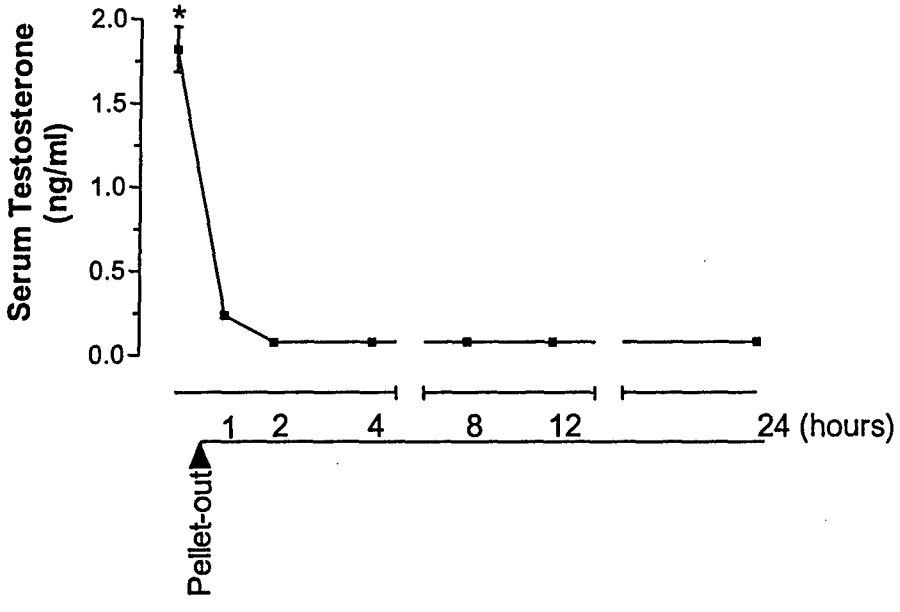


Figure 3: Effect of testosterone depletion on ischemic lesion volume of MCAO.

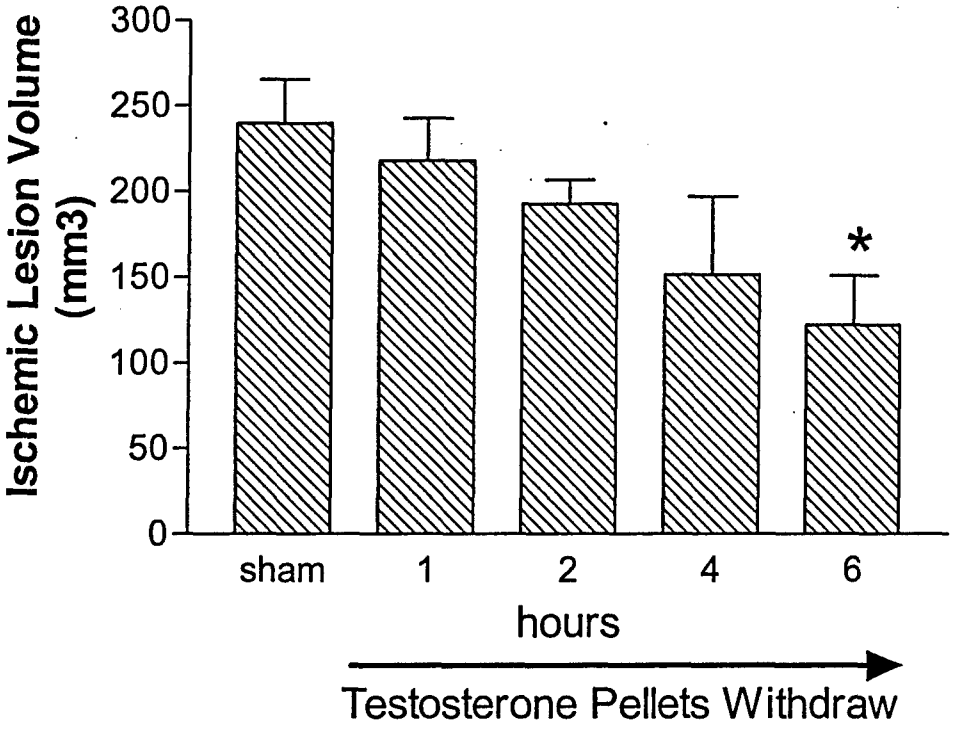


Figure 4: Effect of testosterone depletion on CBF during occlusion and reperfusion.

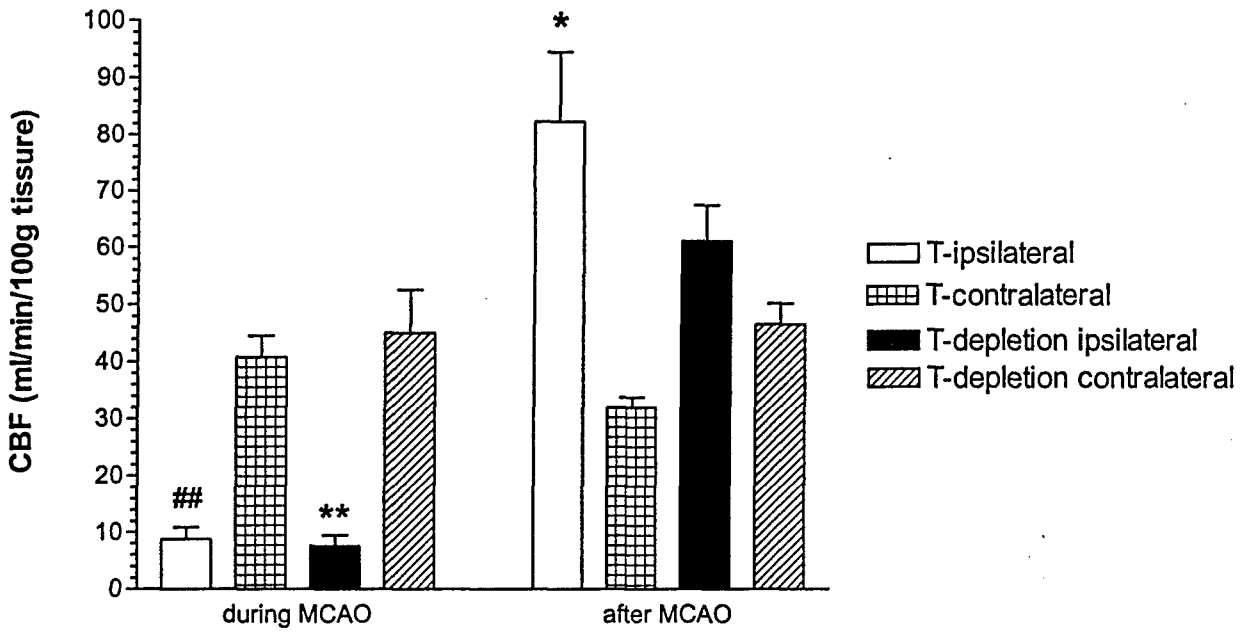
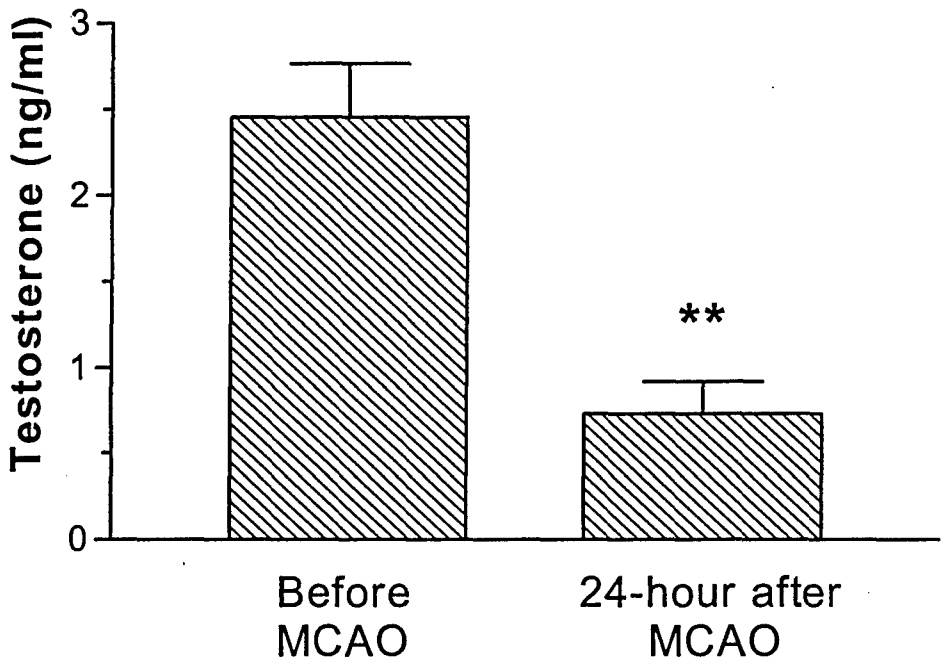


Figure 5: Reduction of testosterone level after stroke.



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Appendix F

Estradiol attenuation of β-amyloid-induced toxicity: A comparison of MTT and calcein AM assays

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Summary

17β-estradiol (βE2) has been shown to attenuate the toxicity of β-amyloid peptides (Aβ) in neuronal cultures with the effective concentration of βE2 ranging from low nM to high μM. This study compares the effective neuroprotective concentration of βE2 against both Aβ-mediated toxicity in a human neuroblastoma cell line, SK-N-SH using cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an endpoint to the effective βE2 concentration obtained using a calcein acetoxymethyl ester (calcein AM) viability assay. The minimum βE2 concentration required for protection varied 1000-fold between the two viability assays with 1 nM βE2 conferring significant protection in the calcein AM assay but 1 μM βE2 required for significant protection in the MTT assay. Interestingly, the maximal inhibition of MTT reduction occured at sub-toxic Aβ concentrations and did not correlate with other markers of cellular viability including calcein fluorescence, dye exclusion (propidium iodide or trypan blue), cellular ATP levels, or reduction of another tetrazolium dye, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium (MTS). By contrast, there was no difference between the MTT and calcein AM assays with respect to H_2O_2 toxicity or the neuroprotective effectiveness of 10 nM βE2 against H_2O_2 toxicity. These results indicate that low concentrations of βE2 can attenuate Aβ and H_2O_2 toxicity in a human neuroblastoma cell line. Further, these results suggest that the MTT assay is not an appropriate assay for the determination of βE2-mediated attenuation of Aβ toxicity.

Introduction

Epidemiological data suggest a role for estrogens in preventing the neurodegeneration associated with Alzheimer's disease (AD) (Tang *et al.*, 1996; Kawas *et al.*, 1997). Estrogens are potent neuroprotective agents in several models of neuronal death (for review see Green and Simpkins, 2000). Of specific interest to AD, 17β-estradiol (βE2) has been shown to attenuate the neurotoxicity associated with aggregated β-amyloid peptides (Aβ). While the role of Aβ in the etiology of AD remains unclear, these 40 to 43 amino acid peptides comprise a key component of senile plaques in AD and can exert toxic effects on neuronal cultures (Yanker *et al.* 1990; Pike *et al.*, 1991).

Colorimetric tests based on the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a purple, formazan product have been widely used to assess both Aβ-induced toxicity (for review see Shearman, 1999) and βE2 mediated neuroprotection (Behl *et al.*, 1995; Bonnefont *et al.*, 1998; Roth *et al.*, 1999). As MTT can be reduced by viable mitochondria (Slater *et al.*, 1963), the Aβ-induced decrease in MTT conversion has been interpreted as evidence for metabolic compromise and an early event in Aβ-induced neuronal death (Shearman *et al.*, 1994; Kaneko *et al.*, 1995). However, the Aβ attenuation of MTT reduction does not correlate with neurotoxic effects in as much as Aβ concentrations in the nM range lead to rapid inhibition of MTT conversion but prolonged exposure to μM concentrations are required for neuronal death as determined by other endpoints (Behl *et al.*, 1994; Shearman *et al.*, 1994).

Liu and Schubert (1997) have shown that Aβ can decrease MTT reduction by enhancing exocytosis of the resulting formazan crystals. Interestingly, they also found that high concentrations of βE2(>10 μM) diminished exocytosis of the formazan product of MTT reduction (Liu and Schubert, 1998). This suggests the possibility that the effects of βE2 seen against Aβ toxicity in the MTT assay may not represent estrogen mitigation of neuronal death. However, βE2-mediated protection against Aβ toxicity has also been demonstrated using other assays including dye exclusion techniques (trypan blue) (Green *et al.*, 1996, 1998), morphological criteria/cell counting (Mook-Jung *et al.*, 1997) and

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vital dyes (calcein AM) (Pike, 1999). Interestingly, these studies reported neuroprotective effects of βE2 in the nM range whereas the studies using MTT reduction as an endpoint required μM concentrations of βE2 to achieve neuroprotection (Behl *et al.*, 1995; Bonnefont *et al.*, 1998; Roth *et al.*, 1999). No conclusion can be drawn from these studies concerning the effect of the viability endpoint chosen on the concentration of βE2 required as different neuronal types, culturing conditions, βE2 treatment paradigms and times of Aβ exposure were used. The present study directly compares βE2-mediated neuroprotection against Aβ and H2O2 toxicity in the MTT assay and calcein AM assay.

Materials and methods

CELL CULTURE

SK-N-SH cells (ATCC, Rockville, MD) are a human neuroblastoma cell line. These cells were maintained in RPMI-1640 media (GIBCO, Gaithersburg, PA) supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT) and 20 μg/ml gentamycin under standard culture conditions (5% CO_2, 95% air, 37° C). Cells used in these experiments were from passages 39–45. Cells were plated at a density of 12,000 to 20,000 cells per well in Nunc 96-well plates 24 h prior to initiation of experiments.

CELL TREATMENTS

1 mg of lyophylized Aβ 25–35 (Bachem, Torrance, CA) was suspended in 1 ml of sterile PBS and incubated for 1 h at 37° C immediately before use. Aβ was diluted to the final concentration (0.05 to 20 μM) in culture media and cells were exposed to Aβ for 24 h.

βE2 (Steraloids, Wilton, NH) was initially dissolved in absolute ethanol at a concentration of 10 mM and diluted to the appropriate concentration (1 nM to 10 μM) in culture media. Exposure to βE2 was initiated 3 h prior to addition of Aβ. Ethanol was used at a final concentration of 0.1% as a vehicle control. This concentration of ethanol had no discernable effects on cell viability or Aβ 25–35 toxicity.

VIABILITY ASSAYS

MTT reduction was determined by incubation with 0.25 mg/ml MTT (Sigma Chemical Co., St. Louis, MO) for 3–4 h at 37° C. Following overnight solubilization of the formazan product in 50% N,N-dimethyl formamide, 20% sodium dodecyl sulfate, pH 4.8, the optical density was determined at 575/690 nm. For the calcein AM assay, cells were rinsed once with PBS and incubated with 25 μM calcein AM dye (Molecular Probes, Eugene, OR) at room temperature for 20 minutes. Fluorescence was determined using an excitation/emission filter set of 485/538 nm. 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium (MTS; Promega Corp., Madison, WI) reduction was determined by incubation with 0.25 mg/ml MTS and 5 μg/ml phenazine methosulfate (PMS) for 3 h at 37° C followed by determination of optical density at 490/690 nm. Cellular ATP levels were determined using the ATP determination kit (Molecular Probes) according to the manufacturer's instructions. Wells containing media without cells or wells containing cells which had been lysed prior to dye addition were used for background determination in both assays.

STATISTICAL ANALYSIS

The significance of differences among groups was determined by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. $p < 0.05$ was considered significant and each group consisted of 4 to 12 wells. All values are expressed as mean ± sem.

Results

THE MTT AND CALCEIN AM VIABILITY ASSAYS ARE DIFFERENTIALLY AFFECTED BY Aβ 25–35

Exposure of SK-N-SH neuroblastoma cells to Aβ 25–35 for 24 h results in a potent inhibition of MTT reduction with an IC_{50} of 115 nM (Fig. 1). Maximal inhibition of MTT conversion averaged 56 ± 3% and was achieved with concentrations of Aβ as low as 0.5 μM. Incubation times up to 96 h did not further increase the Aβ-induced attenuation in MTT reduction (data not shown).

The Aβ-induced reduction in MTT conversion did not correlate with a reduction in viability as determined by the calcein AM fluorescence assay (Fig. 1). Calcein fluorescence requires active cellular esterases (deesterfication of calcien AM) and plasma membrane intergrity (retention of the fluorescent calcein product). No decrease in calcein fluorescence was observed with

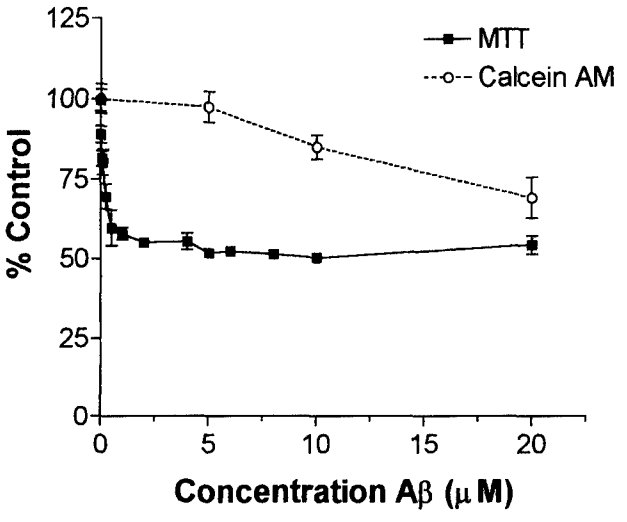

Fig. 1. Comparison of MTT and calcein AM assays for assessment of Aβ 25–35 toxicity in SK-N-SH cells. The indicated concentration of Aβ 25–35 was added 24 h prior to viability assessment. Depicted are mean ± sem for 6–12 wells per group. The data are representative of 7–9 individual experiments. In the MTT assay, $p < 0.01$ versus untreated control cells for all concentrations above 0.1 μM. In the calcein AM assay, $p < 0.05$ versus untreated control cells only for the 20 μM concentration.

Aβ 25–35 concentrations less than 10 μM even with incubation times up to 96 h (data not shown). 24 h exposure to 20 μM Aβ resulted in a statistically significant reduction in calcein fluorescence in 7 out of 12 experiments with the average reduction in fluorescence of 26 ± 6% in these experiments. Similar results were obtained using trypan blue or propidium iodide dye exclusion (markers of plasma membrane integrity) to assess Aβ-toxicity (data not shown).

Of particular interest, Aβ-inhibition of cellular reduction of the tetrazolium dye MTS did not correlate with potent inhibition seen in the MTT assay. MTS is a tetrazolium dye which is similar in function to MTT with the exceptions that the formazan product of MTS is aqueous soluble and MTS conversion requires the addition of an electron coupling reagent (PMS). A 24 h exposure to 20 μM Aβ 25–35 produced a 12 ± 2% reduction in MTS conversion as compared with 56 ± 3% reduction in MTT conversion. Aβ-induced reductions in cellular ATP levels correlated with the reductions in cellular viability rather than reductions in MTT conversion. No reduction in cellular ATP levels was observed with 5 μM or less of Aβ. A reduction in cellular ATP levels of 42 ± 8% and 65 ± 4% was observed following 24 h exposure to 10 and 20 μM Aβ 25–35, respectively.

βE2 ATTENUATES Aβ-INDUCED TOXICITY IN SK-N-SH CELLS

βE2 attenuates Aβ 25–35-induced reduction in both MTT conversion and calcein fluorescence in SK-N-SH neuroblastoma cells; however, the potency of βE2 differs in the two assays (Fig. 2). A 1 μM concentration of βE2 was required to significantly abate the reduction in MTT conversion induced by 5 μM Aβ 25–35. Lower concentrations of βE2 did not diminish the Aβ-induced effect on MTT conversion even at Aβ 25–35 concentrations of 0.1 μM (data not shown). By contrast, 1 nM βE2 attenuated 72 ± 11% of the Aβ-induced reduction in calcein fluorescence and 10 nM βE2 completely abolished the Aβ effect (Fig. 2). Differentiation of SK- N-SH cells with 10 μM retinoic acid for 4 days prior to βE2 addition yielded similar results with a 23 ± 3% reduction in viability with 20 μM Aβ 25–35 exposure and complete protection with 100 nM βE2 (data not shown).

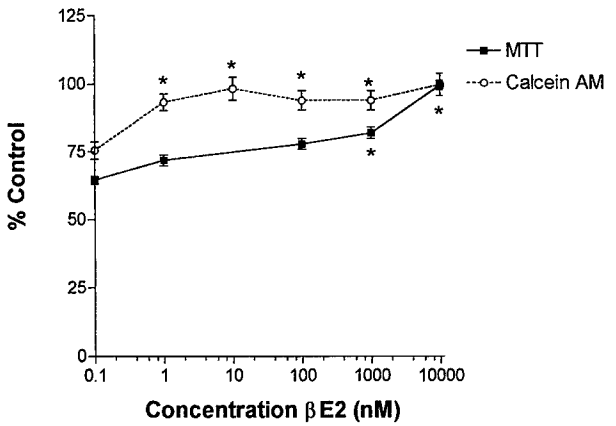

Fig. 2. Comparison of MTT and calcein AM assays for assessment of βE2 attenuation of Aβ 25–35-induced toxicity in SK-N-SH cells. The indicated concentration of βE2 was added 3 h prior to addition of Aβ 25–35. Cells were exposed to Aβ 25–35 (5 μM in the MTT assay and 20 μM in the calcein AM assay) for 24 h prior to viability assessment. Depicted are mean ± sem for 12 wells per group. The data are representative of 4 individual experiments. * = $p < 0.05$ versus the representative control wells.

βE2 ATTENUATES H_2O_2-INDUCED TOXICITY IN SK-N-SH CELLS

In contrast to Aβ-induced toxicity, H_2O_2-induced toxicity showed similar results between the MTT and calcein AM assays (Fig. 3). H_2O_2 exposure of SK-N-SH cells resulted in significant reductions in MTT conversion and calcein fluorescence with IC_{50} values averaging 4.1 μM and 5.2 μM, respectively. Low concentrations of βE2 significantly attenuated the toxicity associated with 5 μM H_2O_2 in both assays with 10 nM reducing

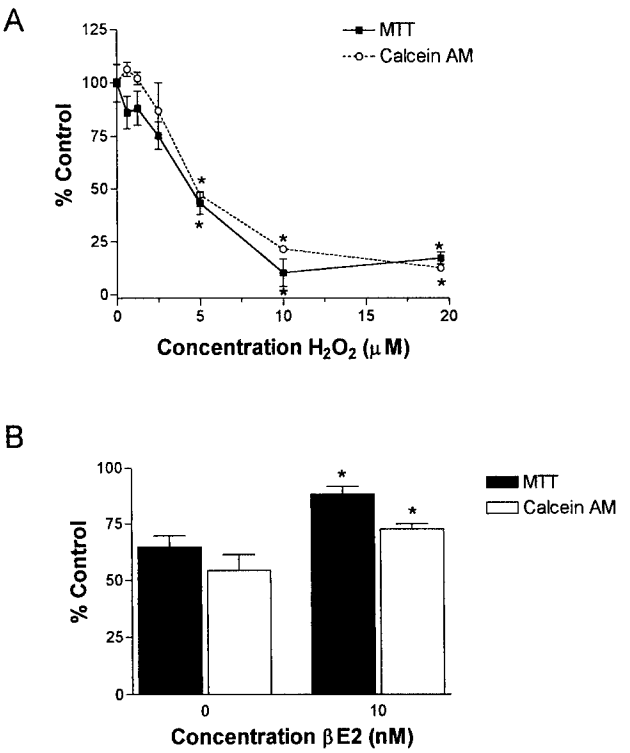

Fig. 3. Comparison of MTT and calcein AM assays for assessment of H_2O_2-induced toxicity and βE2 neuroprotection. Cells were incubated with the indicated concentration (A) or 5 μM (B) H_2O_2 for 24 h prior to viability assessment. In (B), 10 nM βE2 was added 3 h prior to H2O2. Depicted are mean ± sem for 6–8 wells per group. The data are representative of 2–4 individual experiments. * = $p < 0.05$ versus the respective untreated control wells.

H_2O_2-induced toxicity by 24 ± 3% and 18 ± 2% in the MTT and calcein AM assays, respectively. No protective effect of 10 nM βE2 was observed with concentrations of H_2O_2 which resulted in greater than a 70% reduction in cell viability (data not shown).

Discussion

The present study finds that the apparent neuroprotective potency of βE2 against Aβ toxicity differs by more than 1000-fold when assessed by MTT reduction and calcein AM fluorescence. βE2 abated Aβ-toxicity as assayed by the calcein AM assay at low nM concentrations whereas μM concentrations were required to attenuate Aβ inhibition of MTT reduction. Further, in this model, Aβ-induced inhibition of MTT reduction does not correlate with the cytotoxic effects of the peptide as assessed by calcein AM fluorescence, dye-exclusion methods (propidium iodide and trypan blue), other indicators of cellular reducing potential, or cellular ATP levels. No difference was seen in the two assays between either the degree of toxicity or βE2-mediated protection with H_2O_2 exposure.

A lack of correlation between concentrations of Aβ required to induce cytotoxicity and to inhibit MTT reduction has been widely reported (Shearman *et al.*, 1994, 1995; Kaneko *et al.*, 1995; Hertel *et al.*, 1996; Liu and Schubert, 1997). As MTT can be reduced by components of the electron transport chain (Slater *et al.*, 1963), the differences in Aβ concentrations required for inhibition of MTT reduction and cytotoxicity has been interpreted as evidence that inhibition of MTT reduction is an early indicator of Aβ-induced metabolic compromise (Shearman *et al.*, 1994, 1995; Kaneko *et al.*, 1995). However, at the low concentrations at which Aβ peptides lead to inhibition of MTT reduction, we and others (Shearman *et al.*, 1995; Hertel *et al.*, 1996) report no change in cellular redox potential as measured by other tetrazolium dyes. Similarly, no change in cellular ATP levels was observed at the nM concentrations which exerted maximal inhibition of MTT reduction although cytotoxic concentrations (10 μM) of Aβ result in a decrease in ATP levels which has been reported to precede Aβ-induced neuronal death (Zhang *et al.*, 1996; Mark *et al.*, 1997).

Other mechanisms for Aβ inhibition of cellular MTT conversion have been proposed including exacerbation of MTT formazan-induced cell lysis (Hertel *et al.*, 1996) and enhancement of MTT formazan exocytosis (Liu and Schubert, 1997). Liu and Schubert (1998) have also demonstrated that βE2 at concentrations greater than 10 μM attenuate MTT formazan exocytosis (resulting in enhanced MTT reduction) and that this activity may be responsible for the βE2 attenuation of Aβ-induced inhibition of MTT conversion. Our results indicate that the effect of high concentrations of βE2 on Aβ-induced alterations in MTT reduction do not represent the neuroprotective activity of the steroid. Rather, low concentrations of βE2 are capable of attenuating Aβ-induced toxicity in SK-N-SH cells, and this neuroprotection is not detected in assays of cellular MTT reduction. Lobner (2000) has recently reported that although the MTT reduction and lactate dehydrogenase (LDH) release are similarly affected by nifedipine or C2-ceramide toxicity, the MTT assay did not detect the neuroprotective efficacies of cyclohexamide and the caspase inhibitor ZVAD. These results indicate caution is warranted when interpreting neuroprotection data obtained in MTT reduction as a viability endpoint.

Reports of attenuation of Aβ toxicity with low concentrations of βE2 (1 to 30 nM) used viability endpoints other than cellular MTT reduction including trypan blue exclusion (Green *et al.*, 1996, 1998), calcein fluorescence (Pike, 1999), and morphological criteria/cell counting (Mook-Jung *et al.*, 1997). In contrast, studies which used MTT reduction as a viability endpoint required 0.1 to 10 μM βE2 to significantly attenuate the Aβ effect (Behl *et al.*, 1995; Bonnefont *et al.*, 1998; Roth *et al.*, 1999). Together, these studies suggest that high, non-physiological βE2 concentrations are required to alter MTT reduction/exocytosis but low concentrations of βE2 are capable of blocking the neurotoxicity associated with Aβ exposure.

The mechanism by which estrogens exert neuroprotective effects is multi-faceted and remains to be elucidated (for review see Green and Simpkins, 2000). Further study of the neuroprotective effects of estrogens requires accurate methods of assessing cell viability. The present study demonstrates that MTT reduction is not an appropriate endpoint for assessment of Aβ-induced toxicity and the corresponding estrogen-mediated neuroprotection.

Acknowledgments

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Appendix G

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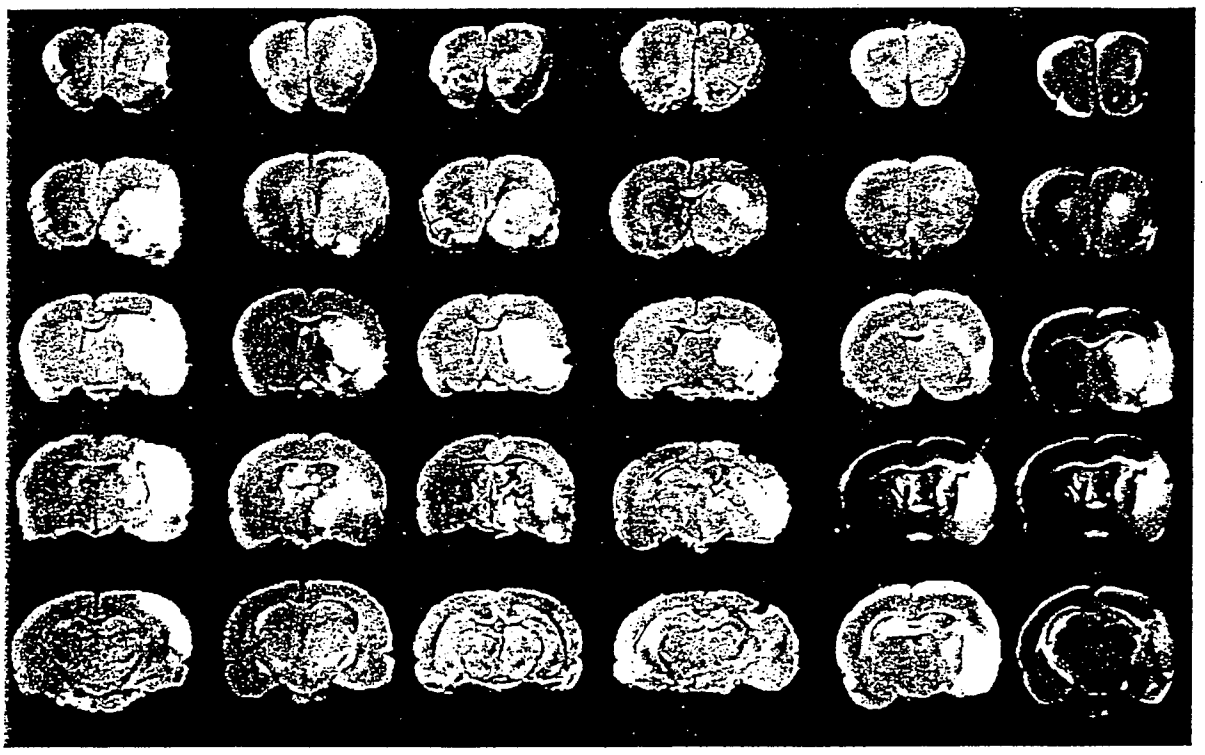

Estrogen Neuroprotective Effects Time Limited

Estradiol Exerts Neuroprotective Effects When Administered After Ischemic Insult

Shao-Hua Yang, MD; Jiong Shi, PhD; Arthur L. Day, MD; James W. Simpkins, PhD

Background and Purpose—17β-Estradiol (E2) has been reported to exert neuroprotective effects when administered before an ischemic insult. This study was designed to determine whether E2 treatment after ischemia exerts the same effects and, if so, how long this therapeutic window remains open, and whether the effects are related to changes in cerebral blood flow (CBF).

Methods—Female Sprague-Dawley rats were subjected to permanent middle cerebral artery occlusion (MCAO). In protocol 1, E2 was administered (100 μg/kg IV followed immediately by subcutaneous implantation of crystalline E2 in a silicone elastomer tube) to ovariectomized females (OVX+E2) at 0.5 (n=8), 1 (n=6), 2 (n=7), 3 (n=6), or 4 (n=9) hours after MCAO. Intact (INT; n=6) and ovariectomized females (OVX; n=12) were subjected to MCAO and received vehicle instead of E2. Two days after MCAO the animals were killed, and ischemic lesion volume was determined by 2,3,5-triphenyltetrazolium chloride staining. In protocol 2, CBF was monitored before and at 1, 24, and 48 hours in a group of animals receiving E2 or vehicle 0.5 hour after ischemia induction (INT, n=6; OVX, n=8; OVX+E2, n=6).

Results—Lesion volume was 20.9±2.2% and 21.8±1.2% in the INT and OVX groups, respectively. E2 was found to decrease lesion volume significantly when administered within 3 hours after MCAO. The lesion volumes were 6.3±0.5%, 10.3±2.1%, 11.8±1.8%, 13.5±1.6%, and 17.9±2.8% when E2 was administered at 0.5, 1, 2, 3, or 4 hours after MCAO, respectively. CBF decreased to 43.1±2.2% and 25.4±1.0% in the INT and OVX animals, respectively, at 5 minutes after MCAO. In comparison to OVX rats, CBF was not different at 1 hour after E2 administration but was increased significantly in the OVX+E2 group 1 and 2 days after E2 administration.

Conclusions—E2 exerts neuroprotective effects when administered after ischemia, with a therapeutic window in a permanent focal cerebral ischemia model of approximately 3 hours. This effect of estradiol was associated with no immediate change in blood flow but with a delayed increase in CBF. (*Stroke*. 2000;31:745-750.)

Key Words: cerebral blood flow ■ estrogens ■ ischemia ■ neuroprotection

Both retrospective and prospective epidemiological studies have demonstrated beneficial effects of estrogen replacement therapy in reducing stroke-related mortality that is associated with stroke in postmenopausal women.[1,2] Recently, several laboratory studies have also emphasized the neuroprotective effects of estrogens.[3–6] Both chronic and acute pretreatment can reduce ischemic damage in focal cerebral ischemia, indicating that estrogens may be a new therapeutic class of drugs to prevent neuronal damage associated with cerebral ischemia.

Presently, it is not known whether postischemic treatment with estrogen is beneficial. The purpose of this study was to determine (1) whether 17β-estradiol (E2) can protect against brain injury when administered after cerebral ischemia; (2) the duration of any therapeutic window offered by E2, and (3) whether any E2 neuroprotective effects are associated with changes in cerebral blood flow (CBF).

See Editorial Comment, page 749

Materials and Methods

Female Charles River Sprague-Dawley rats (225 to 250 g: Wilmington, Mass) were maintained in laboratory acclimatization for 3 days before ovariectomy. Bilateral ovariectomy was performed 2 weeks before middle cerebral artery occlusion (MCAO) under methoxyflurane inhalant anesthesia. All animal procedures were approved by the University of Florida Animal Care and Use Committee.

Middle Cerebral Artery Occlusion

Animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal temperature was monitored and maintained between 36.5°C and 37.5°C during the procedure. MCAO was achieved according to the methods described by others, with the following modifications.[7,8] With the aid of an operating microscope, the left common carotid artery, external carotid artery and internal carotid artery were exposed through a midline cervical skin incision. A 4-0 monofilament suture with its tip

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rounded by heating was introduced into the internal carotid artery via the external carotid artery lumen and advanced until resistance was encountered. The distance between the common carotid artery bifurcation and the resistive point was approximately 1.9 cm. A 6-0 silk ligature was placed around the external carotid artery and tightened around the intraluminal monofilament suture to prevent bleeding and change of the suture position. The common carotid artery and pterygopalatine artery temporary ligatures were then released, and the skin incision was closed.

Measurement of Regional CBF

A laser-Doppler flowmeter was used for CBF measurements. The scalp was incised on the midline, and bilateral 2-mm burr holes were drilled 1.5 mm posterior and 4.0 mm lateral to the bregma. The dura was left intact to prevent cerebrospinal fluid leakage. Laser-Doppler flowmeter probes held in place by a micromanipulator were stereotaxically advanced to gently touch the intact dura mater. CBF was measured before and within 1.5 hours after MCAO. The incision was stapled, and the animals were then returned to their home cages. At 1 and 2 days after MCAO, the animals were reanesthetized with ketamine (60 mg/kg IP) and xylazine (10 mg/kg IP), and stable CBF recordings were obtained bilaterally at the same sites for at least 10 minutes. The CBF values were calculated and expressed as a percentage of the baseline values. CBF values reported represent the mean±SEM for the average of the CBF recordings obtained.

Measurement of Lesion Volume

Each group of animals was decapitated 2 days after MCAO, and the brain was removed and placed in a metallic brain matrix for tissue slicing (Harvard) immediately after decapitation. Five slices were made at 3, 5, 7, 9, and 11 mm posterior to the olfactory bulb. Each slice was incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride in physiological saline at 37°C and then fixed in 10% formalin. The stained slices were photographed by a digital camera (Sony MVC-FD5) and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 3.0.1). Ischemic lesion volume was calculated as the sum of the areas of the ischemic lesion across the 5 slices divided by the total cross-sectional area of these 5 brain slices.

E2 Administration and Serum Concentration

To obtain a prompt and sustained elevation in serum E2 concentration, intravenous injection of an aqueous soluble E2 preparation combined with simultaneous implantation of a silicone elastomer pellet containing the steroid was used. To assess serum concentrations of E2 after this treatment regimen, 6 OVX animals were anesthetized with methoxyflurane inhalant, and a control blood sample was taken via the jugular vein. Then E2 (100 μg E2/kg body wt) complexed with hydroxypropyl-β-cyclodextrin (E2-HPCD, Sigma), which was dissolved in 0.9% normal saline, was administered via tail vein injection, and a 5-mm-long silicone elastomer tube (1.57 mm ID; 3.18 mm OD) containing crystalline E2 was immediately implanted subcutaneously. The animals were put back into their cages, and blood samples were then taken via the jugular vein at 5 minutes and 0.5, 1, 2, 4, 6, 12, 24, and 48 hours after steroid administration, under methoxyflurane inhalant anesthesia. Serum was separated from blood by centrifugation and stored frozen (−20°C). Serum E2 concentrations were determined with the use of duplicate serum aliquots in a radioimmunoassay (ultrasensitive estradiol kit, Diagnostic Laboratory).

Protocol 1

To determine whether E2 exerts any beneficial neuroprotective effect when administered after the ischemic insult and the duration of any therapeutic window, E2 was administered (100 μg/kg, by tail vein injection combined immediate with subcutaneous implantation of an E2-containing silicone elastomer tube) in ovariectomized female rats (OVX+E2 group) at 0.5 (n=8), 1 (n=6), 2 (n=7), 3 (n=6), or 4 (n=9) hours after MCAO. As controls, ovariectomized females (OVX group: n=12) and intact females (INT group: n=6) were treated with equivalent volumes of saline and empty pellets at 0.5, 1, 2, 3, or 4 hours after MCAO.

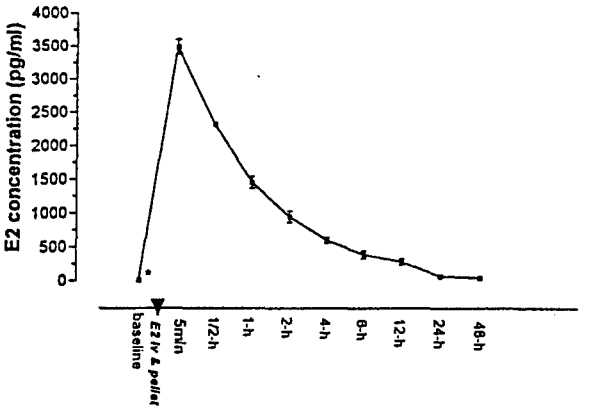

Figure 1. Effect of simultaneous intravenous injection of E2 (100 μg/kg complexed in hydroxypropyl-β-cyclodextrin) and subcutaneous implantation of an estradiol-filled silicone elastomer pellet on serum estradiol concentration in OVX rats. At the time indicated, 0.5-mL blood samples were obtained for subsequent E2 concentration analysis. n=6 rats per time point. Mean±SEM values are depicted. *$P<0.05$ vs all other sample times.

Protocol 2

To determine whether any neuroprotective effects of E2 were associated with blood flow changes, a laser-Doppler flowmeter was used to monitor CBF. After a baseline CBF reading was obtained, CBF was continuously recorded for 1.5 hours after MCAO induction. E2 was administered (100 μg/kg tail vein injection and subcutaneous implantation of an E2 pellet) 0.5 hour after MCAO induction (OVX+E2 group; n=6), and CBF was obtained for 1 hour thereafter and at 24 and 48 hours after MCAO. Intact females (INT group: n=6) and ovariectomized females (OVX group: n=8) received equivalent volumes of saline and empty pellet as controls.

Statistical Analysis

Statistical analyses were performed with SigmaStat 2.0 Software (Jandel Scientific). All data were expressed as mean±SEM. The lesion volumes in each group comparison were analyzed with 1-way ANOVA. The CBF values in each group were analyzed among groups at each sampling time with 1-way ANOVA and multiple comparisons. The difference for each comparison was considered significant at the $P<0.05$ level.

Results

Effects of E2 Administration on Serum E2 Concentration

In young cycling female rats, serum levels of E2 vary between 11±1 pg/mL at diestrus and 41±5 pg/mL at proestrus. Serum E2 concentrations increased and peaked at 3487±110 pg/mL 5 minutes after E2 administration, then decreased to 76±16 pg/mL 24 hours after administration (Figure 1). With the slow release from the E2 pellet, serum E2 concentration remained high at 45±5 pg/mL 48 hours after administration, compared with 13±4 pg/mL in OVX animals.

Therapeutic Window of E2

E2 treatment after the ischemic insult exerted neuroprotective effects (Figures 2 and 3). The ischemic lesion volume was significantly reduced in the OVX+E2 group when E2 was administered at 0.5, 1, 2, or 3 hours after the ischemic insult, with lesion volumes of 6.3±0.5%, 10.3±2.1%, 11.8±1.8%,

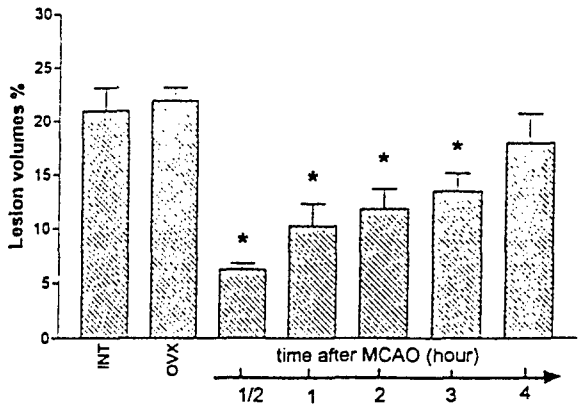

Figure 2. Effects of E2 treatment on lesion volume after MCAO in OVX and OVX+E2 rats. E2 was administered by simultaneous intravenous injection (100 μg/kg) and subcutaneous implantation of an E2 pellet at 0.5 (n=8), 1 (n=6), 2 (n=7), 3 (n=6), or 4 (n=9) hours after MCAO. Mean±SEM values are depicted. *$P<0.05$ vs OVX and INT.

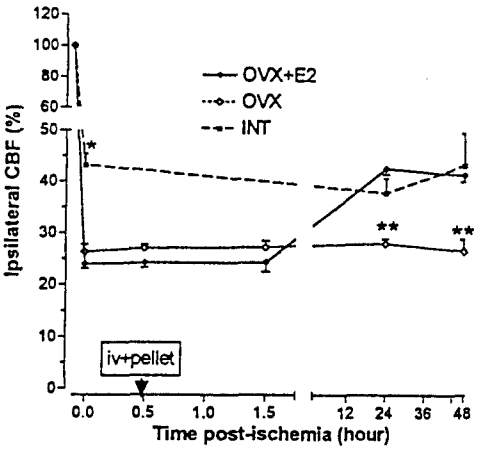

Figure 4. Effect of ovarian status and E2 replacement on ipsilateral CBF after MCAO. E2 was administrated by an intravenous injection (100 μg/kg) and subcutaneous implantation of an E2 pellet at 0.5 hour after MCAO. CBF was measured before and within 1.5 hours after MCAO (OVX, n=8; OVX+E2, n=6; and INT, n=6) and at 24 and 48 hours after MCAO. Mean±SEM values are depicted. *$P<0.05$ vs OVX and OVX+E2; **$P<0.05$ vs INT and OVX+E2.

and 13.5±1.6%, respectively ($P<0.05$), indicating a therapeutic window of up to 3 hours in permanent focal cerebral ischemia. No significant difference of lesion volume was noted between OVX and INT groups (21.8±1.2% and 20.9±2.2%, respectively).

Effect of E2 on CBF

The ipsilateral CBF was higher immediately after MCAO in the INT group compared with the OVX and OVX+E2 groups: values for INT, OVX, and OVX+E2 groups were 43.1±2.2%, 26.2±1.5%, and 23.9±0.9%, respectively ($P<0.01$). After E2 administration, ipsilateral CBF increased at 1 and 2 days after E2 administration but not at 1 hour (Figure 4). The effects of MCAO on the contralateral CBF were similar in all groups and were independent of the estrogen status of the animal.

Discussion

This study demonstrates 3 potentially important clinical effects of E2. First, E2 exerts neuroprotective effects even when administered after the onset of an ischemic insult, with a therapeutic window up to 3 hours. Second, the neuroprotective effects of E2 are not associated with an immediate blood flow augmentation effect but with a later improvement in CBF. Third, at the dose used, neuroprotective effects of E2 are flow independent and in this permanent focal cerebral ischemia model are only observed with exogenous E2.

Several studies have demonstrated that E2 is a potent neuroprotective agent that decreases focal ischemia–induced lesion size by approximately 50% with E2 chronic pretreatment.[3–6] E2 also exerts neuroprotective effects when administered immediately before occlusion.[9] The present study, for the first time, systematically defines the therapeutic window of E2 in a model of permanent focal ischemia when the drug is administered after the ischemia has been induced.

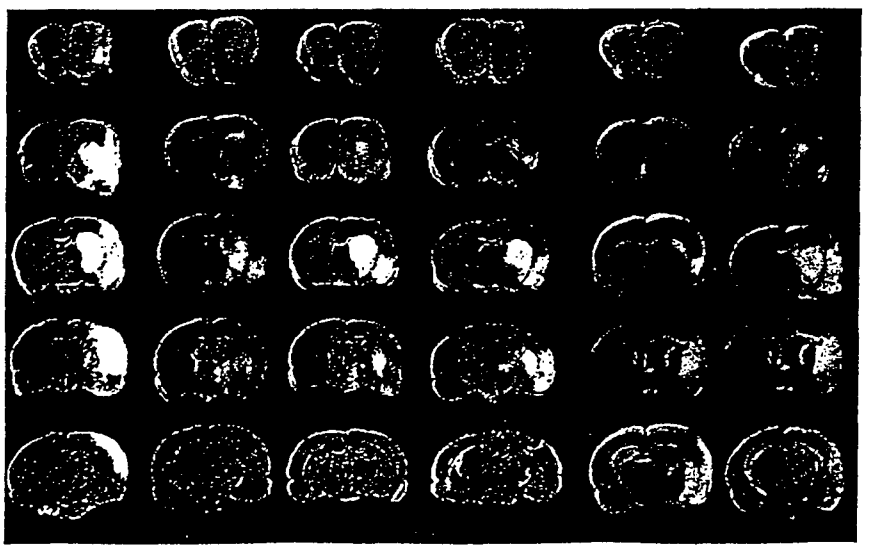

Figure 3. Photographic display of representative brain sections from OVX and OVX+E2 rats at different time points (0.5 hour, n=8; 1 hour, n=6; 2 hours, n=7; 3 hours, n=6; and 4 hours, n=9). Note the increasing infarct size with greater delays in drug administration.

The neuroprotective mechanisms of E2 are not yet elucidated, although both direct neuroprotective action on neurons and indirect effects on the cerebral vasculature are possible. Direct effects can include reduction in reactive oxygen species that accumulate during ischemia,[10] blockade of excitatory amino acid toxicity,[11,12] modulation of calcium homeostasis,[13–15] induction of neurotrophins and their receptor and intracellular signaling pathway,[16,17] induction of antiapoptotic protein,[18,19] and/or enhancement of brain glucose uptake.[20] E2 could also improve the outcome of cerebral ischemia through a protective effect on brain vascular endothelial cells,[20] resulting in the presently observed delayed improvement in CBF in E2-treated rats.

E2 has been shown to act on both of the peripheral and intracranial vascular systems.[21–24] In young cycling female rats, serum levels of E2 varied between 11±1 pg/mL on diestrus and 41±5 pg/mL on proestrus.[25] In our study, deprivation of endogenous ovarian steroids resulted in low residual CBF ipsilateral to the MCAO.[5,26] Acute administration of exogenous E2 (in which serum levels of E2 vary from 3487±110 to 45±5 pg/mL) increased ipsilateral CBF after stroke, but this effect was delayed until 1 to 2 days after occlusion. It appears that low levels of endogenous ovarian steroids resist the ipsilateral CBF effects after permanent MCAO. Acute treatment with high doses of E2 caused a delayed preserving effect on CBF, an effect that only occurred in the side ipsilateral to the MCAO.

The mechanism of any blood flow–preserving effects of E2 is still not well known, but 3 possibilities have been proposed. First, we have found that exposure of endothelial cells to E2 helps to maintain their viability during an ischemic episode.[20] Findings in this experiment suggest that the delayed effect of E2 on CBF maybe be secondary to a vascular cytoprotective action of the hormone. Alternatively, estrogen could induce vasodilation in cerebral arteries.

Second, E2 has been found to modulate serum lipid levels, reducing aggregation of platelets and the thrombotic and vasoconstrictive effects of thromboxane.[27,28] E2 withdrawal after ovariectomy increases the sensitivity of the rabbit basilar artery to serotonin.[29] Using a mouse carotid model, Sullivan et al[23] found that physiological levels of E2 replacement could significantly suppress the response of the carotid artery to injury. The endothelium produces a variety of vasoactive mediators such as prostacyclin and endothelium-derived nitric oxide, both of which have roles in regulating not only vascular tone but also smooth muscle cell proliferation.[21] Goldman et al[30] have also reported that within 10 minutes of injection of a supraphysiological dose of E2, CBF increases to most regions of the brain. In contrast, our study showed that the blood flow–preserving effects of E2 are not immediate but occur from 1 to 24 hours after E2 administration. These blood flow–preserving effects could be likely due to a slower genomic effect, since the cellular effects of E2 on gene expression occur hours to days after any insult.[31]

Finally, E2 could cause a delayed improvement in CBF through angiogenic mechanisms. Recently, Morales et al[32] found that E2 exerted angiogenic effects in peripheral vessels. While angiogenic effects of E2 may play a potential role in protecting against cerebral ischemia, we are not aware of studies demonstrating that estrogens can induce angiogenesis within 2 days of steroid replacement. However, by promoting neovascularization and collateral formation, E2 could restore cerebral perfusion in ischemic areas and hence lessen the impact of occlusion.

Both low and high circulating concentrations of E2 have been reported to exert neuroprotective effects in the temporary cerebral ischemia model in E2 pretreatment studies.[5,33] Both low and high physiological levels of E2 have exerted similar effects in a 1-day permanent cerebral ischemia study when administered before ischemia.[6] The present study showed that E2 neuroprotective effects could be induced by high-level exogenous E2 in 2-day permanent cerebral ischemia when administered after ischemia. Subsequent assessment of the dose dependence of this neuroprotection is clearly needed.

Assessments of efficacy also need to be conducted in both male and female rats. E2 has been found to exert neuroprotective effects in males, although in males the effects are dependent in part on the suppression of testosterone secretion.[34] Additionally, the neuroprotective effects of estrogens do not appear to be mediated by an estrogen receptor mechanism. 17α-Estradiol, a very weak estrogen, exerts neuroprotective effects equivalent to E2 both in vitro and in vivo.[3,35] Additionally, we have recently reported that ent-estradiol, the enantiomer of E2 that lacks estrogenic activity, is as potent as E2 in protecting cerebral tissue from MCAO.[36] These data indicate that several nonfeminizing estrogens that lack classic genomic-mediated estrogenic effects are potential clinical candidates for stroke neuroprotection.

In summary, our study demonstrates that E2 exerts neuroprotective effects when administered after an ischemic insult, with a therapeutic window of approximately 3 hours. The neuroprotective effect has a delayed CBF-preserving component and a blood flow–independent component. This study raises the possibility that estrogen compounds could be a useful therapy in preserving brain tissue, even if administered after the ischemic insult.

Acknowledgments

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Editorial Comment

Clinical studies have demonstrated that chronic estrogen use reduces stroke-related mortality.[1,2] Along similar lines, animal models of cerebral ischemia have demonstrated that the presence of estrogen in physiological amounts is protective.[3–8] Although much evidence exists that estrogen reduces stroke-related morbidity and mortality when present at the time of injury, it has been unclear whether estrogen is of therapeutic utility when administered after an ischemic event has occurred. To be of utility in the treatment, as opposed to the prevention, of stroke, estrogen must exert a protective effect when given within a reasonable time window after the ischemic event. The article by Yang et al demonstrates that postischemic administration of estrogen affords protection against ischemic damage similar to preischemic administration and that it acts within a clinically useful therapeutic window. However, this postischemic protection only occurs at supraphysiologic doses of estrogen. Another study[5] has suggested that preischemic administration of supraphysiologic doses of estrogen lacks the neuroprotective activity exhibited by physiological doses of estrogen. Differences between the mechanisms of action of physiological and pharmacological amounts of estrogen must be determined to account for the differing actions when estrogen is administered before or after the ischemic event. It remains to be seen whether the mechanism by which supraphysiologic doses of estrogen exert a protective effect is a novel one or represents a nonspecific action of estrogen at a previously described neuroprotective site.

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Appendix H

Biology of Menopause

With 58 Figures

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10

Neuroprotective Effects of Estrogens

JAMES W. SIMPKINS, PATTIE S. GREEN, KELLY E. GRIDLEY, JIONG SHI, AND EILEEN K. MONCK

Postmenopausal estrogen replacement therapy (ERT) is associated with numerous overall health benefits, including reduced risk of osteoporosis and a decrease in mortality (1). Of particular interest to neurodegenerative disease, ERT correlates with a decreased incidence of Alzheimer's disease (AD) (2,3), reducing the onset of the disease by as much as 10 years in one study (3). Further, several small clinical studies support a role for estrogen therapy in improving cognitive function in AD patients (4–6). Epidemiological studies have also demonstrated a beneficial effect of ERT in reducing the mortality and morbidity associated with myocardial infarction and stroke in postmenopausal women (7–10).

Estrogen, specifically the naturally occurring 17β-estradiol (βE2), is a potent neuroprotective agent in multiple experimental models of neurotoxicity and neurodegeneration. In this chapter, we will first describe neuroprotective effects of estrogens in both cell culture and animal models, and then discuss possible cellular mechanisms of this protection.

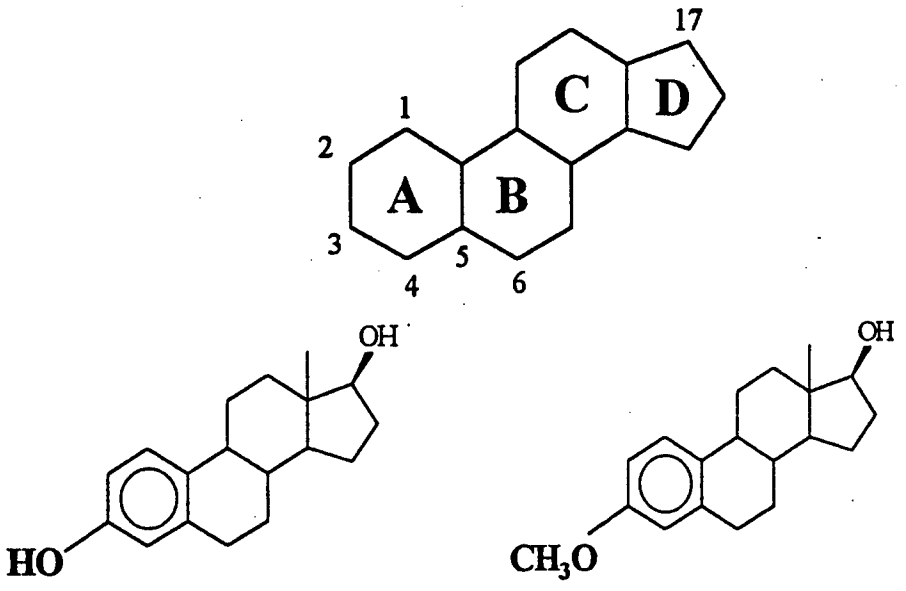
Estrogen and Neuroprotection

In Vitro Models

Our laboratory first demonstrated that physiological doses of the potent estrogen, βE2, could exert direct cytoprotective effects on a neuronal cell line using a human neuroblastoma cell line, SK-N-SH, under the conditions of serum-deprivation (11). Treatment with βE2 did not increase ^{3}H-thymidine uptake in these cells (11), verifying that this is a cytoprotective rather than a mitogenic effect of βE2. In addition, βE2 attenuates oxidative stress-induced toxicity, such as exposure of neurons to the Alzheimer plaque associated β-amyloid peptide (Aβ) (12–14) or exposure to H_2O_2 (12). βE2 treatment also attenuates cell death in rat primary hippocampal and cortical neurons due to

excitotoxic insults, such as glutamate exposure (13,15,16) and anoxia/reoxygenation (Zaulynov et al., unpublished observations).

Several lines of evidence suggest that these neuroprotective effects of βE2 are not mediated by a classical estrogen-receptor (ER) mediated mechanism. First, the structure–activity relationship for the neuroprotective effects of estrogens differs markedly from the structure–activity relationship for binding to the ER. We have demonstrated the 17α-estradiol (αE2) has similar neuroprotective efficacy and potency to the potent estrogen, βE2 (17), although αE2 binds only weakly to the ER and the αE2–ER complex binds only transiently to the estrogen responsive element (18,19). Both we (20) and Behl et al. (15) have demonstrated that estrogens with a hydroxyl group in the C3 position of the A ring (estratrienes) are neuroprotective (Fig. 10.1). If the phenolic nature of the A ring is removed by a 3-O-conjugation, the neuroprotective effects are abolished. The necessity of the phenolic A ring is demonstrated by the diphenolic estrogen mimic, diethylstilbesterol (DES). DES is neuroprotective, and retention of a single hydroxyl function on an aromatic ring is sufficient to retain neuroprotective activity (20). The di-O-methyl ether of DES does not demonstrate protective activity. Furthermore, steroids that lack a phenolic A ring, such as testosterone, progesterone, or cholesterol, do not demonstrate protective effects (20). This suggests that it is the possession of a phenolic A ring rather than binding to the ER that confers neuroprotective potential to estratrienes.



β-estradiol β-estradiol-3-o-methyl ether

FIGURE 10.1. A structural representation of the cyclopentaphenanthrene ring (top), the neuroprotective estrogen, 17β-estradiol, and the nonprotective 17β-estradiol-3-methyl ether. The bold letters indicate ring designation, and the numbers are the carbon positions in the molecule. A hydroxyl group at the 3 position and an aromatic A ring are necessary for neuroprotective activity.

Second, ER antagonists fail to block the neuroprotective effects in SK-N-SH cells (17,21). Exposure to 2 nM 17β-estradiol (βE2) during 48 hours of serum deprivation increases live cell number by an average of two- to three-fold over vehicle controls and concurrent treatment with a 100-fold excess of tamoxifen, which is a mixed ER agonist/antagonist, does not significantly alter the degree of protection conferred by βE2 (17). Tamoxifen alone has no effect on cell viability in this assay system. Further, ICI 187,780, which is a pure ER antagonist that contains a phenolic A ring, is itself protective against Aβ toxicity (21). These results indicate that antagonism of the ER does not antagonize the protection conferred by estratrienes.

Finally, estrogens have been shown to protect a neuronal cell line that lacks an estrogen receptor. The HT-22 cell, which is a mouse hippocampal cell line, does not demonstrate specific ^{3}H-βE2 binding in crude nuclear extracts or whole cell preparations (22). Further, when HT-22 cells are transfected with an ERE- reporter plasmid construct, no increase in reporter plasmid expression was seen with estrogen exposure (12). Estrogens have been shown to protect these neuronal cells from the toxic effects of Aβ, glutamate, buthionine sulfoximine, and H_2O_2 (12,15,22). Further, we have shown that this protection can be achieved with physiologically relevant doses of estrogens (2 nM) (22).

Animal Models

In animal models, we and others have demonstrated that estrogens protect against events associated with ischemia (23–27). We have reported that the treatment with various forms of estrogens at a variety of time points exerts neuroprotective effects against the damages associated with middle cerebral artery (MCA) occlusion-induced focal ischemia in female rats. Twenty-four-hour pretreatment of ovariectomized rats with βE2-CDS, the brain-targeted chemical delivery system, or βE2 reduced post-MCA occlusion mortality by more than 50% compared with vehicle treated ovariectomized rats (23). In a separate study, ovariectomy similarly decreased 24-hour postocclusion survival from 87.5% in intact female rats to 76.5% (24). Pretreatment with the presumed inactive estrogen, αE2, consistently reduced mortality from 36 to 0%. The reduction in the ischemic lesion size may underlie this remarkable reduction in mortality. Twenty-four-hour pretreatment with βE2-CDS or βE2 in ovariectomized rats caused a reduction in ischemic lesion sizes from 25.6 ± 5.7% in ovariectomized rats to 9.1 ± 4.2% and 9.8 ± 4%, respectively.

Pretreatment with aE2 similarly reduced the ischemic lesion sizes by 55–81%. Of greater importance, treatment of ovariectomized rats with bE2 after onset of either temporary or permanent occlusion continued to reduce ischemic lesion sizes by about 50% (23; Shi et al., unpublished observations). βE2 treatment also reduced ischemic lesion size from 17 to 8% in intact male rats (25).

Potential Mechanisms of Action

Antioxidant Effects

Phenolic A ring estrogens may exert their neuroprotective actions through a antioxidant mechanism because lipophilic phenols are well known to be an tioxidants (28). Mukai et al. have demonstrated that estrogens, specificall phenolic A ring estrogens, are potent antioxidants (29). 17β-estradiol als not only protects neurons from oxidative insults, such as hydrogen peroxid (12) and β-amyloid peptide (12–14) toxicity, but prevents the increase i lipid peroxidation that accompanies these toxicities (13,30). This is signifi cant as increased lipid peroxidation is associated with a variety o neurodegenerative diseases, including ischemic/anoxic insults (31–32) an Alzheimer's disease (33–34).

Synergism with Glutathione

In attempts to reconcile our previous work, in which low concentrations o estradiol (2 nM) were protective against βAP 25-35-induced toxicity (14) with others where protection was obtained using estrogens at much highe concentrations (12,13), we identified that our work was done with reduce glutathione (GSH) present in the cell culture milieu, whereas others use culture media where GSH was absent. Further experimentation showed tha when GSH was absent in the extracellular milieu, the EC_{50} for βE2 protectio against Ab toxicity was 126 ± 89 nM for SK-N-SH cells (21) and 3.2 ± 408 μM for HT-22 cells (22). The presence of GSH (3.25μM) shifted the EC_{50} for βE2 neuroprotection to 0.03 ± 0.031 nM in SK-N-SH cells (21) and 5 ± 2 nM i HT-22 cells (22). We also evaluated the effect of GSH on the protection conferred by aE2 and estratriene-3-ol, which are two classically weak estrogens that we have formerly demonstrated to be as potent as βE2 in neuroprotection assays. GSH increased the neuroprotective potency of these estrogens by approximately 400-fold (22).

The low concentrations of GSH used in the aforementioned studies did not have any effect on cell viability in the absence of estrogen; however, GSH also has neuroprotective properties and protected SK-N-SH cells from Aβ toxicity with an ED_{50} of 82.6 ± 60 μM in the absence of estrogens. A physiologically relevant dose of βE2 (2 nM) significantly potentiated the neuroprotective potency of GSH to an ED_{50} 0.04 ± 0.02 μM (21). This implies a synergistic interaction as the neuroprotective potency of both molecules are markedly shifted by the presence of the other. We performed similar experiments in rat primary cortical neurons and obtained similar results (21). Although differences exist in the magnitude of the GSH-induced shift in the neuroprotective potency of E2 in these cell types, intracellular concentrations of GSH may play a role because we have determined that primary rat cortical neurons have higher intracellular GSH concentrations (172 ± 12 μM)

than do SK-N-SH cells (15 ± 2) (21). In addition, this effect appears to be independent of the type of cytotoxic insult used because results obtained using serum deprivation and zinc toxicity (unpublished observations) were comparable to the data obtained from βAP 25-35 (21,22) and βAP 1-40–induced (22) toxicities.

The specificity of estrogens and glutathione for synergism in neuroprotection is supported by several lines of evidence. First, there are no apparent interactions noted between estrogen and the other thiols tested, lipoic acid or taurine, or any other antioxidants tested, ascorbic acid or α-tocopherol (21). Second, oxidized glutathione (GSSH) works in this model (21), and lends credence to the idea that the glutathione-estrogen interaction may involve the glutathione peroxidase/reductase system.

Estrogens are lipophilic and likely to partition to membrane constituents. They should associate their phenolic A rings with the charged hydrophilic head groups of the membrane phospholipids (Fig. 10.2). As a result, estratrienes are well placed to attenuate lipid peroxidation, and we predict the hydroxyl hydrogen of estradiol is donated to prevent the peroxidative cascade. This is further substantiated by the aforementioned structure-activity relationship with neuroprotection (15,17,20). Further, high potency of estratrienes may result from their ability to donate hydrogen ions from several positions on the A ring (35). An oxidized form of estrogen could result from this hydrogen ion donation that would be relatively stable, and glu-

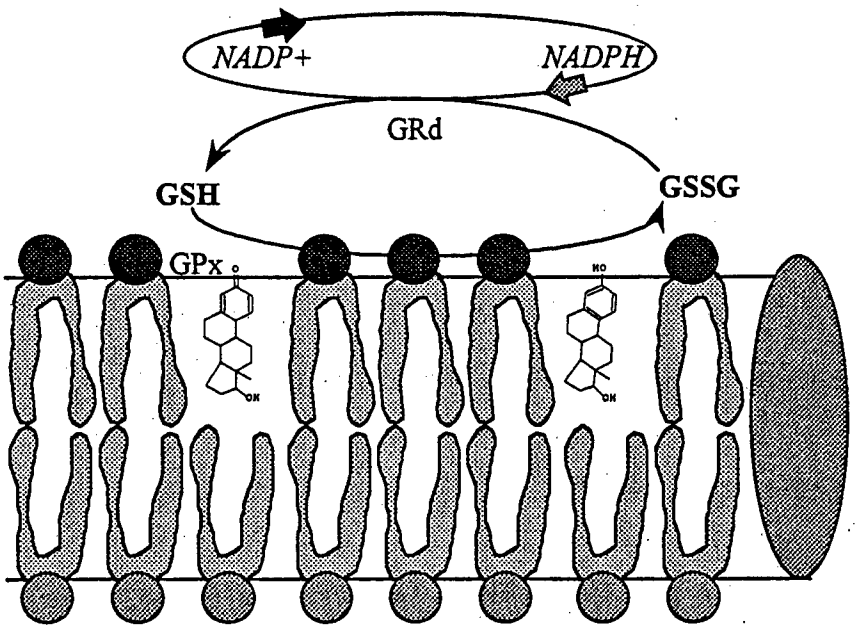


FIGURE 10.2. A schematic representation of the proposed relationship between glutathione and estradiol. The reduced and oxidized forms of estradiol and the reduced and oxidized forms of glutathione (GSH and GSSG, respectively) are depicted. Oxidative stress drives estradiol to the oxidized form, thereby protecting membrane lipids. The proposed role of glutathione is to reduce estradiol. GSSH is then reduced by glutathione reductase (GRd) using NADPH as a substrate.

tathione peroxidase would likely regenerate the reduced form of estroge by using GSH as a substrate, thus explaining the synergy between the tw molecules.

Effects on CREB

Estrogen's neuroprotective effects may also be mediated via interaction wit cyclicAMP (cAMP) response element-binding protein (CREB), a constitu tively expressed transcription factor that is activated by phosphorylatio Two independent studies have suggested that CREB may play an importa role in neuroprotection. Walton et al. (36), using a hypoxic–ischemic injur model, demonstrated a decline in phosphorylated CREB (PO_4–CREB) in munoreactivity in CA1 pyrimidal cells, which do not survive mild hypoxia ischemia injury. In contrast, the more resistant dentate granule cells an cortical cells showed an increase in PO_4–CREB immunoreactivity. We hav previously reported that hypoglycemic seizure results in a selective redu tion of CREB immunoreactivity (37). This decline in CREB immunorea tivity does not appear to be due to cell loss because the rats were sacrifice 90 minutes postseizure. It is interesting to note, however, that the regions CREB decline correlated with regions that have previously been shown have massive cell loss at 1 week after hypoglycemic seizure (38).

Estrogen replacement has been shown to reduce the seizure-induced d cline in CREB immunoreactivity (38). We have examined the effect of s rum deprivation on both CREB and PO_4–CREB levels in SK-N-SH cel (Green et al., unpublished observations). These cells showed a decline both PO_4-immunoreactivity and CRE binding capacity at 4–6 hours of s rum deprivation that was normalized by 12 hours. No loss of cell viability determined by propidium iodide exclusion was detected at these time point It is interesting that 10 nM βE2 prevented this decline in PO_4–CREB imm noreactivity and CRE binding capacity.

Zhou et al. (39) demonstrated that estrogen treatment of ovariectomize rats increases CREB phosphorylation within 15 minutes of injection in t preoptic area (POA) and bed nucleus of the stria terminalis. This rapid r sponse suggests a non–ER-mediated effect of estrogen. We have demo strated similarly that βE2 rapidly increases phosphorylation of CREB SK-N-SH cells (unpublished observations). We observed a 50% increase PO_4–CREB immunoreactivity within 1 hour using a physiological dose βE2 (2 nM) (Fig. 10.3). We did not see a change in CREB immunoreactivity this time point with βE2 doses of up to 10 μM. This rapid βE2-induced pho phorylation of CREB may be due to increased cAMP production because βE has been shown to activate adenylate cyclase activity in MCF7 breast tum cells (40).

These data collectively suggest the possibility that estrogen regulates t phosphorylation state of CREB by interactions with signal transduction pat ways other than the traditional estrogen receptor.

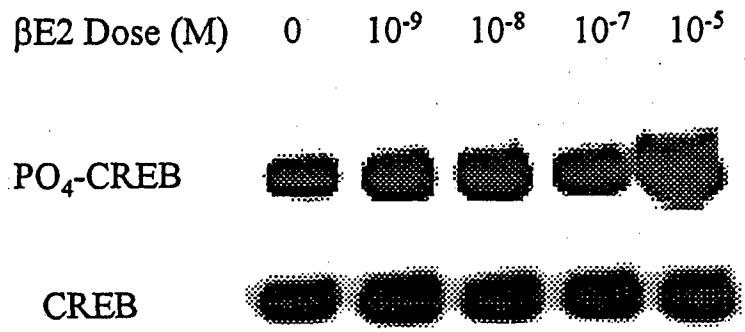


FIGURE 10.3. Western blot analysis of CREB phosphorylation by 17β-estradiol. SK-N-SH cells were treated with the indicated dose of steroid for 1 hour. Nuclear protein was extracted and analyzed by Western blot using an antibody specific to PO_4–CREB (upper blot) and an antibody for CREB. 17β-estradiol increases immunoreactivity of PO_4–CREB while having no effect on overall CREB immunoreactivity.

Conclusion

Neuronal death due to normal aging, neurodegenerative diseases, and acute conditions (e.g., ischemia) is not currently treatable. There is mounting evidence that estrogens are potent cytoprotective compounds against a variety of neurotoxic insults. These actions of estrogens do not appear to require an ER and may involve antioxidant properties of the estratriene molecule as well as interaction with signal transduction pathways. Further, these neuroprotective actions may contribute to the beneficial effects of estrogens seen in both clinical trials and epidemiological studies of AD and/or stroke. As such, estrogen use should be further evaluated for treatment of various neurodegenerative conditions.

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Definition of the anterior choroidal artery territory in rats using intraluminal occluding technique

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Appendix I

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Definition of the anterior choroidal artery territory in rats using intraluminal occluding technique

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Abstract

This manuscript delineates the territory of the anterior choroidal artery (AChA) in rats, as defined by the induction of an AChA infarction. By advancing a 0.24-mm surgical suture up the internal carotid artery (ICA) to a point 0.5–2 mm proximal to the middle cerebral artery (MCA) origin, the AChA could be occluded and a reliable AChA distribution infarction was produced in 62% (23/37) of animals. The infarct volume, as defined by TTC staining, was 55 ± 7 mm^3. Maps of the infarction, generated by measuring the entire area of overlapping coronal slices, demonstrated that the internal capsule was always damaged. Other areas that might be affected included the hippocampus, thalamus, amygdaloid complex, piriform cortex, dorsal caudatoputamen, and lateral ventricular wall. Positioning the coated suture proximal to the AChA produced a much smaller infarct involving the medial and lateral hypothalamus, preoptic region, optic chiasm, and marginal region of the internal capsule near to the lateral hypothalamus exempt from AChA territory damage. A causative relationship between AChA occlusion and a deep cerebral infarct centered on the internal capsule was further established by: (1) identifying the AChA on the non-ischemic side with colored silicone perfusion, and subsequent similar delineation on the ischemic side, and (2) delineating infarction in the silicone perfused AChA region using hematoxylin and eosin staining and the TUNEL method. The AChA usually originated from the ICA (91% of cases), 1.75 ± 0.12 mm proximal to the MCA bifurcation. Approximately 27% of the AChAs had periamygdaloid branch(es) on its initial segment.

Keywords: Anterior choroidal artery; Middle cerebral artery; Intraluminal thread; Focal cerebral ischemia

1. Introduction

Intraluminal middle cerebral artery (MCA) occlusion has gained increasing acceptance as an experimental rat model of focal cerebral ischemia [1]. Advantages of this technique over other methods include avoidance of a craniectomy and the easy ability to establish reperfusion. Disadvantages, however, include variation in infarct sizes. When 4–0 nylon surgical sutures are obtained from two separate companies, infarct size may vary because of difference in suture diameter and thread quality [2]. More severe ischemia results from a thread coated with silicone, which alters the diameter and the quality of the thread, compared to those without silicone coating [3].

Intraluminal MCA occlusion is generally performed by inserting a nylon thread into the internal carotid artery (ICA) and advancing the tip to the bifurcation at the anterior communicating artery [4]. The final thread posi-

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tion at least partially obstructs the entire course of the ipsilateral anterior cerebral A-1 segment and the ICA, raising the possibility that the thread introduced via the ICA can produce ischemia in the anterior hypothalamus and preoptic area of male Wistar rats [5]. Based on the vascular anatomy of the rat hypothalamus, this ischemia localization has been attributed to occlusion of the anterior cerebral and anterior choroidal arteries (AChA) [6,7]. We have observed similar results in male Sprague–Dawley rats [8].

Differentiation of infarction subtypes is essential to the treatment and prevention of recurrences [9]. Recent advanced imaging techniques have renewed interest on AChA infarct territory [10–12]. The AChA may supply portions of many anatomical structures [13], leading to discrepancies on the definition of its territory. The pathogenesis of AChA territory stroke is also uncertain [14]. Proposed causes of spontaneous AChA-territory infarction include hypertensive or diabetic-related intrinsic vascular disease of the artery, thromboembolism, and vasospasm after subarachnoid hemorrhage, or surgery for ICA aneurysms [15]. Several subtypes of lacunar stroke, as classified by Fisher [16], involve the AChA territory. The pathogenesis of lacunar stroke is not fully understood, and establishing an experimental model of selective AChA occlusion may provide important clinical and pathological information needed for clarification.

Surgical AChA occlusion has been attempted in dogs, but the operative trauma confused the experimental results [17]. Coyle [6] and Seremin [7] have provided principal information about the AChA territory in rats using vascular cast, cerebral section, and ink injection techniques. Their studies, however, have not led to the conclusions whether selective occlusion of the AChA will absolutely cause AChA territory infarction and which structure(s) in the territory will be affected consistently in case of occlusion of the AChA, because there exists rich anastomosis between the AChA and the posterior choroidal artery, the longitudinal hippocampal artery, and the dorsal thalamic artery, respectively [6,7]. Our recent report demonstrated that advancing an occluder up to 0.7–1.9 mm proximal to the MCA in the intracranial segment of the internal carotid artery (ICA) produced a small infarct in the AChA territory [18]. However, the successful rate of the model is less striking (33%). In the present study, we tested whether a variation in the placement of the intraluminal thread could cause AChA territory infarction without affecting the MCA territory with rather higher successful rate. We used infarct overlap maps to define the AChA territory infarction using 2-mm coronal brain slices stained by 2,3,5-triphenyltetrazolium chloride (TTC). Furthermore, We used perfusion of colored silicone followed by microsurgical dissection to define the AChA on the non-ischemic side, and to guide the identification of AChA occlusion on the ischemic side. Perfusion-fixed samples were then examined using 5 μ coronal slices stained by hematoxylin and eosin (H&E) and the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick labeling (TUNEL) method to delineate the ischemic lesion.

2. Materials and methods

The experimental protocols have been approved by the Institutional Animal Care and Use Committee of the National Cardiovascular Center Japan and the University of Florida, USA. Female Wistar rats, 10-week old and 223–265 g, were purchased from Oriental Yeast Co., Japan and Charles Rivers Laboratories, (Wilmington, MA), USA and a total of 74 rats were used. Five rats each were kept in a cage and given laboratory chow and tap water ad libitum under a fixed light–dark cycle for 2–3 weeks prior to experimentation to allow acclimation to the laboratory condition.

2.1. Experimental design and surgical procedures

Rats were anesthetized with 2% isoflurane together with a mixture of 30% oxygen and 70% nitrous oxide. Body temperature during the surgical procedure was monitored with a rectal probe and controlled by a heating pad set at 37.0°C. The time necessary for introduction of cerebral ischemia was 25 min or less. Post-operatively, each rat was kept in a separate cage and allowed to recover from anesthesia at room temperature (21±1°C).

Five of 37 rats subjected to attempted AChA occlusion (experiment 1), together with five rats each from the sham-operated and positive control groups, were randomly selected for determination of arterial physiological parameters and blood pressure. The femoral artery in these rats was cannulated for continuous recording of arterial blood pressure until 10 min after the onset of cerebral ischemia. Blood sampling was performed twice, at rest before surgery and at 10 min after the onset of ischemia, for pH, $PaCO_2$, and PaO_2 measurements.

Body temperature was measured by a probe temporarily inserted 2 cm into the rectum. Measurements were recorded before, 0, 1 and 2 h following surgery in hand-handled, non-anesthetic rats. The resting level temperature before surgery was measured at 3:00±0:30 p.m. in all animals. To evaluate sensorimotor function, the postural reflex test was carried out at the same time as body temperature determination as described previously [18].

2.1.1. Experiment 1

This experiment was designed to induce an AChA territory infarct without affecting the MCA territory, using a variation of the intraluminal technique [8,18]. The protocol was designed to advance an occluder to the position about 1 mm proximal to the MCA bifurcation (Fig. 1). Briefly, under an operating microscope, a 3–0 nylon surgical suture (diameter 0.24 mm) coated with 0.1%

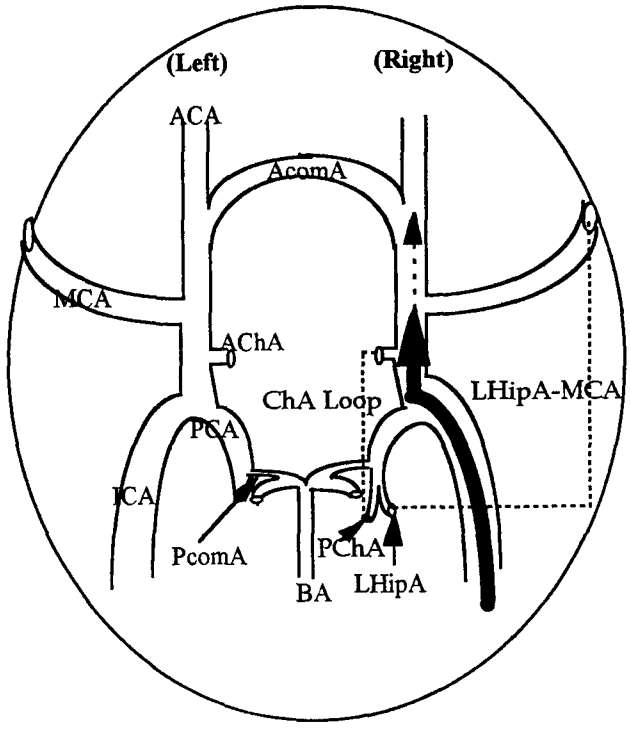

Fig. 1. A schematic illustration of intracranial arteries showing the position of the occluder for intraluminal occlusion of the anterior choroidal artery (AChA). The surgical suture was advanced up to about 1 mm proximal to the middle cerebral artery (MCA). ACA, anterior cerebral artery; AcomA, anterior communicating artery (It is also used to represent anterior common artery in rats); BA, basilar artery; ICA, internal carotid artery; PCA, posterior cerebral artery; PcomA, posterior communicating artery. The large arrow with the thick solid line represents the position of an occluder for occlusion of the ICA segment proximal to the MCA bifurcation, while the arrow with the dotted line indicates the usual position of the occluder for MCA occlusion by the conventional intraluminal technique. The choroidal artery (ChA) loop with the dotted line represents an arterial loop connecting the AChA to the posterior lateral choroidal artery. The LHipA-MCA with the dotted line indicates the anastomosis between the longitudinal hippocampal artery (LHipA) and the MCA.

poly-L-lysine, was introduced through the right common carotid artery (CCA) and advanced intracranially in the ICA until resistance was encountered. When the advanced distance from the CCA bifurcation was shorter than 18 mm, the occluder was retreated by 1–1.5 mm. If the advanced distance from the CCA bifurcation was longer than 18 mm without feeling resistance, the occluder was retreated to a position 15.5, 16 or 16.5 mm from the CCA bifurcation. Measured another way, the distance varies between the CCA bifurcation and the base of the front teeth ($\leq$32, 32–34 or $\geq$34 mm, respectively), since there was subtle variation in the location of the CCA bifurcation. A total of 37 animals were subjected to attempted AChA infarction. Another 16 were used as positive controls ($n=11$) by advancing the coated suture more than 18 mm from the CCA bifurcation or until resistance was felt. Sham-operated controls ($n=5$) were prepared by advancing the suture 13 mm from the CCA bifurcation. In all groups, the occluder was fixated intraluminally, and rats were allowed to live for 72 h.

Since the induction of isolated deep cerebral ischemia was done blindly, at least four results could be predicted: no infarction or infarction in the hypothalamic artery (HTA), AChA, and MCA territories, as pointed out in our previous study [18]. If abnormal postural reflex did not appear within 1–2 h after surgery (supposing that the manifestation indicates AChA territory ischemia), the rat was subjected to a second operation, advancing the occluder 0.5–1 mm further.

2.1.2. Experiment 2

Eleven rats were used to define the AChA and to establish a causative relationship between AChA occlusion and a small deep infarct involving the internal capsule. These rats were perfused with colored silicone after perfusion of 100 ml of saline and 100 ml of 10% of phosphate-buffered formalin, respectively, 72 h after the onset of ischemia. The circle of Willis was then exposed using microsurgical dissection. Photography was used to validate that the contralateral AChA was filled well with colored silicone, and that the vessel on the ischemic side was obstructed by the occluder. Brain samples were harvested and subjected to H&E staining and the TUNEL method to identify any AChA territory infarction.

2.2. Assessment of the infarct size and volume

At 72 h after ischemia onset, each animal was re-anesthetized with pentobarbital (50 mg/kg body weight) and sacrificed by decapitation. In Experiment 1, the occluder tip position relative to the MCA bifurcation was verified by naked eye visualization before each brain was removed from the cranial base. Photographs were taken in some cases to verify the occluder tip position.

Each brain was then coronally sectioned into seven 2-mm-thick slices, starting from the frontal pole, and stained by TTC. Areas not stained red with TTC in the ipsilateral cerebral structures were considered infarcted. Rats in Experiment 1 were divided into sub-groups according to whether the tip of the occluder obstructed the MCA, and whether the infarct was limited to the subcortex involving the internal capsule. We defined a lesion involving the caudatoputamen and adjacent neocortex as an infarct in the MCA territory, even though the lesion might extend to the structures in the non-MCA territory such as the thalamus, dorsal hippocampus, and those supplied by the AChA. We also defined a lesion encompassing the hypothalamus and internal capsule without affecting the MCA territory as AChA territory infarct.

Infarct size of each slice was determined using computer programs as described previously [19]. The total infarct volume was calculated by numerical integration of the infarct volume from all brain slices (Σ(the slice thickness$\times$(the infarcted area on the rostral surface+the

infarcted area on the caudal surface)/2)). Similarly, the calculation of the infarct volume in the non-neocortical structures was determined by subtraction of the value of the neocortex from that of the total area. For convenience of analysis, the cerebral cortex above the rhinal fissure was considered as neocortex, while the piriform cortex was defined as non-neocortical structure. Overlap maps of the infarct area were constructed to find common infarct areas and regions at risk. Common infarct area was identified by demarcating the infarct area spatially overlapped in all rats, while the risk infarct area was outlined by demarcating the area encompassing all infarct areas on the overlap maps in all rats. Infarct frequency in respective cerebral regions was calculated by counting each cerebral region with ischemic damage on the overlap maps.

2.3. H&E staining and in situ detection of TUNEL-positive cells

The formalin perfusion-fixed brain samples, cut into seven 2-mm-thick coronal sections, were paraffin-embedded, then cut further into three to six slices of 5 μm-thick from the caudal surface of each section. Adjacent sections were used to evaluate histologic changes with H&E staining and TUNEL method.

The presence of TUNEL-positive cells was assessed in situ by direct immunoperoxidase detection of digoxigenin-labeled 3′-OH DNA strand breaks using the TUNEL method with the Apop Tag In Situ Apoptosis Detection Kit-Peroxidase (Oncor Technical Assistance Inc. MD 20877 USA). Sections confirmed by color silicone perfusion with an infarct limited to the AChA territory ($n=5$) were stained with H&E to clarify the extent of ischemic injury. Lesions were further defined in closely adjacent slices by the TUNEL method [18]. Anatomically matching areas localized contralateral to areas within the occluded vascular territory were used as internal controls for each brain slice.

2.4. Statistical analysis

The Mann–Whitney and Chi-square tests were used to compare the infarct area and volume between experimental groups. The statistical software used was Statview-J 4.1 (Abacus Concepts, Inc., Berkeley, CA). A P-value <0.05 was considered significant.

3. Results

3.1. Experiment 1

A total of 23 of 37 rats had an AChA infarct. The fate of the other 14 rats included a MCA infarct (4), an incomplete MCA infarct involving the caudatoputamen but with little effects on the adjacent neocortex (5), a lacunar-like infarct limited to the hypothalamus (3), and death before histological assessment (2). All 11 positive controls developed MCA infarcts but four of them died before evaluation of histological changes. All negative controls showed normal TTC brain staining.

Body temperature was 37.0–38.0°C before surgery. During surgery, it decreased to 32.5–35.5°C due to anesthesia. Body temperature spontaneously increased to 37.6–39.3°C 1–2 h after induction of cerebral ischemia. A total of 32 rats had abnormal postural reflex after 2 h of cerebral ischemia (eight of 37 rats received the second operation).

Slight increases in blood pressure and subtle changes in pH and $PaCO_2$ were noted after CCA occlusion and insertion of surgical suture (Table 1). The tip of the suture was advanced to a position 0.5–2.0 mm proximal to the MCA bifurcation in all rats with an infarct in the HTA or AChA territories, and about 0.5–1 mm across the MCA

Table 1
Arterial physiological parameters and blood pressure (BP) before and after the onset of focal cerebral ischemia[a]

	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	pH	BP (mmHg)	
				Systolic	Diastolic
Pre-occlusion					
Control	40±4	198±16	7.39±0.02	122±3	88±3
AChAI	36±1	214±7	7.41±0.01	122±3	85±5
MCAI	40±2	193±15	7.40±0.02	129±5	81±3
10 min after occlusion					
Control	42±3	189±18	7.36±0.03	140±4	92±6
AChAI	39±1	211±7	7.40±0.02	140±7	97±5
MCAI	45±3	195±15	7.36±0.02	145±6	92±6

[a] Values are expressed as mean±S.E.M. based on four to five rats in each group. A 3–0 nylon surgical sutre coated with 0.1% poly-L-lysine was used for induction of cerebral ischemia by intraluminal occlusion. In the Control group, the occluder was advanced to 13 mm from the carotid artery bifurcation (CAB) via the internal carotid artery (ICA). In the AChAI group, the occluder was similarly advanced until resistance was felt, and then retreated by 1.5 or 3 mm, if the advanced distance from the CAB was less than 18 mm, or equal to or more than 18 mm, respectively (see text for details). In the MCAI group, the occluder was advanced to the cranium via the ICA until resistance was felt.

bifurcation in four rats with an MCA territory infarct. Incomplete MCA blockage by the occluder tip (five rats) produced an infarct involving the caudatoputamen but had little effect on the adjacent neocortex. Withdrawing the occluder tip to a position more than 3 mm proximal to the MCA bifurcation caused no infarct (sham controls).

Fig. 2 shows the infarct area (Fig. 2a) and volume (Fig. 2b) on 2-mm-thick coronal slices in the AChA and MCA infarct groups. Since no significant differences in infarct area or volume between those animals in this group with inadvertent MCA infarct ($n=4$) and MCA infarction in positive controls ($n=7$), the data from these two groups were pooled as a single group. The total infarct area on each slice and the infarct area in non-neocortical structures on all slices in the MCA infarct group were significantly larger than those in the AChA infarct group. The AChA infarct group volume was 55 ± 7 mm^2, which constituted about 15% of the total infarct volume and 31% of the non-neocortical infarct volume, respectively, compared to that produced by conventional MCA intraluminal occlusion.

Positioning the coated suture proximal to the AChA produced a much smaller infarct involving the medial and lateral hypothalamus, preoptic region, optic chiasm, and marginal region of the internal capsule near to the lateral hypothalamus exempt from AChA territory damage (data not shown).

Intraluminal MCA occlusion produced infarction not only in the ipsilateral caudatoputamen and adjacent neocortex but also in the ipsilateral hippocampus, hypothalamus, thalamus, amygdaloid complex, piriform cortex, dorsal caudatoputamen, and lateral ventricular wall and internal capsule, even though the latter structures are generally not supplied by the MCA (Fig. 3, the lower two lines). Withdrawal of the suture to a position 0.5–2 mm proximal to the MCA bifurcation produced an infarct only encompassing non-neocortical structures, namely an AChA infarct (Fig. 3, the upper two lines). The lesion was limited to the two slices 8 and 10 mm from the frontal pole in 17 of 23 rat brains. The infarction might involve the amygdaloid complex, internal capsule, or part of the caudal caudoputamen located to the margin of the MCA territory. The AChA territory lesion was almost always associated with hypothalamic infarction, although generally limited to the lateral hypothalamic area. In the remaining six rats, lesions expanded to slices 2–4 mm rostral and 2 mm caudal to the 8 and 10 mm slices. Additional structures involved in these infarcts included the rostral caudoputamen, hippocampus, and part of the thalamus.

Overlap maps for infarct areas were constructed on coronal slices 8 and 10, because all brain samples with an infarct in the AChA territory showed lesions on these two surfaces regardless of staining methods (TTC or H&E). Since MCA infarcts were only evaluated by TTC staining, AChA infarct images stained by H&E or the TUNEL method were not used for making overlap maps. The

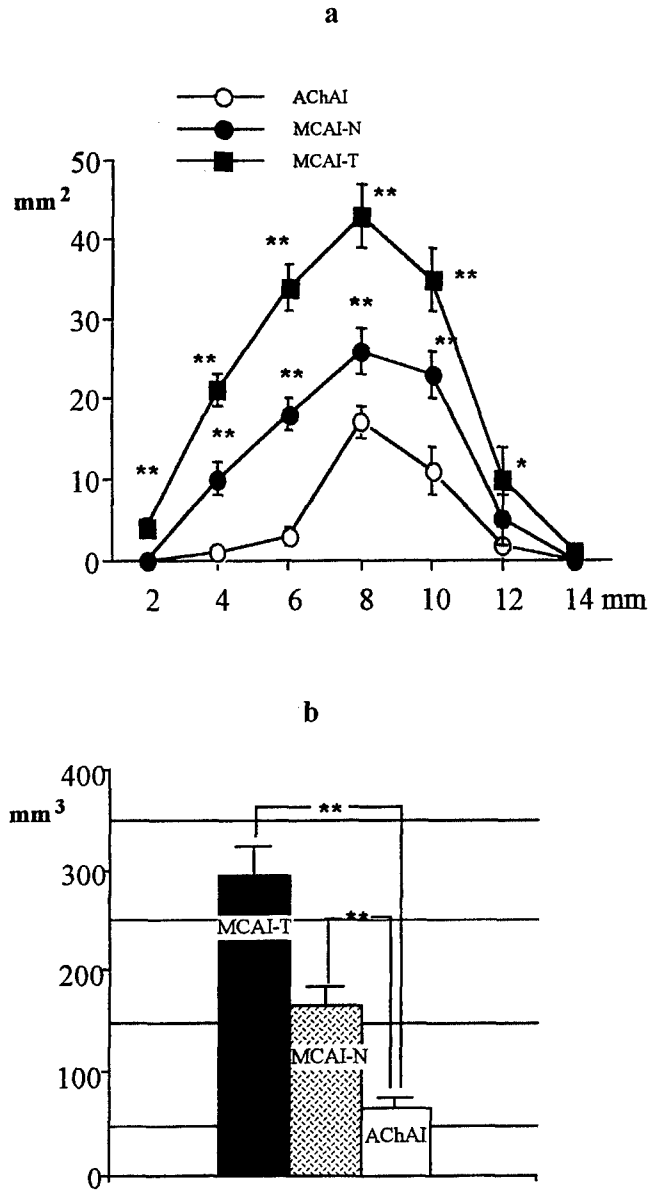

Fig. 2. The infarct area (a) on 2-mm-thick coronal slices, starting from the frontal pole, and the infarct volume (b) after 72 h of focal cerebral ischemia induced by insertion of a 3–0 nylon surgical suture coated with 0.1% of poly-L-lysine via the internal carotid artery (ICA). Anterior choroidal artery infarct (AChAI, see text for details) was successfully produced in 23 of 37 rats. Middle cerebral artery infarct (MCAI) was erroneously produced in four rats. The infarct data in these rats were pooled together with the positive control group ($n=7$), for which the MCA was occluded by advancing the occluder across the MCA. T-MCAI represents the total infarct area or total infarct volume including both neocortex and non-neocortical structures. N-MCAI indicates the infarct area or infarct volume of the non-neocortical structure. Bars represent S.E.M. $^*P<0.05$, $^{**}P<0.01$ vs. the AChAI group.

common (100%) infarct area was located only on the 8 mm slice (about 6 mm^2), while the risk infarct area on overlap maps made from the 8 and 10 mm slices measured about 42–48 mm^2. Structures constantly affected by intraluminal AChA occlusion were the internal capsule and

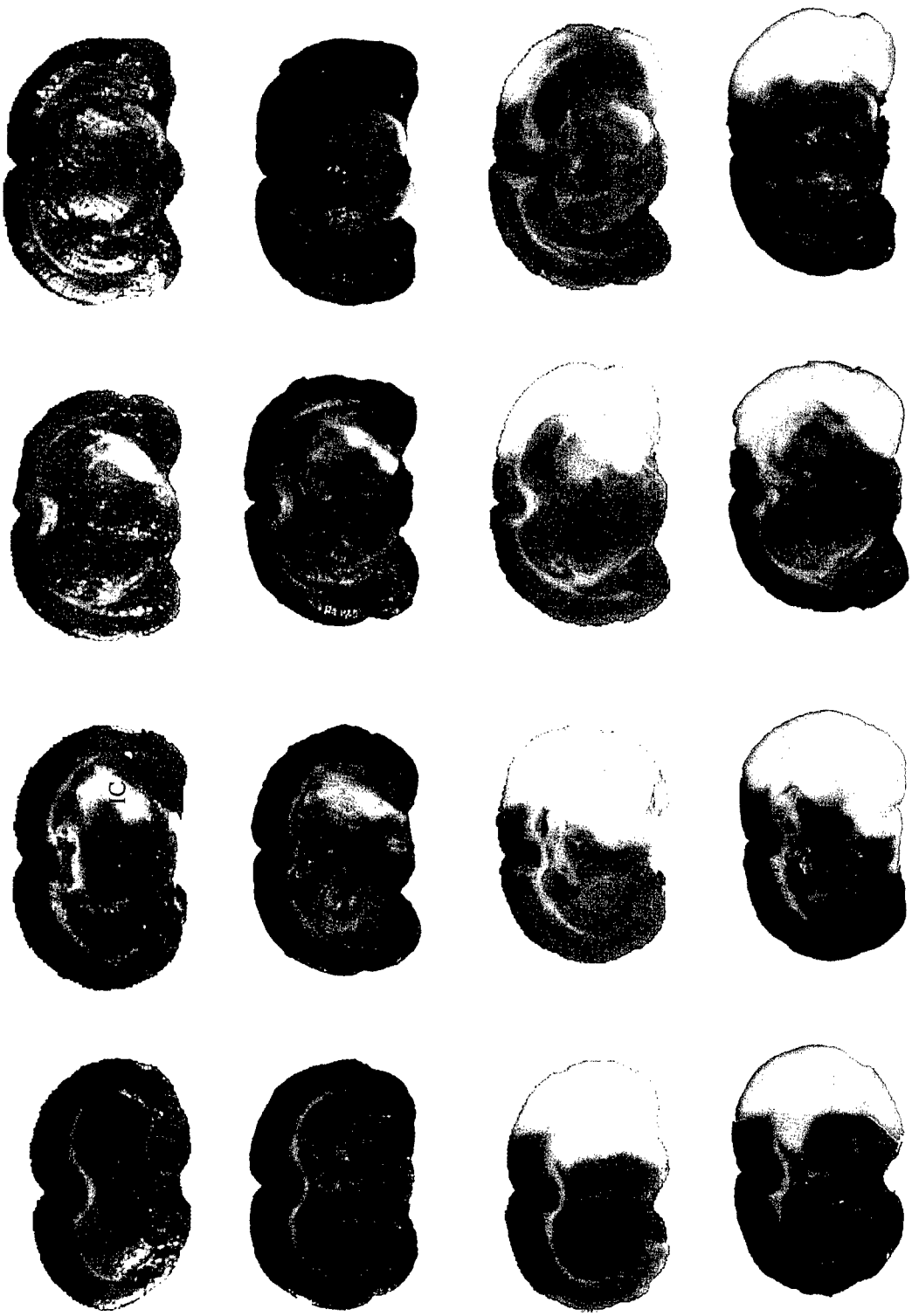

Fig. 3. Four representative rat brains with four slices (2 mm-thick) each between 6 and 12 mm from the frontal pole were stained by 2,3,5-triphenyltetrazolium chloride immersion method. The areas without staining (white) represent infarction on right side of the slices. The upper two lines show a small deep infarct produced by intraluminal occlusion of the anterior choroidal artery (AChA). The internal capsule (IC) was always affected. Intraluminal occusion of the middle cerebral artery (MCA) caused a huge infarction (shown in the lower two lines) encompassing the ipsilateral dorsal hippocampus (Hip), thalamus (Th), hypothalamus (HT) and the AChA territory which are outside the area supplied by the MCA. CPU: caudatoputamen.

endopeduncular nucleus. Part of the caudal caudatoputamen and the lateral hypothalamus were affected in 83% of rats having an infarct in the AChA territory, while the frequency of involvement in the entire caudal caudatoputamen was 13% (Fig. 4a). In contrast, all aforementioned structures and the cortex were infarcted on the 8 mm slice in the MCA infarct group (Fig. 4b).

On the 10 mm slice, the risk area following AChA infarction included part of the thalamus (70%), dorsal hippocampus (17%), amygdala and piriform cortex (17%)

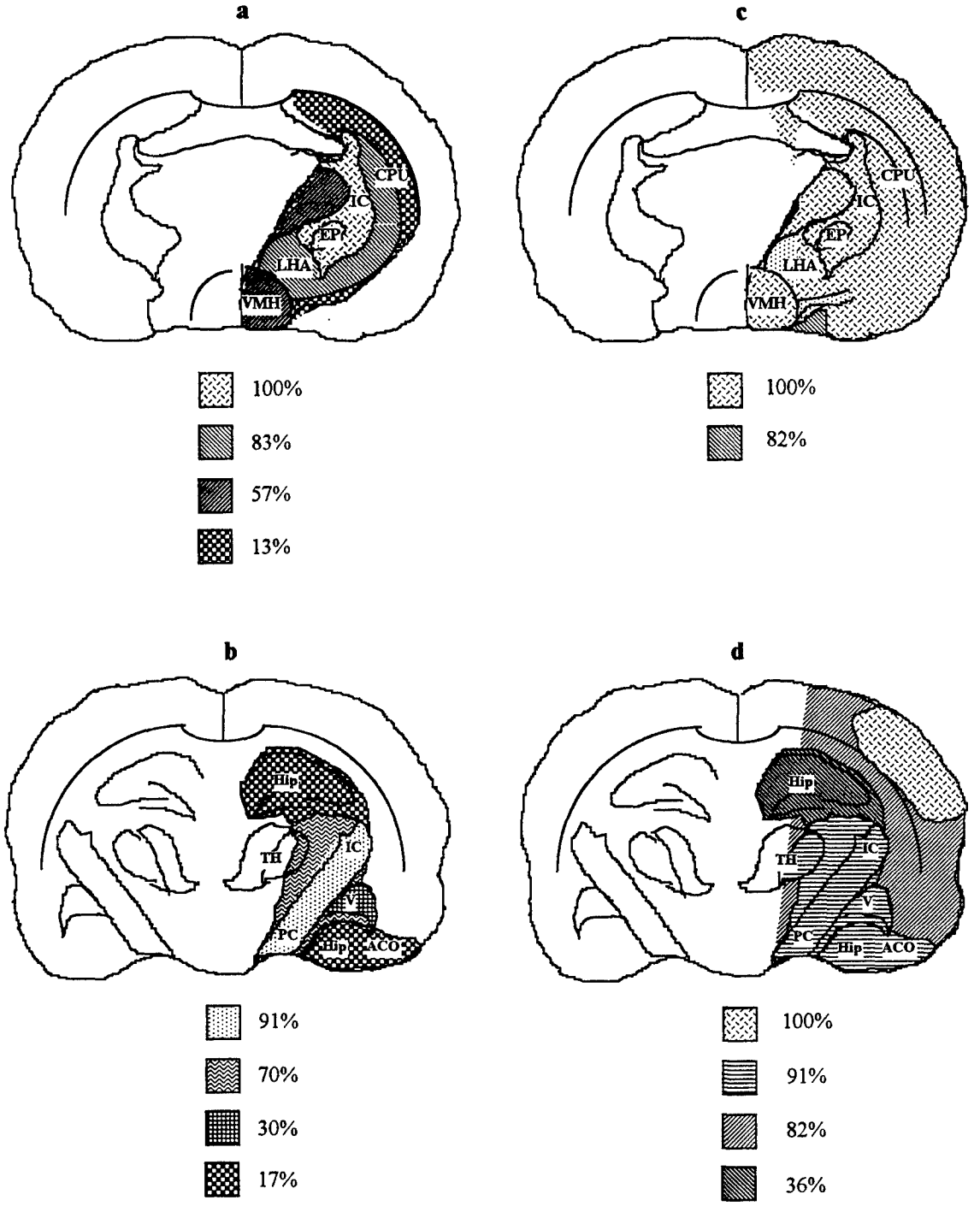

Fig. 4. Infarct area overlap maps of the coronal slices were made from 23 brains with anterior choroidal artery (AChA) infarct (a, b) and from 11 brains with middle cerebral (MCA) infarct (c, d). The slices are 8 (a, c) and 10 mm (b, d) from the frontal pole, respectively. The common infarct area in AChA infarct was confined to the slice shown in (a) and involved the internal capsule and endopeduncular nucleus according to Pellegrino et al. [37]. Infarct frequency in the marginal regions such as cortical amygdaloid nucleus was very low in the AChA infarct group, while intraluminal occlusion both the AChA and MCA significantly increased the infarct frequency. CPU, caudoputamen, IC, internal capsule, EP, endopeduncular nucleus, LHA, lateral hypothalamic area, VMH, ventromedial nucleus of the hypothalamus, Hip, hippocampus, TH, thalamus, PC, cerebral peduncle, V, lateral ventricle, ACO, cortical amygdaloid nucleus.

(Fig. 4c). Intraluminal MCA occusion increased infarct frequency to 80–90% for the thalamus, 91% for the amygdaloid and piriform cortex, and 36% for the hippocampus (Fig. 4d).

3.2. Experiment 2

Perfusion with colored silicone on the non-ischemic side demonstrated the AChA from its ICA origin to the point where the vessel entered parenchyma (Fig. 5a and b). The distance between the AChA and MCA origins from the ICA was 1.74±0.12 mm, with a range from 0.85 to 2.64 mm. Several variations were identified: (1) the AChA might stem from the posterior cerebral artery (1/11); (2) branch(es) to the periamygdaloid cortex might originate from the ICA (8/11), and (3) and/or the AChA had two branches (3/11), one of which passed to the periamygdaloid cortex, the other entered the brain parenchyma. Obstruction of the AChA on the ischemic side was defined by: (1) a distance between the occluder tip and the MCA between 0.5 and 2 mm; (2) the AChA was not filled with colored silicone; and (3) blood residue remained in the occluded AChA (not always evident). AChA occlusion specimens showed ischemic lesions in the internal capsule both on H&E staining and TUNEL method (Fig. 6a and b). Withdrawing the occluder proximal to the AChA caused a much smaller infarct limited to the hypothalamus and sparing the AChA territory (Fig. 6c and d). Damage in marginal regions rarely supplied by the AChA such as the hippocampus could be readily identified by H&E staining and TUNEL method (Fig. 6e and f)

4. Discussion

Ischemic infarction accounts for about 75% of all strokes [20], and those involving the AChA territory comprise about 3% of all hospitalized infarct patients [21]. The use of physiologically regulated and reproducible ischemic brain injury animal models is crucial in the understanding of both the mechanisms governing occurrence and potential therapeutic strategies [22]. We herein report that a suture advanced to a position 0.5–2 mm proximal to the MCA bifurcation obstructs the initial segment of the AChA and produces a small deep infarction in the AChA territory of rats. The brain regions affected by this maneuver were limited to non-neocortical structures. Confirmed by mapping of the infarct area, the internal capsule was constantly affected, suggesting that the ischemic core in AChA occlusion involve the structure.

The role of the AChA in the variation of infarct size produced by intraluminal MCA occlusion: The AChA is the largest branch of the intracranial ICA between the MCA and posterior cerebral artery (PCA), and originates from the ICA at a position 1.75±0.12 mm proximal to the MCA bifurcation as demonstrated in the present study. This gap makes it possible to advance an occluder up to a position between the AChA and the MCA origins. If the occluder diameter is large enough to effectively close the entire intracranial ICA lumen at that position, then flow into adjacent ICA branches could cease, while MCA flow could be maintained through retrograde anterior cerebral artery collaterals. The 3–0 surgical suture, coated with poly-L-lysine, was ideal in achieving this goal. The coating process does not increase the diameter of the suture, but appears to increase adhesive forces around the suture, leading to effective occlusion of the target site by adhesion of the occluder to the surrounding vascular endothelium [23].

Furthermore, a mild abnormality of the postural reflex (grade ≤2) with or without increase of body temperature during the first 30–60 min following surgery was highly predictive of an AChA infarct [18]. Although the passage of the suture is done "blindly", the present protocol produced AChA territory infarction in 62% of rats (the AChA originates from the PCA in 9% of cases herein). The infarct caused by the AChA occlusion constituted 15% of the total infarct volume induced by conventional intraluminal MCA occlusion.

Our model of AChA occlusion eliminates some of the shortcomings of intraluminal occlusion of the MCA, in which other branches of the ICA are involved in addition to the MCA. By advancing an occluder proximal to the AChA, we excluded the possibility that the medial and lateral hypothalamus, preoptic region, optic chiasm, and marginal region of the internal capsule near to the lateral hypothalamus area are supplied by the AChA. Using color silicone perfusion followed by microsurgery technique, the medial, lateral and abdominal aspects of the ICA segment between the MCA and the PCA were well shown, and in general, the AChA, branch to optic nerve, branch to periamygdaloid cortex, and HTA originate from the segment. Since the branch to periamygdaloid cortex originates from the AChA in about 30% frequency, its domain could be defined to the AChA territory. The territory of the branch to optic nerve is readily excluded according to its location and definition. Although we can not role out the other branches originating from the dorsal segment of the ICA between the HTA and the MCA, the results of the present study are consistent with the works of Coyles [6] and Seremin [7], as well as the results conducted in dog [17], indicating that our model provides, at least, a mimic of selective occlusion of the AChA alone.

4.1. AChA territory

In humans, as established by autopsy injection techniques in postmortem specimens and modern imaging techniques, the AChA gives off branches to the medial segment of the globus pallidus, the piriform cortex, amygdala, anterior hippocampus and dentate gyrus. In addition, the AChA supplies the middle third of the

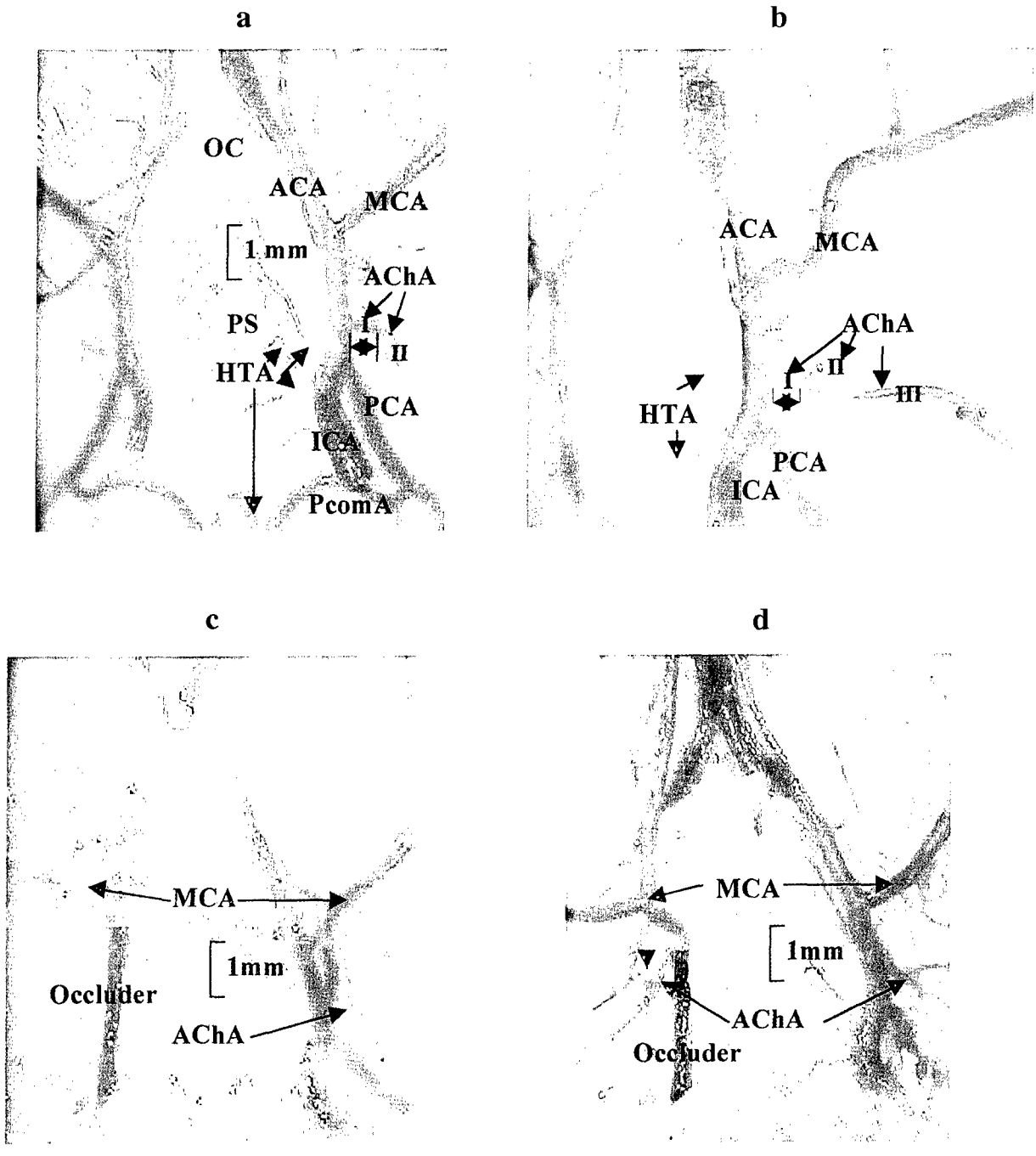

Fig. 5. The arterial circle and its branches at the brain base and occlusion of the anterior choroidal artery (AChA) in rats. To define the AChA and to establish causative relationship between occlusion of the AChA and a small deep, rats were subjected to a surgery advancing an occluder via the internal carotid artery (ICA) up to about 1 mm proximal to the middle cerebral artery. A period of 72 h later, the rats were perfused with colored silicone following perfusion of 100 ml of saline and 100 ml of 10% of phosphate-buffered formalin, respectively. The circle of Willis was exposed by microsurgical dissection. The normal arterial circle with its branches at the brain base was shown on panel (a). Panel (b) shows that the AChA is divided into three parts, I, II and III from the same sample of panel (a). The common trunk is named as segment 'I', which can be seen from a ventral view because the AChA courses laterally on the surface of the brain before entering the brain parenchyma. The superficial branches are named as the segment 'II'. The segment of the AChA entering the parenchyma is named 'III'. On the panel (b), the overlying brain tissue of segment 'III' was dissected. Advancing an occluder about 1 mm proximal to the MCA led to obstruct the AChA (c, d), which originated from the ICA 1.74±0.12 mm (n=11) proximal to the MCA. ACA, anterior cerebral artery; HTA, hypothalamic artery; OC, optic chiasm; PCA, posterior cerebral artery; PcomA, posterior communicating artery; PS, pituitary stalk.

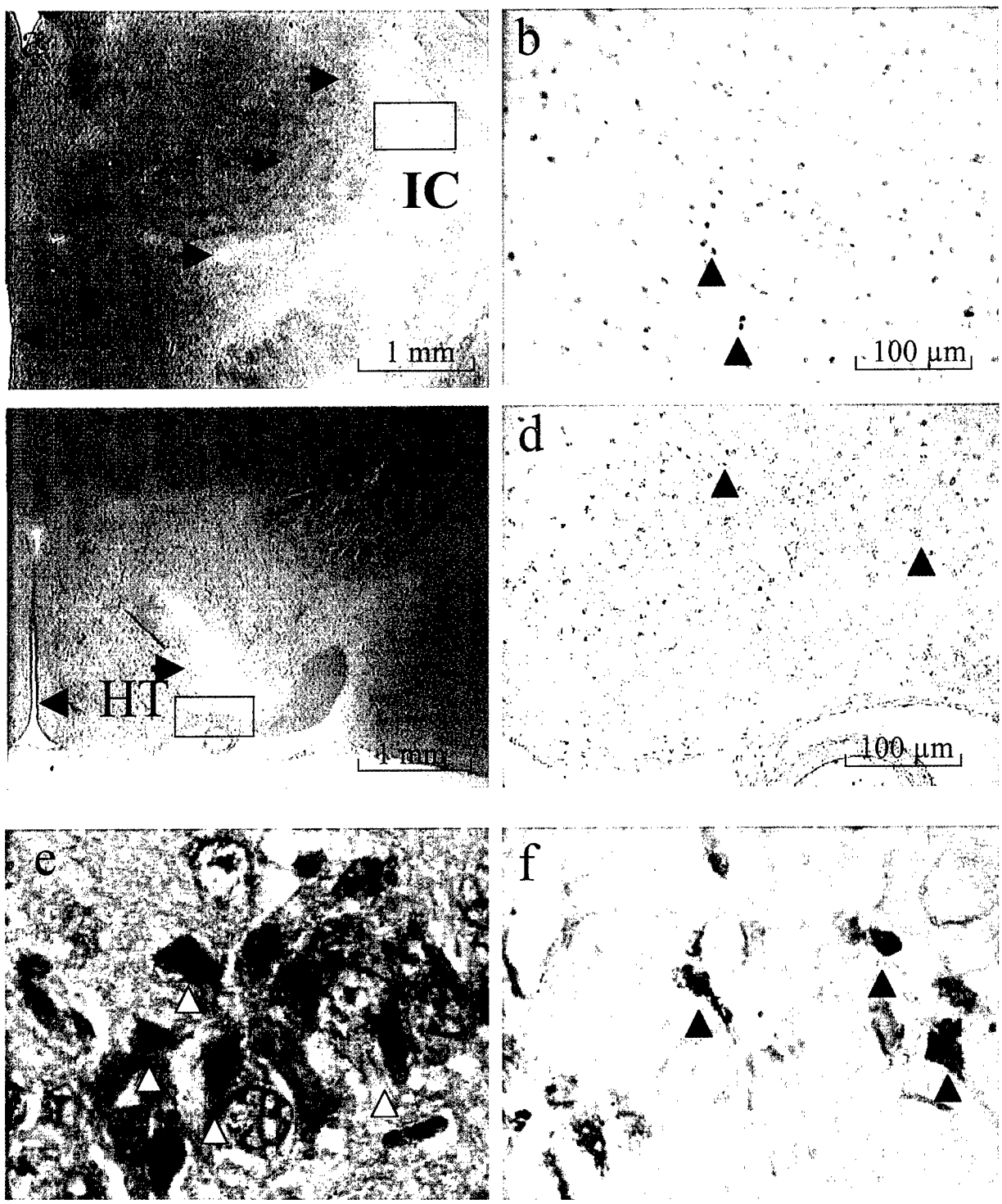

Fig. 6. Representative histologic findings showing small infarcts in anterior choroidal artery (AChA) territory. Ischemic damage was visualized by hematoxylin and eosin (a, c, e) staining and the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick labeling (TUNEL) method (b, d, f). Intraluminal occlusion of the AChA caused a small deep infarct centered on the internal capsule (a). Advancing the occluder proximal to the AChA produced a much smaller infarct limited to the hypothalamus exempt from AChA territory (c). The square notes the regions where staining with the TUNEL method on the closely adjacent slices showed TUNEL-positive cells (b, d). Large arrow heads indicates the wall of the third ventricle (a, c). Large arrows indicates infarction in (a, c). The damaged cells in the hippocampal CA1 region are shown as high magnification (630×, Panel: e, f). Small white triangles indicate the damaged cells stained by hematoxylin and eosin (e). Small black triangles indicate TUNEL-positive cells (b, d, f).

cerebral peduncle, subthalamus, part of the thalamus, and part of the internal capsule before it terminates in the choroid plexus [11,15]. AChA occlusion in dogs produces gross infarction in a similar pattern [17]. To our knowledge, an isolated AChA occlusion model in rats has not been reported.

The arterial patterns in the rat brain are remarkably similar to those in humans [6,24,25], and the similar distribution of infarction in our study strongly suggests that AChA occlusion is responsible for much of the deep ischemia seen in our model. That a suture withdrawn to the position 3 or more mm proximal to the MCA bifurcation failed to produce any infarct also argues that AChA occlusion contributed to ischemia in those structures. Although the AChA has many variations in branching in humans, the most constant branches are those to the optic tract, cerebral peduncle, posterior limb of the internal capsule, and choroid plexus [26,27]. The common infarct structures produced by AChA occlusion in the present study, verified by the infarct area maps, encompassed the internal capsule and endopeduncular nucleus, suggesting that the AChA give off constant branches to these structures in rats.

The AChA territory in humans and dogs does not include the hypothalamus. Although 57–83% of AChA infarcts in the present study showed ischemic damage in the medial and/or lateral hypothalamus, our ability to produce a hypothalamic lesion independent of AChA territory infarction argues that at least one artery branch to the hypothalamus originates from the ICA proximal to the AChA.

4.2. Collateral circulation of the AChA

The AChA may not supply the hippocampus or caudoputamen in rats [6,24,28], and any damage in the caudal caudatoputamen may be due to occlusion of branches from the caudal part of the circle of Willis [28]. The AChA anastomoses with the MCA, posterior communicating artery and PCA. An intraluminal thread tip in the position just proximal to the MCA bifurcation may interfere with other ICA branches to the PCA or small direct perforators [29]. An arterial loop is generally present between the posterior lateral choroidal artery (a branch of the PCA) and the AChA (Fig. 1) [6]. Anastomoses are also present between the MCA and the longitudinal hippocampal artery (a branch of the PCA, Fig. 1) and between the MCA and AChA in the amygdaloid region. As demonstrated on infarct area overlapping maps, intraluminal MCA occlusion markedly increases infarct frequency of the amygdaloid complex. Therefore, whether infarction occurs in the hippocampus and amygdalar region may depend on the extent of collaterals. Furthermore, because the branch to the periamygdaloid cortex directly originates from the ICA in most cases, advancing the tip of occluder up to a position between the branch to periamygdaloid cortex and the AChA might produce an infarct sparing damage of the amygdaloid complex. Another possibility is the presence of "steal phenomenon", since the choroidal artery loop may divert blood supply from the PCA to the AChA when the AChA origin is occluded.

4.3. The advantages and disadvantages of the present model

It is thought that the brain vasculature in rats is different between not only rat strains but also bleeders, from the fact of the probability of successful forebrain ischemia in 4-vessel-occlusion model. In Experiment 1, we used rats purchased from a Japanese company and in Experiment 2, we used an American bleeder. Furthermore, we have tested both sexes of another stain, Sprague–Dawley rat, and demonstrated that advancing the occluder proximal to the MCA to obstruct the AChA caused an infarct involving the internal capsule.

The advantage of the present model has been already used in our recent study, demonstrating that a correlation between abnormal postural reflex and AChA territory infarction involving the internal capsule [18,30]. The findings are consistent with the report conducted in human beings, in which axonal injury in the internal capsule correlates with motor impairment after stroke [31]. Human stroke usually affects cerebral white matter. To our lower case knowledge, this is the first focal cerebral ischemic model in rodent that selectively affects the white matter. Since white matter consists of axons and glia but not synapses, ischemic injury of white matter presumably is mediated by nonsynaptic cellular mechanisms, which are different from those of neuron death. Our model provides a tool suitable for investigating the mechanisms of ischemic damage in the white matter.

The disadvantage of the present model is the large variability in infarct size. For example, this model may not be suitable for investigating interventions aimed at reducing infarct size. On the other hand, the model addresses that the variation is the nature of vasculature. For example, using vascular perfusion followed by micro-dissection to investigate the supply of the hypothalamus, it was reported that "the optic area is supplied by vessels that originate in the anterior cerebral arteries and run upwards and backwards at both sides of the third ventricle" [32]. Our recent study demonstrated that advancing the occluder proximal to the AChA in the ICA caused optic area infarction in approximately 15% of rats [18], indicating a variation that this region may be supplied by the vessels that originates from the ICA proximal to the AChA. High frequency of ischemic damage in the thalamus in the present study (Fig. 4b) suggests that a part of the thalamus be supplied by the AChA in rats. The results are consistent with the work of Scremin [7]. However, an endocast and scanning electron microscopic study argues against that the thalamus is

supplied by the AChA [29]. The vasculature variation may explain for the discrepancy.

The territorial variation of the AChA may also include the hippocampus. In the present study, 17% of rats with an AChA infarct had damage in the hippocampus using TTC staining to delineate ischemic lesion. The frequency of damage in the hippocampus should increase when H&E staining and the TUNEL method are employed (Fig. 6e and f) [18]. This finding is consisted with those reported both in dog and humans. Several hypotheses have been proposed to explain hippocampal damage following intraluminal MCA occlusion in rats [33]. They include edema-related elevation of intracranial pressure [33], loss of trophic support [34], excessive excitation of hippocampal neurons resulting from loss of the inhibitory input, overactivation of the excitatory input from the ischemic core or penumbra projecting to the hippocampus [35], and opening of the blood–brain barrier following MCA occlusion, which may expose hippocampal neurons to blood-born molecules such as iron-containing heme protein [36]. Our findings argue a much simple explanation for the damage: shortage of blood flow from branches of the AChA that can supply the hippocampus.

In conclusion, intraluminal occlusion of the MCA in rats may produce an infarct beyond the MCA territory by obstructing other ICA branches, including the AChA. An occluder tip placed 1–2 mm proximal to the MCA origin is capable of producing a small deep infarct in AChA territory in rats. The internal capsule is absolutely damaged in such cases, while wider territories of AChA infarction depends on variation of AChA branching and its collaterals.

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Appendix J

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Neuronal nitric oxide synthase activation mediates neuroprotective effects of estradiol in SK-N-SH neuroblastoma cells

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ABSTRACT

Estrogens exert neuroprotective activity in both *in vivo* and *in vitro* model systems. We have previously observed a lack of structure-activity relationships for estradiol mediated neuroprotection which differed markedly from that for estrogenicity. This suggests that this activity of estrogens is independent of classical estrogen receptor mediated transcriptional activity. In the present study we report that both 17 β-Estradiol (17 β-E2) and low concentrations of nitric oxide (NO) attenuate hydrogen peroxide (H_2O_2) toxicity in SK-N-SH cells that express only the neuronal isoform of nitric oxide synthase (nNOS). Using DAF2-DA, a cell permeable NO specific fluorescent probe, to examine the effect of 17 β-E2 on NO production, 17 β-E2 induced an increase in NO levels within 10 min, with an EC_{50} of 0.1 ± 0.1 nM. L-NNA, a partial nNOS inhibitor reduced the protection of 17 β-E2. CGMP, a major mediator of the biological function of NO, also protected against H_2O_2 induced toxicity. NO's protection was attenuated by ODQ, a soluble guanyl cyclase (sGC) inhibitor. These data indicate that both 17 β-E2 and NO can attenuate oxidative stress in SH-N-SH cells and further suggest that increased activity of nNOS may mediate neuroprotection conferred by 17 β-E2 in a manner which is independent of estrogen's classical transcriptional activity.

Key words:

estradiol, estrogen, nitric oxide, neuroprotection, oxidative stress

INTRODUCTION

Epidemiological evidence shows an association between estrogen replacement therapy and a reduction of Alzheimer's disease incidence in post-menopausal women (Grady et al., 1992; Paganini-Hill and Henderson, 1996; Kawas et al., 1997). Numerous reports have demonstrated estrogen-mediated neuroprotection from a variety of *in vitro* insults; (Green and Simpkins, 2000) including serum deprivation (Bishop and Simpkins, 1994; Green et al., 1997), oxidative stress (Behl et al., 1995), excitotoxicity (Behl et al., 1995; Singer et al., 1996; Singer et al., 1998), and amyloid-β peptide (Behl et al., 1995; Goodman et al., 1996; Green et al., 1996). Further, estrogens can reduce ischemic infarct size following middle cerebral artery occlusion (Simpkins et al., 1997; Wise, 2000; Yang et al., 2000) and subarachnoid hemorrhage (Yang et al., 2001) in rodent models. Collectively these studies indicate that estrogens are potent neuroprotective agents.

Nitric oxide (NO) is an important mediator in many cellular functions (Faraci, 1993; Ignarro et al., 1999). Interestingly NO is associated both with initiating apoptosis in a variety of cell types (Messmer and Brune, 1994; Nishio and Watanabe, 1997), as well as inhibition of apoptosis (Kim et al., 1997; Tzeng et al., 1998; Rossig et al., 1999).

Among the three known isoforms of nitric oxide synthase (NOS), epithelial NOS (eNOS) and neuronal NOS (nNOS) are regulated by intracellular calcium and produce physiological concentrations of NO (Bredt, 1999), while inducible NOS (iNOS) is regulated by transcription and produces pathological concentrations of NO (Licinio et al., 1999). Estrogen is reported to activate eNOS in many epithelial cell lines through a non-genomic pathway (Ruehlmann and Mann, 2000), probably by regulating transient calcium currents (Stefano et al., 2000a), activation of the PI3 kinase/akt pathway

(Hisamoto et al., 2000), or affecting an unidentified cell surface receptor (Stefano et al., 2000b). We now report that estrogen can rapidly increase NO concentrations and NO may play an important role in mediating estrogen's potent neuroprotection.

MATERIALS AND METHODS

Cell Cultures

SK-N-SH, human neuroblastoma cells (ATCC, Rockville, MD), were cultured and maintained in RPMI 1640 media (GIBCO-BRL, Gaithersburg, MD, USA) containing 10% charcoal stripped fetal bovine serum (Hyclone, Logan, UT), and 20 μg/ml gentamycin under standard cell culture conditions (5% CO_2, 95% air, 37° C). Cells used in these experiments were from passages 39-45. Cells were plated at a density of 12,000 –20,000 cells/well in Nunc 96-well plates or 1 X 10^6 –1 X 10^7 cells per 100 mm dishes 24 hours before treatments were added.

Chemicals:

17 β-E2 (Steraloids, Wilton, NH) was initially dissolved in absolute ethanol as a 10 mM stock solution, and stored in –20 °C. All working concentrations of 17 β-E2 were diluted into culture medium from stock solution before experiments. All final concentrations of ethanol were ≤0.1%. Therefore, all control wells were treated with 0.1% ethanol.

(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1- ium-1,2-diolate (NOC-18), and N^G-Nitro-L-arginine (L-NNA), were purchased from Calbiochem (San Diego, CA). 1H-[1, 2, 4]-Oxadiazolo-[4,3-a]-quinoxalin-1-one (ODQ) and 8-Br-cGMP were purchased from Biomol (Plymouth Meeting, PA).

Caspase-3 like Activity Assay:

Cells were plated at 100,000 cells per well in 24 well plates. Cells were exposed to various combinations of H_2O_2 (0 to 20 μM), 17 β-E2 (0 to 10,000 nM), and/or L-NNA (10 μM) for 8 h. Cells were lysed in lysis buffer (10 mM Tris-HCl, 1% Triton-X-100, 10 mM NaPPi, 10% 10X PBS), at 2-10 million cells/ml, with moderate shaking on ice for 1 h. 20 μM caspase-3 fluorogenic substrate (Ac-DEVD-AMC) (Pharmingen, San Diego, CA) in assay buffer (20 mM HEPES, 10% glycerol, 2 mM DTT) was then added, and the mixture was allowed to incubate for 60 min at 37 °C. Fluorescence was measured with a spectrofluorometer, at excitation/emission wavelengths of 380/440 nm. Purified caspase-3 (Pharmingen, San Diego, CA) was used as a positive control.

Viability Assays:

We used Calcein AM based viability assay. Calcein AM is a cell permeable non-fluorescent compound, which can be quickly hydrolyzed by intracellular esterases to a highly fluorescent molecule, calcein. This assay measures the total intracellular esterase activity as a marker of cell viability (Rat et al., 1994). SK-N-SH cells were plated in 96-well plates at about 20,000 cells per well. Cells were exposed to H_2O_2 (1 to 50 μM), 17 β-E2 (0-10 μM), L-NNA (0-10 μM), NOC-18 (0-1 mM) or their combination for 24 h. Medium was discarded and cells were rinsed with PBS. 25 μM Calcein AM (Molecular Probes, Eugene, OR in PBS buffer was added to each well. Plates were wrapped with aluminum foil and incubated for 20 min at 37 °C. Fluorescence was measured at

excitation/emission wavelenghts of 495/538 nm. Wells without cells were used to determine background fluorescence.

Nitric Oxide Production Imaging Analysis

Real time *in situ* determination of NO production was achieved using the NO specific fluorescence dye DAF2-DA (Calbiochem, San Diego, CA). SK-N-SH cells were loaded with DAF2-DA (10 μM) in culture medium for 30 minutes at 37 °C. DAF2-DA was washed out with phenol red free medium before initial treatment. Culture medium containing 100 nM 17 β-E2 was administered at time zero. Green fluorescence was observed with an excitation wavelength compatible with FITC. Photomicrographs were taken at the appropriate exposure time (2 sec) and magnification (40X). To photograph cells, medium was removed briefly to reduce background, then replaced.

Nitric Oxide Fluorescence Quantitative Assay

SK-N-SH cells (20,000 cells/well) in 96-well plates were loaded with 10μM DAF2-DA 30 min prior to measurement. Phenol red free RPMI 1640 medium with 100 nM 17 β-E2 was administered at time zero. After 30 min at room temperature, fluorescence was determined by a 96-well compatible fluorometer using an excitation/emission pair at 470/520 nm. Cells preloaded with L-NNA were used as control.

RT-PCR:

RT-PCR was carried out using hNOS isoform specific primers from Biomol (Plymouth Meeting, PA). One-step RT-PCR kit was purchased from Qiagen (Valencia, CA). Specifically 100 ng total RNA was used in each reaction. Cycling parameters were 30 minutes at 50 °C first strand synthesis, 15 minutes initial activation at 94 °C, followed by a three-step cycle method: 15 second denature at 94 °C, 30 seconds anneal at 58 °C, 1 minute extension at 72 °C. The final product was resolved in 1.2% agarose gel. Actin mRNA was amplified in the same reaction tube as a positive control to insure the quality of RNA and RT-PCR conditions.

STATISTICAL METHODS

The significance of differences among groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $P < 0.05$ was considered significant and each group consisted of 4 to 12 replications. All values are expressed as mean ± SEM. KD values were obtained by one-site binding nonlinear regression using Prism software.

RESULTS

1: Effect of estrogen on NOS activation.

We achieved an *in situ*, real-time detection of NO with DAF2-DA (Fig.1). DAF2-DA is a non-fluorescent probe that binds specifically to nitric oxide and the fluorescence of the bound probe is directly proportional to NO production (Blute et al.,

2000; Brown et al., 2000). 17 β-E2 exposure resulted in an increase in fluorescence within 10 min, the first sampling time, and the NO accumulated thereafter (Fig. 1). Quantitative analysis of NO response induced by 17 β-E2, as determined at 30 min of exposure, revealed a dose-dependent increase in NO with an EC_{50} of 1.1 ± 0.3 nM and a maximal effect at 100 nM (Fig. 2).

2: Identification of NOS isoforms in SH-N-SH cells

We performed RT-PCR with primer pairs specific for each isoform of hNOS. We could only detect the neuronal NOS in SK-N-SH cells (Fig. 3).

3: Dose-response and time-course evaluation of H_2O_2 toxicity in SK-N-SH cells

In our assessment of H_2O_2 toxicity, we conducted dose-response (Fig. 4a) and time-course evaluations (data not shown) to determine an optimal concentration and time of treatment for assessing cell death. A 5 μM H_2O_2 treatment was slightly toxic to cells while a 50 μM H_2O_2 treatment killed most of the cells after 24 h of treatment. At 20 μM H_2O_2, cell death began at about 8 h and there was substantial microscopic evidence of cell death at 24h. Most sick and dying cells showed the morphology of apoptosis as there was evidence of cell shrinkage and many cells detach from the surface of the wells. We determined that treatment with 10 to 30 μM H_2O_2 for 24 h is optimal for evaluation of protective effects of compounds as this dose and time of exposure caused approximately 30%-70% cell death (Fig. 4a).

As it is reported that apoptosis is highly associated with cell death induced by H_2O_2 (Teramoto et al., 1999; Huang et al., 2000), we used a caspase-3 like activity assay as a quantitative measurement of apoptosis. In these studies we also performed dose-response (Fig. 4b) and time-course assessments (data not shown). At a similar

concentration that caused about 50% cell death at 24 h (10-30 μM H_2O_2), caspase-3 like activity reaches its peak at about 8 h of exposure. At 24 h, caspase-3 like activity decreased to basal level, a likely result of H_2O_2 induced cell death.

4: Estrogen's neuroprotection against H_2O_2 induced toxicity.

17 β-E2 caused a dose-dependent protection of SK-N-SH cells from 20μM H_2O_2 toxicity. Significant neuroprotection of 17 β-E2 was seen at 1nM and the ED_{50} was close to 6 ± 3 nM (Fig. 5). To determine that if NOS activation was involved in estrogen's protection signaling, we conducted 17 β-E2 protection (against H_2O_2 toxicity) in the presence of L-NNA (10 μM) (Fig. 5). L-NNA alone had no effect on cell numbers. However, treatment with L-NNA markedly blunted the protective effects of 17 β-E2, as evidenced by an increase in the ED_{50} of 17 β-E2 protection to 140 ± 80 nM.

Similar effects of 17 β-E2 and L-NNA were observed when caspase-3 like activity was measured (Fig. 6). At 20μM H_2O_2, treatment with 17 β-E2 alone reduced caspase-3 like activity by 60% whereas treatment with L-NNA alone had no significant effect. H_2O_2 induced caspase-3 like activity by 7.5-fold. 17 β-E2 treatment reduced the H_2O_2 response by 40 %. L-NNA partially antagonized this protective effect of 17 β-E2.

5: Effects of NO donor on cell viability.

To assess the effects of NO on cell viability, we used NOC-18, a novel NO donor with a half-life of approximately 3,400 minutes (Shibuta et al., 1996). The slow release of NO from NOC-18 has the advantage of avoiding the sharp fluctuation in NO concentration often seen in other NO donors, such as SNAP (Shibuta et al., 1996). NOC-

18 caused a biphasic cell viability response (Fig. 7). At low concentrations, NOC-18 protects cells from H_2O_2 toxicity, while at higher concentrations the protection was lost. Maximal protection from H_2O_2 toxicity was seen at 10 μM NOC-18 (Fig. 7).

6: cGMP may mediate NO's protective role.

The best known physiological mediator of NO is cGMP. NO is well known to activate soluble guanylyl cyclase (sGC) and results in the subsequent accumulation of cGMP in many cell types including neurons (Fessenden and Schacht, 1998). To determine if the cytoprotective action of NO is mediated through cGMP, we used caspase-3 like activity assay to measure the anti-apoptotic effect of NO (Fig. 8). NOC-18 and 8- Br-cGMP, a cell membrane permeable analog of cGMP, did not have significant effects on basal caspase-3 like activity. As seen previously, H_2O_2 caused a 7-fold increase in caspase-3 like activity. Both NOC-18 and 8-Br-cGMP, but not the soluble guanylyl cyclase inhibitor, ODQ, reduced caspase-3 activation induced by H_2O_2. Also, the protection offered from NOC-18 was blocked by ODQ.

DISCUSSION

The present study provides the first evidence for NO mediation of the neuroprotective effects of estrogen in a neuronal cell model. Estrogen rapidly induced NO elevation, an effect that occurs at low concentrations. The further induction of cGMP level or the inactivation of caspas-3 may mediate estrogen's neuroprotection. The rapidity

of this 17 β-E2 activation suggests that the mechanism of estrogen protection against H_2O_2 induced toxicity is independent of ERE-related transcriptional activity.

Estrogens have a variety of cellular effects that appear to be independent of their interaction with genomic estrogen receptors and subsequent transcriptional activities. There are several reports that estrogen's neuroprotection is independent of classical transcriptional activity (Green et al., 1997; Moosmann and Behl, 1999). Many estrogen-induced cellular responses are observed within a relatively short time course, and the pathways involved are generally regulated by membrane receptors and protein kinase activity rather than transcriptional activity. Among these rapidly activated signaling pathways are the growth factor-mediated Ras/Raf/MAPK pathway (Castoria et al., 1999; Singh et al., 2000); the cAMP/PKA/CREB pathway (Zhou et al., 1996; Panickar et al., 1997; Segal and Murphy, 1998); modulation of glutamate receptors (Regan and Guo, 1997; Woolley, 1998); and activation of PI3-kinase/Akt pathways (Hisamoto et al., 2000; Honda et al., 2000). Many of these activities have been proposed to account for the potent neuroprotective activity of estrogens. The present study is the first to indicate that NO may mediate the neuroprotective activity of estrogens.

NO is produced by NOS through the convertion of arginine to citruline. In SK-N-SH cells, nNOS is the only isoform that can be detected using RT-PCR technique to amplify mRNA (Fig. 3). NO plays a dual role in cell viability. At low concentration, it functions as a signaling molecule and seems to be protective against insults and oxidative stress, while it is toxic at high concentrations (Tanaka et al., 1999). Our data show that estrogens can induce nNOS within 10 minutes and cause a potent and dose-dependent activation of nNOS which may contribute to estrogen's neuroprotection. The time-course

of this response suggests that this activation is independent from estrogen's classical transcriptional activity, It is also noteworthy that the increase in NO induced by estrogen is small (2-fold) suggestive of a regulatory role rather than a toxic NO production. This induction of nNOS activity by estrogen contributes to the neuroprotective activity of estrogen, as we can protect cells with the NO donor, NOC-18, and reduce estrogen's protection with the nNOS inhibitor, L-NNA. This indicates the increase of NOS activity is involved in the neuroprotective activity of estrogen.

Our observation that NOC-18, like 17 β-E2, can reduce caspase-3 like activity, suggests that the mild, rapid stimulation of NO production by estrogens sends a protective signal. This effect may be mediated by known activity of low level induction of NO, including activation of sGC with the result of cGMP production, (Laitinen et al., 1994; Giulivi, 1998; Denninger and Marletta, 1999; Medina et al., 2000). Alternatively NO could inhibit caspase-3 through S-nitrosylation which inactivate this pro-apoptotic enzyme (Rossig et al., 1999). These activities individually or in combination may account for the protective signals sent by estrogen induced NO production.

Estrogen can cause a rapid calcium dependent NOS activation (Duran et al., 2000; Hisamoto et al., 2000), and NO can rapidly spread and elevate intracellular cGMP level both within the same cell, and in the neighboring cells. In our study 8-bromo-cGMP, a cell permeable cGMP analog (Goodman and Weigle, 1981; Dillingham and Anderson, 1988), protected against H_2O_2 induced toxicity, and ODQ, a sGC inhibitor blocked NOC-18's protecting activity. This suggests that a cGMP signaling pathway mediates NOC-18's neuroprotection. Among the potential mechanisms of this cGMP action are the

activation of PKG, further phosphorylation of IP_3 receptors and the resulting increase in intracellular calcium (Pryzwansky et al., 1995; Walsh et al., 1995).

The ED_{50} for 17 β-E2 induced increase in NO was approximately 1 nM and for protection from H_2O_2 induced toxicity was about 6 nM. These values are higher than circulating 17 β-E2 (0.1-0.4 nM), but within the ranges of estrogen concentrations needed during post-menopausal estrogen replacement therapy (up to 10 nM). Therefore activation of nNOS could play an important role in the neuroprotection during post-menopausal estrogen replacement therapy.

In summary this is the first report which shows that direct, rapid activation of nNOS mediates at least part of estrogen's neuroprotection, and providing yet another piece of evidence for a rapid estrogen receptor independent mechanism of neuroprotective action of this ovarian steroid.

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Figure legends

Fig. 1 *In situ* real time detection of NO. All cells were loaded with 10 μM DAF 2-DA (a cell membrane permeable fluorescent dye activated by NO). All photographic images were set at constant exposure time of 2 sec and 40X magnification.

Fig. 2 Dose-dependent effects of 17 β-E2 on nitric oxide concentrations in SK-N-SH cells. The values are expressed as a percentage of control values. Data are shown as mean ± S.E.M. Replication number ≥ 16. * indicates $P<0.01$ between treatment and control groups.

Fig. 3 Identification of NOS isoforms in SK-N-SH cells. 100ng total RNA from normal SK-N-SH cells was analyzed with isoform specific primer pairs for human NOS. The lower actin bands were used as internal positive control.

Fig. 4a The dose-dependent effects of H_2O_2 on cell viability in SK-N-SH cells. Cells were treated with H_2O_2 at the indicated concentrations for 24 h. Relative Fluorescence Units (RFU) were used as a measurement of cell viability. The RFU values are expressed as a percentage of control values. All control cells are treated with 0.1% ethanol as a vehicle. Data are shown as mean ± S.E.M. Replication number ≥ 10. * indicates $P<0.05$ between treatment and control groups.

Fig. 4b The dose-dependent effects of H_2O_2 on caspsase-3 like activity in SK-N-SH cells. Cells were treated with H_2O_2 at the indicated concentrations for 8 h and caspase-3 like

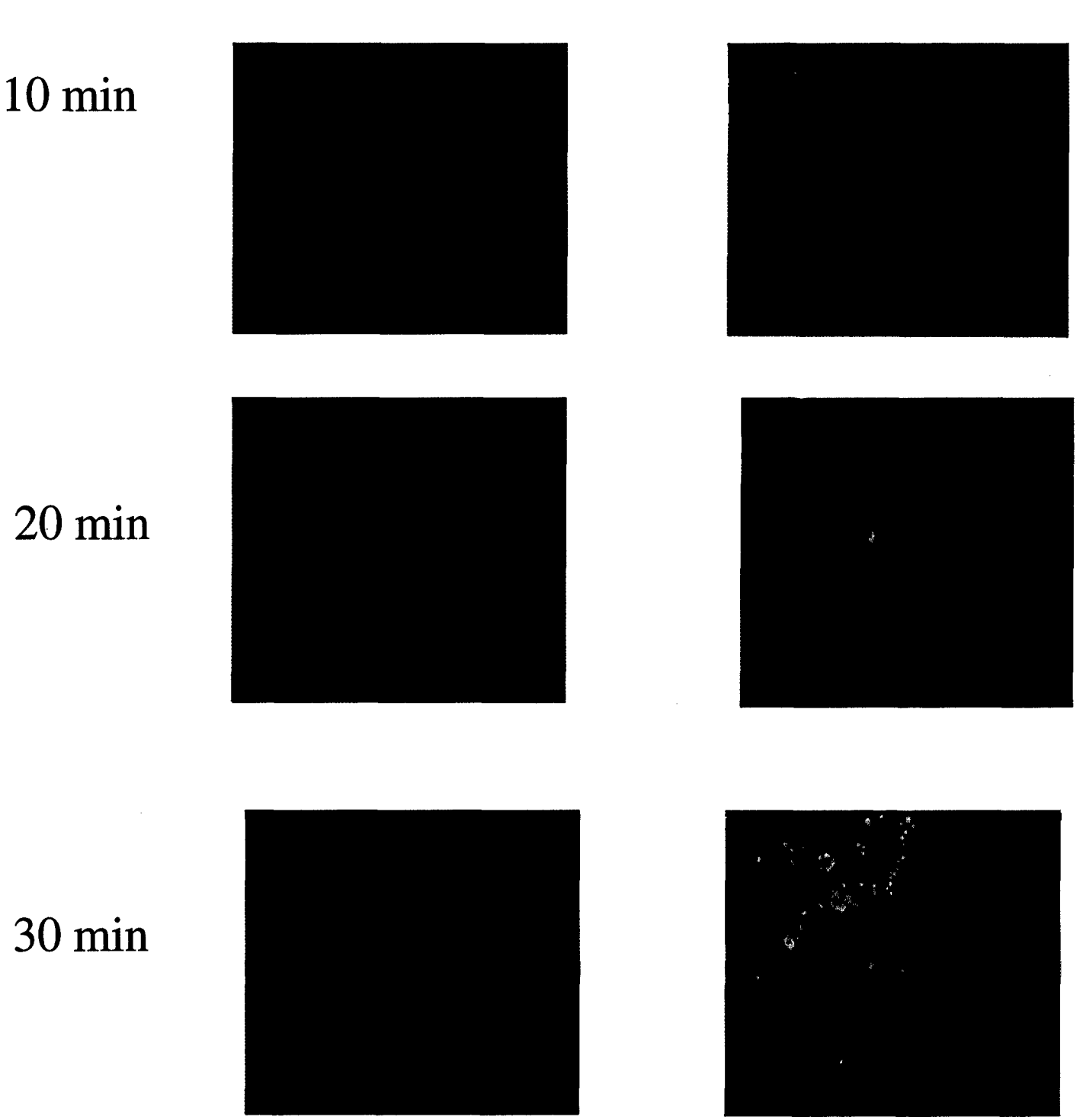
activity was assayed. The values are expressed as a percentage of control values. All control cells were treated with 0.1% ethanol as a vehicle. Data are shown as mean ± S.E.M. Replication number ≥ 4. * indicates $P<0.05$ between treatment and control groups

Fig. 5 The effects of L-NNA on the protective effect of 17 β-E2 in SK-N-SH cells. Cells were treated with 20μM H_2O_2 and the indicated concentrations of 17 β-estradiol for 24 h with or without 10μM L-NNA, a partial specific nNOS inhibitor. The assays are expressed as a percentage of control cells. All control cells were treated with 0.1% ethanol as a vehicle. Data are shown as mean ± S.E.M. Replication ≥ 10. * indicates $P<0.05$ for comparison between L-NNA treated and untreated groups.

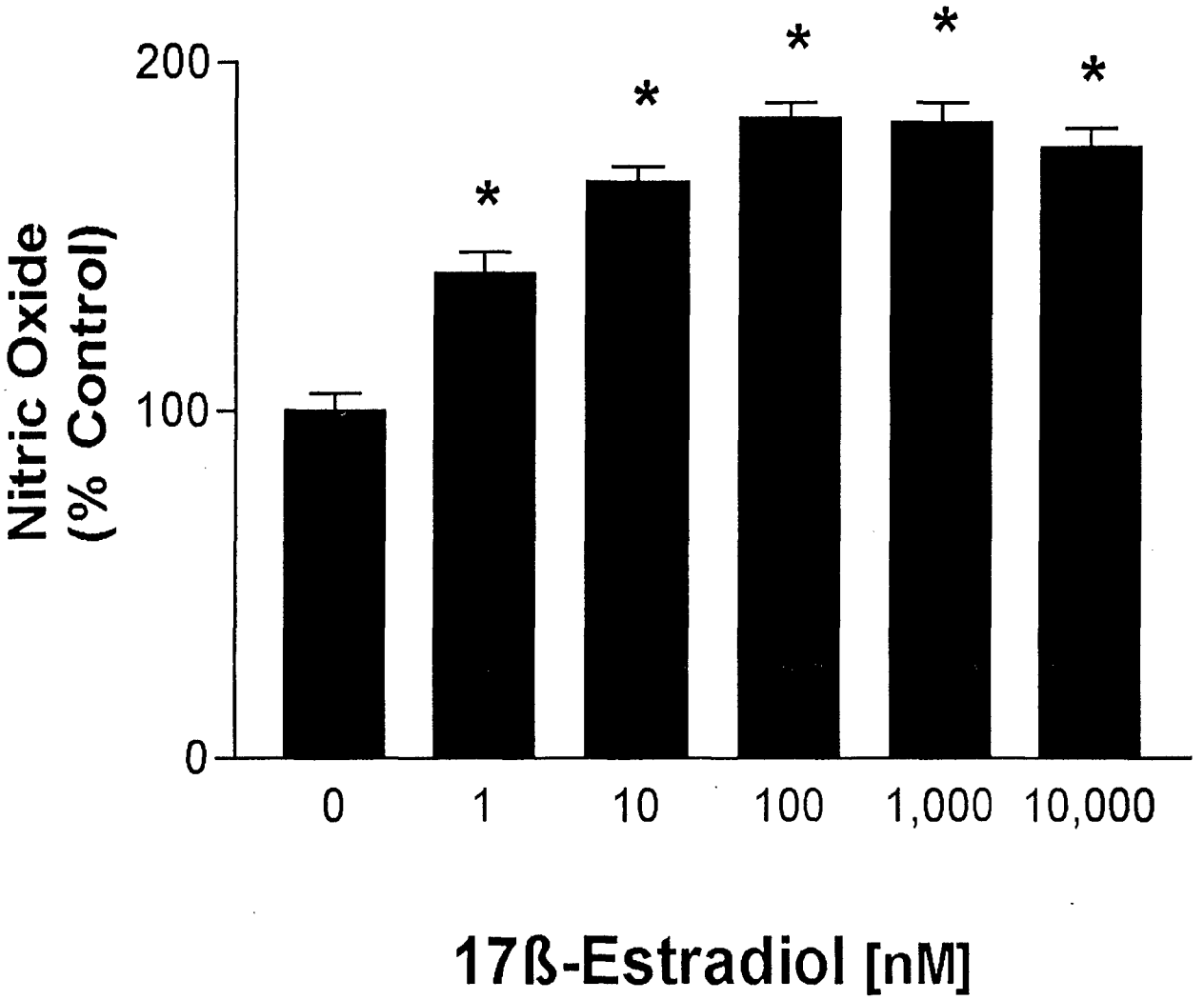
Fig. 6 The effects of H_2O_2, 17 β-E2, L-NNA and their combinations on caspase-3 like activity. Cells were treated with 20μM H_2O_2, 100nM 17 β-E2, and/or 10μM L-NNA as indicated. The assays are expressed as a percentage of control cells. Data are shown as mean ± S.E.M. Replication number ≥ 4. * indicates $P<0.05$ between indicated groups.

Fig. 7 The dose-dependent effects of NOC-18 on cell viability. Cells were treated with 20μM H_2O_2 and the indicated concentration of NOC-18. The assays are expressed as a percentage of control cells. All control cells were treated with 0.1% ethanol. Data are shown as mean ± S.E.M. Replication number ≥ 10. * indicates $P<0.05$ between treatment groups and control groups.

Fig. 8 The effects of H_2O_2, NOC-18, Br-cGMP and/or ODQ on caspase-3 like activity. Cells were treated with 20μM H_2O_2 , 10μM NOC-18, 10μM Br-cGMP and/or 10μM ODQ as indicated. Data are shown as mean ± S.E.M. Replication number ≥ 4. * indicates $P<0.05$ between treatment and H_2O_2 group.



Wen et al Fig.1



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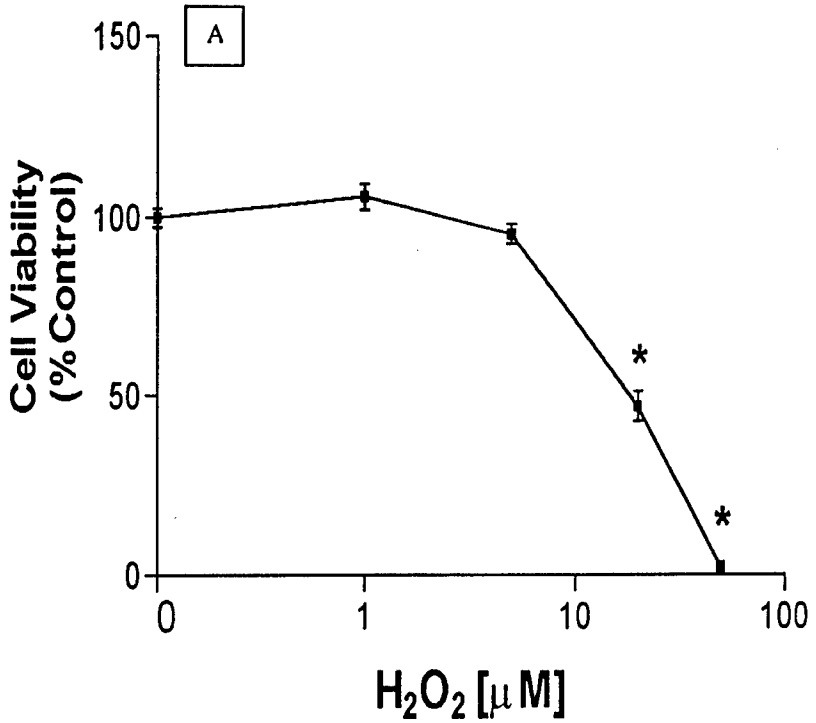

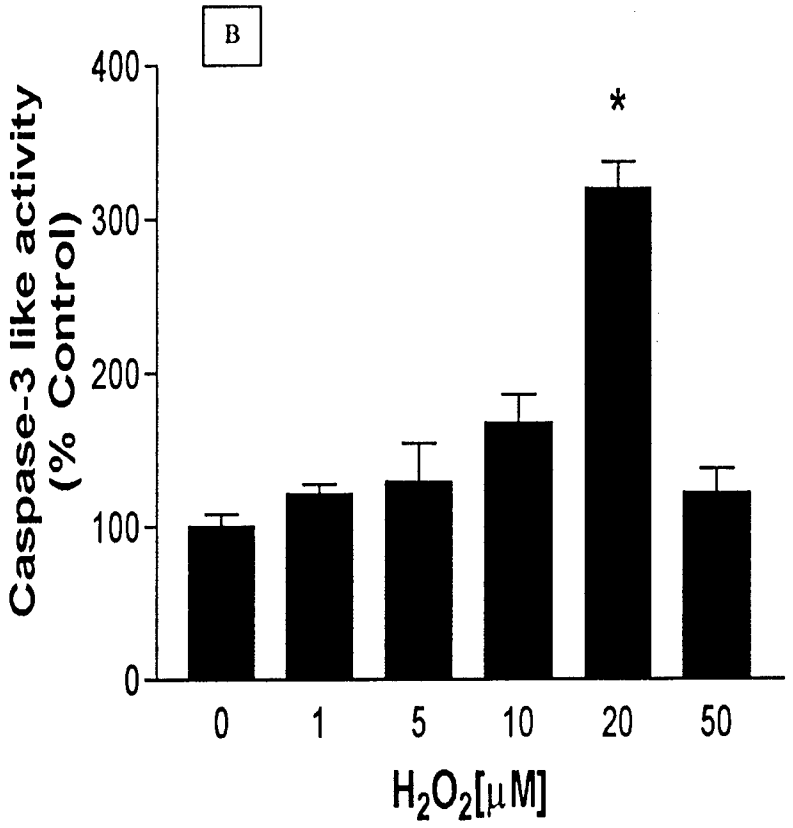

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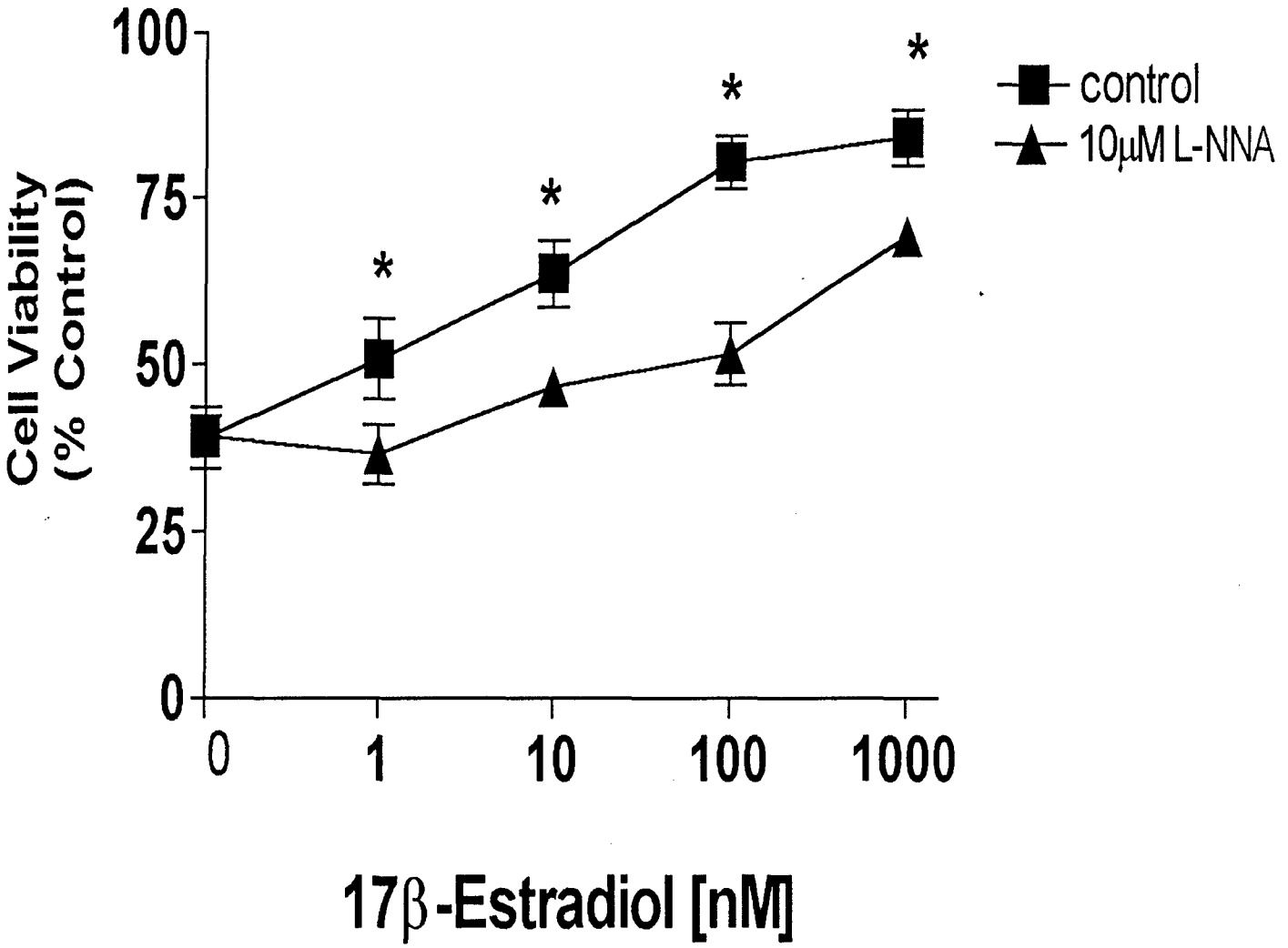

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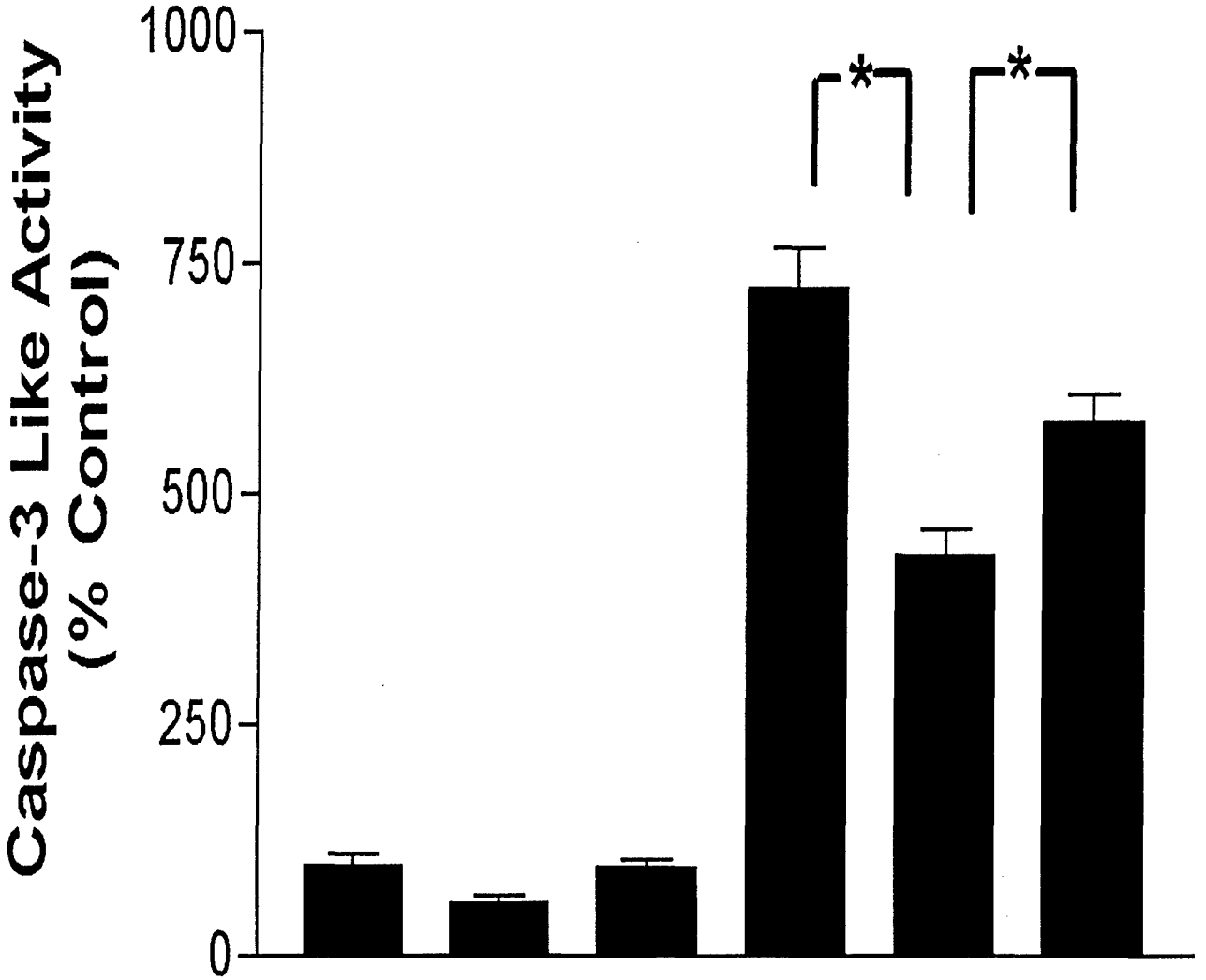
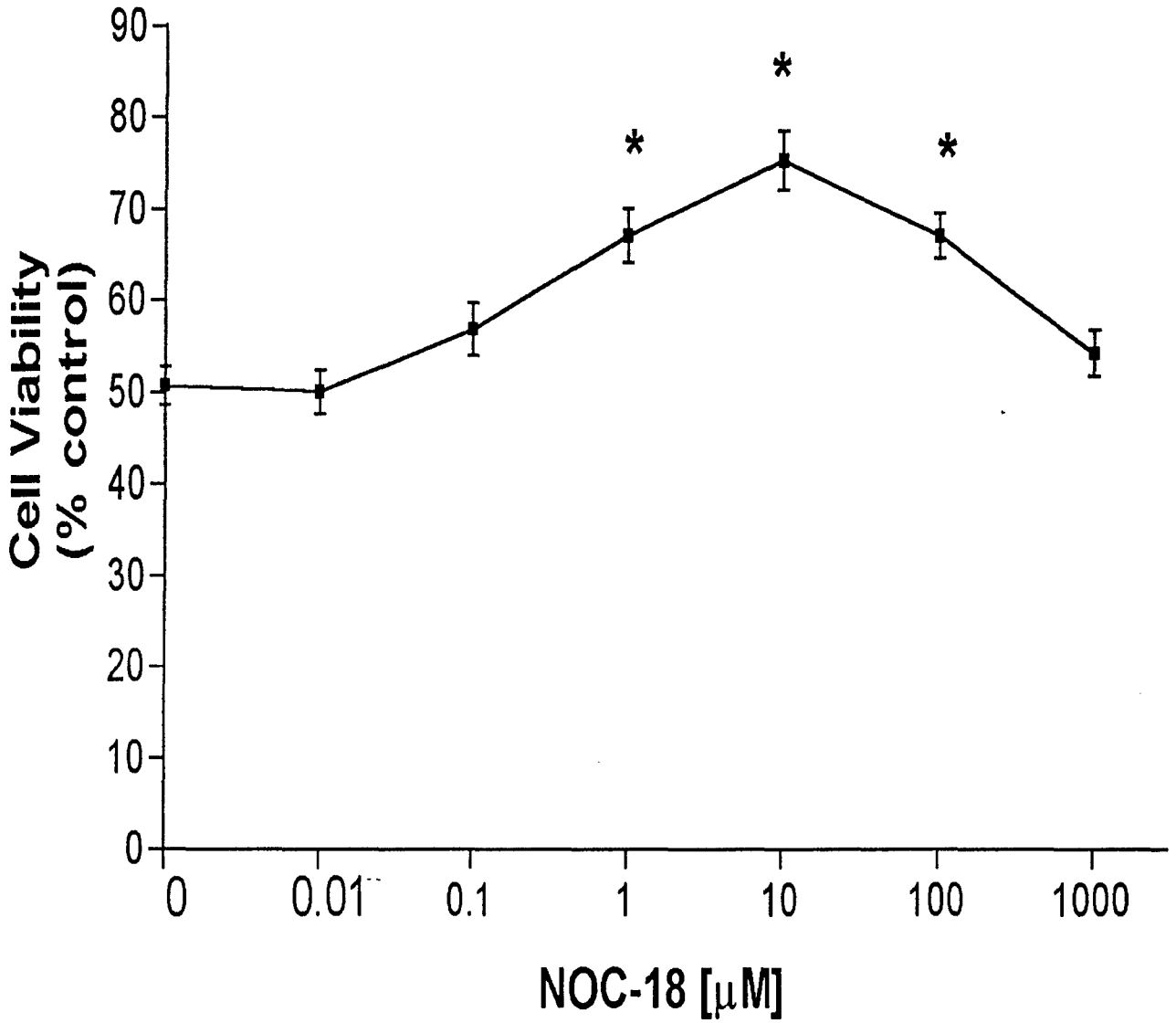
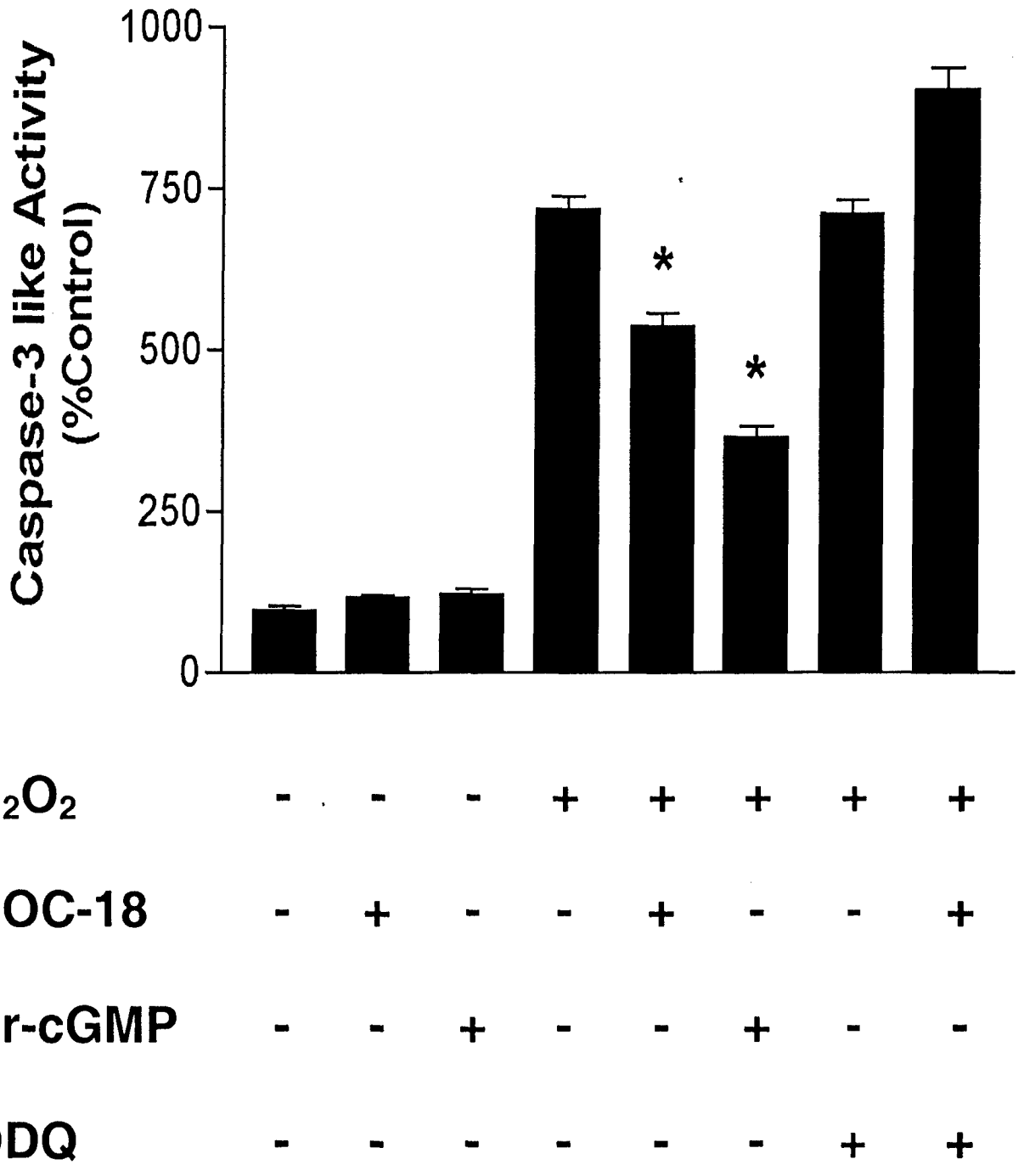


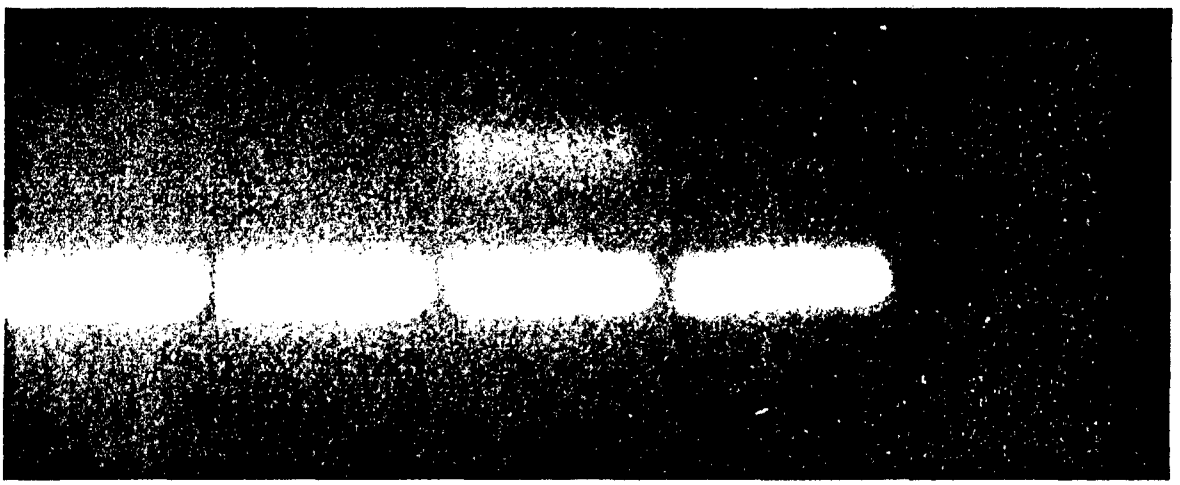
H_2O_2	-	-	-	+	+	+
E_2	-	+	-	-	+	+
L-NNA	-	-	+	-	-	+

Wen et al Fig.5



Wen et al Fig.6


Caspase-3 like Activity
(%Control)
1000
750
500
250
0
*
*
H2O2 - - - + + + + +
NOC-18 - + - - + - - +
Br-cGMP - - + - - + - -
ODQ - - - - - - + +



NOS

β-Actin

eNOS iNOS nNOS blank

Wen et al Fig.8

Proestrus Levels of Estradiol during Transient Global Cerebral ischemia Improves Histological Outcome of the Hippocampal CA1 Region: Perfusion-Dependent and -Independent Mechanisms

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Key words: Caspase-3, Cerebral blood flow, Histology, Proestrus estradiol, Rats, Transient global ischemia

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Abstract

We conducted this study to determine whether high physiological levels of estradiol (proestrus) could protect the hippocampal CA1 neurons following transient global ischemia. Ovariectomized or ovary-intact female rats were subjected to 20 minutes of ischemia and allowed to survive for 96 hours. Estradiol was administered subcutaneously in a group of ovariectomized rats 24 hours before ischemia induction. Ending serum estrogen levels were correlated to cerebral blood flow (CBF), histologic assessment and immunofluorescent caspase-3 active peptide (C-3AP) positive cell count. Estradiol administration significantly improved CBF in the hippocampus (compared with intact or ovariectomized rats) but not in the parietal cortex. No significant differences in CBF between intact or ovariectomized rats were noted. Estradiol administration maintained serum levels of the steroid in estradiol-treated rats-about 10 times that of intact animals and more than 20 times that of ovariectomized animals. Morphologically, live cell counts in estradiol-treated rats were significantly higher than in intact or ovariectomized rats. Live cell counts were also significantly higher in intact than ovariectomized rats. C-3AP positive cell counts were much higher in ovariectomized rats than in intact and estradiol-treated rats. In conclusion, proestrus levels of 17β-estradiol protect hippocampal CA1 neurons against transient global ischemia, through mechanisms that appear to involve improvement of perfusion and inhibition of caspase-3 activity.

Introduction

Clinical observations indicate that estrogens may be naturally occurring neuroprotectants. Extensive experimental evidence indicates that estrogens are neuroprotective against both focal and forebrain ischemia [1-13]. One critical consideration concerning estrogen replacement therapy is the effective dose range. Some investigations suggest a narrow therapeutic dose range within physiological levels [8,13], while the others indicate that high doses were necessary for estrogen to exert the neuroprotective effects against oxidative stress and/or excitatory amino acids [14-16].

Another important issue concerning estrogen replacement therapy is the mechanism(s) by which these compounds act against ischemia. Several studies demonstrated that estrogen does not change cerebral perfusion during focal cerebral ischemia [5,8,17] despite significantly reducing infarct volume; others, however, using the forebrain or global ischemia models, indicate that estrogen administration does improve CBF [13-18]. None of studies clarify histologic outcome relative to CBF or plasma estradiol concentration. Experimentally induced transient global ischemia causes selective, delayed neuron death in the hippocampal CA1 region [19-21], a phenomenon attributable to programmed cell death or apoptosis [22,23]. Whether apoptotic mechanisms play a role concerning the neuroprotective effect of estrogen against cerebral ischemia in vivo remains uncertain.

Caspase-3 protein is present at low level in the adult brain [24,25]. Expression of both caspase-3 mRNA and protein in the brain is markedly up-regulated in response to ischemia [26]. In addition, ischemia induces proteolytic cleavage of the precursor protein into two subunits, one of which contains the catalytic site, i.e. caspase-3 active. Several lines of evidence indicate that the active caspase-3 serves as a key effector of the apoptotic process, contributing wholly or partially to the proteolytic cleavage of may critical proteins involved in apoptosis. Caspase-3 knock-out mice appear to lack programmed neuron death [27]. Inhibition of caspase-3 leads to reduction of apoptotic neuron death induced by cerebral ischemia [26]. Therefore, investigation of caspase-3 activation pathway may provide an important insight regarding apoptotic mechanisms and estrogen-related neuroprotection.

In the present study, we examined whether proestrus estradiol levels could protect hippocampal CA1 neurons in rats subjected to transient global ischemia, and correlated neuron death with ending (96-hour following ischemia) serum 17β-estradiol concentrations and cell death relative to caspase-3 activity.

Materials and Methods

Animals: The experimental protocols were approved by the University of Florida Institutional Animal Care and Use Committee. Sprague-Dawley female rats weighing 225-250 grams were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in pairs and acclimated with a

laboratory diet and tap water ad libitum under a fixed light-dark cycle for 1-2 weeks before experimentation. A total of 53 animals were divided into Non-ischemia (N), vehicle-treated (V), estrogen-treated (E) and intact (I) groups. The N (n=3), V (n=16), and E (n=18) groups were subjected to bilateral ovariectomies 1-2 weeks before induction of cerebral ischemia, while the I group (n=16) group received a sham-operative ovariectomy.

17β-estradiol administration and concentration determination: Estradiol was administered subcutaneously by implanting two 5-mm steroid containing silicone elastomer pellets (4mg/ml) 24 hours before ischemia onset in the E group. Corn oil pellets were implanted in the V, I and N groups. Two-pellet-implantation was selected because one estradiol pellet did not maintained serum concentration up to or above proestrus level for the duration of the experiment. The ending serum 17β-estradiol concentration was measured 96 hours following the onset of cerebral ischemia and just prior to sacrifice. Blood samples were taken via the jugular vein, and the serum was separated from blood cells by centrifugation and stored frozen (-20 °C). Serum estradiol concentration was determined using duplicate serum aliquots in an ultrasensitive estradiol radioimmunoassay kit (Diagnostic Systems Lab, Webster, TX, USA).

Cerebral ischemia Production: Transient incomplete global ischemia was produced as described previously [28]. Briefly, through a midline occipital-suboccipital incision, both vertebral arteries (VA) were exposed between the first

and second cervical vertebra and cauterized under operating microscope magnification using a digital bipolar cauterizer. Both common carotid arteries (CCA) were occluded for 20 minutes in the E, I and V groups through an anterior cervical incision 24 hours after the VA occlusion. All surgical procedures except for CCA occlusion were performed in the N group. Body temperature was monitored and maintained at 36.5-37.5 ^{o}C using a heating lamp during surgery, ischemia and early reperfusion time. All rats were allowed to survive 96 hours following the onset of cerebral ischemia.

Arterial physiological parameter Determination: The animals were anesthetized using 2.5% halothane in air supplemented with 10% pure oxygen delivered via a face-mask. Once the carotid arteries were temporarily occluded, the halothane was discontinued for the first 5 minutes of global ischemia, and then re-adjusted to approximately 1% in air (or less) during the last 15 minutes of global ischemia. Ten rats (3-4 animals in each of the V, I and E groups) were used to determine mean arterial blood pressure (MABP), pH, PO_2, PCO_2, hematocrit, sodium, potassium and calcium. One femoral artery was cannulated and blood samples was taken before and 15 minutes following the onset of ischemia. MABP was monitored using a pressure transducer (Harvard Apparatus, Holliston, Massachusetts, USA) and blood parameters were determined using i-STAT portable clinical analyzer (Abbott Laboratories Inc., East Windsor, NJ, USA).

Local Cerebral blood flow measurement: Cerebral blood flow (CBF) was measured in 22 rats (E group, n=8; I group, n=7; V group, n=7) using the hydrogen clearance method (the Digital UH meters of Type MHG-D1, Unique Medical Co., LDT, Chifu-shi, Tokyo, Japan) as described previously [29,30]. Animals were anesthetized by intraperitoneal ketamine (60mg/kg) and xylazine (10mg/kg) injection, and the head was fixed in a stereotaxic frame. One teflon-coated platinum electrode was stereotactically inserted to a depth 3 mm from the brain surface into the right hippocampus through a burr-hole placed 4.5 mm posterior to the Bregma and 2.5 mm lateral to the midline. A second electrode was inserted to a depth 1 mm from the brain surface into the left parietal cortex through a burr-hole 2 mm posterior to the Bregma and 2 mm lateral to the midline (Fig.1). CBF was measured 10 minutes before ischemia, 10 minutes into the ischemia, and 10 and 30 minutes after reperfusion was re-established.

Histologic and immunofluorescent assessments: Twenty-one rats (n=6 in each of the E, I, and V groups and n=3 in the N group) were used for histologic and immunofluorescent assessments. The anesthetic protocol was identical to that used for determining arterial physiological parameters. After survival for 96 hours, animals received intraperitoneal ketamine (60mg/kg) and xylazine (10mg/kg), and intraarterial perfusion with 100 ml of saline followed by 100ml of 4% buffered paraformaldehyde phosphate.

A 3 mm-thick tissue block 5.5 - 8.5 mm from the frontal pole was taken from each brain and coronally sectioned and paraffin-embedded. Four 5-μm-

thick slices were cut from each block, each containing cross-section of the dorsal hippocampus. Adjacent 5-μm slices were evaluated using hematoxylin and eosin (H&E) staining and immunofluorescent staining of caspase-3 active peptide (C-3AP).

The sections were deparaffinized in xylene, rehydrated through graded ethanol, and washed with phosphate buffered solution (PBS)-Tween 20. After incubated in 4% normal goat serum diluted by PBS-Tween 20, the slides were incubated overnight at 4 ^{o}C with C-3AP rabbit antibody (R&D Systems, Minneapolis, MN, USA) 1:200 diluted in 4% normal goat serum PBS-Tween 20. After incubation with Alexa Flur^{TM} 488 goat anti-rabbit IgG (1:200) for 75 minutes, the slides were stained using 100 μM DAPI. Sections were examined under fluorescence optics using excitation and barrier filters appropriate for selectively visualizing FITC and DAPI, respectively. A rabbit isotype control IgG (ZYMED Laboratories Inc., South San Francisco, CA, USA) was used to replace the first antibody as a negative control. In processing the staining in the V group, samples from group N were included as a further negative control.

Live cell and C-3AP positive cell counts were performed on the middle of the hippocampal CA1 sectors bilaterally (Fig. 1). The average cell number in each hippocampal CA1 sector was derived from 5 view fields under a 400x microscope magnification lens using a 10x10 mm grid. The mean cell number was generated by averaging the cell counts from both sides of the hippocampus. The live cell ratio was used as a histologic measurement of viability. This ratio

was produced by dividing mean cell number of live cells in the V, I and E groups by that in the N group.

Histologic and immunofluorescent assessments were not performed in the same rats used for CBF and arterial blood parameter determination. The former showed surgical damage due to electrode insertion, and the latter were administered heparinized saline to keep the arterial cannula open and to compensate for blood loss due to sampling. These treatments might have complicated the immunofluorescent results.

Statistical Analysis: Fisher's ANOVA was used for comparison of blood physiological parameters, blood pressure, cerebral blood flow, 17β-estradiol concentration, the live cell ratio and C-3AP positive cell counts between groups. The statistical software Statview-J 4.1 (Abacus Concepts, Inc) was used. A p value <0.05 was considered significant.

Results

The experimental protocol and its components are documented in table 1. Three rats in both the I and V groups and four rats in the E group stopped spontaneous respiration between 3-6 minutes following global ischemia onset, each of which was anesthetized with ketamine and xylazine. These animals were to be used for measuring CBF changes, and all (total n=10) were excluded from

further analysis. No rats stopped breath or died before histologic assessment when the halothane-anesthetic protocol was employed.

Physiological Parameters. No significant differences in arterial blood pressure, pH, PO_2, PCO_2, hematocrit, sodium, potassium and calcium were noted between the V, I and E groups before the onset of ischemia (data not show). The MABP in all groups increased by 48-53 mmHg by 15 minutes following ischemia. Cerebral ischemia-related hyperventilation and halothane cessation followed by reinstitution of a reduced halothane supply resulted in an increased arterial pH and decreased PCO_2 at 15 minutes following ischemia, with no significant differences between groups.

Estrogen Levels: The ending serum concentrations of 17β-estradiol were 3±0 and 9±3 pg/ml in the V and I groups, respectively (Fig. 2.). The concentration in the N group was similar to that in the V group (3±0 pg/ml). Estradiol administration raised the steroid levels to 87±15 pg/ml, about a 10-fold increase over that of intact rats and more than 20-fold that of ovariectomized rats.

Changes in CBF: CBF in the parietal cortex was 37-40 ml/100g/min in the V, I and E groups before the onset of cerebral ischemia (Fig. 3a). Global ischemia resulted in decreased CBF to 7-11% of the pre-ischemia resting state. Following reperfusion, the CBF initially recovered to close to or higher than the resting state at 10 minutes, but then dropped to 60-87% of resting levels in all groups.

No significant CBF differences in the parietal cortex were noted at any time between groups.

Hippocampal CBF ranged from 29-42 ml/100g/min, with no significant differences between groups before the onset of cerebral ischemia (Fig. 3b). Marked CBF reduction was noted following ischemia onset and 30 minutes after circulation was re-established in the V and I groups but not in the E group. The CBF in the E group was significantly higher than V and I group during the ischemia, and was higher than the I group after 30 minutes of reperfusion. No significant CBF differences between the I and the V groups were noted at any time.

Histologic and immunofluorescent assessments: Twenty minutes of four-vessel-occlusion induced severe cell loss in the V group (Fig. 4. A-D), although the damage was not homogenously distributed on both sides. Five of six rats in the V group showed much more severe damage in the medial and middle sectors of the hippocampal CA1 region on one side and in the medial sector on the opposite side. Damage to the middle sector on opposite side and both lateral sectors was rather mild. Four rats displayed severe cell loss on right side and only one on the left.

Two of six rats in the I group exhibited similar damage as that observed in the V group (non-homogeneous severe cell loss in the CA1 region limited to one side). The other animals in this group exhibited clustered cell loss as shown in Fig. 4E-H. 17β-estradiol provided robust protection. None of six rats in the E

group showed severe damage in any sectors of the hippocampal CA1 region, and most exhibited only spotty cell lose (Fig. 4I-L).

Immunofluorescent images on slices adjacent to those used for histologic analysis demonstrated that the morphology of C-3AP positive cells and their numbers paralleled the severity of the histologic ischemic damage. In the V group, the C-3AP immunoreactivity was primarily localized in the deformed nuclei (Fig. 5C&F). In the I group, the immunostaining was often observed in both the disfigured nuclei and the cytoplasmic compartment (Fig. 5E). Some cells in the E group also exhibited mixed nuclear-cytoplasmic compartment immunostaining (Fig. 5D), althouth the nucleic deformation labeled with C-3AP immunoreactivity was rather mild in the estrogen treated rats.

The histologic and immunofluorescent assessments are summarized on Fig. 6. The live cell ratio, calculated in reference to the N group, was 35±3% in the V group, a value which was significantly lower than that of both in the I (46±3%, $p<0.05$) and the E groups (56±2%, $p<0.001$). The ratio was also lower in the I group compared to the E group ($p<0.05$). The C-3AP positive cell number was significantly higher in the V group than in the I and E groups ($p<0.05$ and $p<0.01$, respectively).

Discussion

The present study demonstrates that proestrus levels of 17β-estradiol ameliorate ischemic damage in the hippocampal CA1 region of rats subjected to

transient global ischemia. A higher neuron survival rate in the estrogen treated rats as compared with that in rats with or without bilateral ovariectomies was accompanied by improvement of CBF and reduction of C-3AP positive cell numbers. These neuroprotective effects may be linked to both perfusion-dependent and perfusion-independent mechanisms. The neuroprotective effects exhibited in ovary-intact rats were much weaker than in the estrogen replaced group, perhaps attributable to the estradiol concentration and the CBF level approximately close to those in estrogen-depleted group.

Physiological plasma 17β-estradiol levels in rats range between 10 and 30 pg/ml, and may increase up to 80-140 pg/ml during proestrus period [31-33]. Pellet implantation leads to an immediate increase of the serial 17β-estradiol concentration, reaching the summit 1 hour following the implantation in ovariectomized, no ischemic rats [34]. One-pellet implantation (24 hours before production of cerebral ischemia) provided slow release of the steroid at least for 8 days, maintaining the concentrations of 92±12, 35±10 and 37±7 pg/ml at 72, 120 and 192 hours, respectively, and these levels only delayed neuron death [35]. To approach those levels seen during the proestrus period, we used two-pellet implantation in the present study. The neuron-sparing effects demonstrated in this study extends our previous report, showing that following transient global ischemia, proestrus estrogen levels provides substantial neuroprotection. Some neuroprotective effects were also seen in intact rats in which the ending serum 17β-estradiol paralleled metestrus level. The discrepancy may be ascribed to a synergetic effect provided by other ovarian

hormones. For example, progesterone has membrane stabilizing effect that may reduce the damage caused by lipid peroxidation [36], and may ameliorate ischemic damage following middle cerebral artery occlusion [37,38].

Pelligrino et al [13] argued against any neuroprotection with supraphysiological estrogen levels. Discrepancies in estrogen dosage and models limit direct comparison of our data with theirs. Mean arterial blood pressure was subjectively controlled in their study (unilateral right carotid occlusion with hemorrhagic hypotension) but not in ours. In addition, their model might involve anti-coagulating agents gaining access to the ischemic area when cerebral circulation is re-established by recirculating and transfusing the blood back into the carotid artery. Their death rate was as high as 32-67% [13] but zero in our histologic assessment group; such disparate survival rates raise the possibility that some other undefined bias has been introduced between groups within these studies.

The hydrogen clearance method provides absolute measurements of local CBF. The CBF in rats anesthetized with katamine and xylazine was similar to those anesthetized with amobarbital in our previous studies [29,30]. The anesthetic route and agents was selected because we already had extensive experience using it for both focal and global ischemic models [1,9,35], and also because the method facilitates stereotaxic frame fixation to insert the CBF measuring electrodes. On the other hand, 43-50% of rats in each group using katamine and xylazine stopped spontaneous respiration between 3-6 minutes following bilateral CCA occlusion. Inhibition of the respiratory control center in

the brain stem due to both global ischemia and the anesthetics may account for this phenomenon. Nevertheless, our observed reduction in parietal cortex CBF following global ischemia was consisted with those using laser-Doppler flow meter reported by Pelligrino et al [13].

The reduction of hippocampual CBF during bilateral CCA occlusion was very profound in our study (decreased by 60-62 % in the V and I groups) compared with those using the model of Pulsinelli et al [20], in which CBF in the hippocampus decreased by only 42% [39]. Estrogen administration improved CBF during the occlusion in the hippocampus but not in the parietal cortex. During the first 30 minutes of reperfusion, the parietal CBF showed biphasic changes (hyperperfusion followed by hypoperfusion) in all three occlusion groups. Similar changes were noted in hippocampal flow in the V and I groups, but not in estrogen-treated rats, where the CBF gradually recovered to the level similar to that prior to the ischemia. A much higher serum level of 17β-estradiol in estrogen treated rats as compared to that in other two groups is presumably responsible for these differences.

Hippocampal neurons undergo delayed death following transient global ischemia, providing a potential in vivo apoptotic cell death model. The key elements of apoptosis include chromatin condensation, DNA fragmentation, caspase activation or processing, and selective substrate cleavage. Our study was consistent with the report conducted by Chen et al [26] by showing that at least some of the neurons in the hippocampal CA1 region died through a caspase-3 activating pathway. The novel findings in our study is that the

attenuation of ischemic damage in the hippocampal CA1 region due to 17β-estradiol administration was associated with reduction of the C-3AP positive cell numbers.

Chen's study also indicated that caspase-3 immunoreactivity in the hippocampal CA1 region increased in the neuron cytoplasm at 8-24 hours, followed by both cytoplasmic and nuclear localization at 72 hours following global ischemia [26]. Permanent focal cerebral ischemia appears to accelerate the shift from cytoplasmic to nuclear localization within 24 hours [40]. In our study, the C-3AP positive staining was primarily localized to the nucleus in ovariectmized rats, while a mixed nuclear-cytoplasmic compartmentalization was often observed in ovary-intact and estrogen treated rats, indicating that estrogen might modify or delay the translocation.

Our study did not assess how estrogens affect caspase-3 activation. In addition to perfusion-dependent effects, estrogen inhibited caspase-3 activity in cardiac myocytes induced by staurosporine but not basal caspase-3 activity [41], indicating that the steroid may affect caspase-3 activation through an indirect pathway. Estrogen may up-regulate anti-apoptotic factor Bcl-2 [7], which in turn suppresses cytochrome c release and the ensuing caspase-3 activation. Estrogen may also suppress intracellular oxygen radicals and exert neuroprotection against oxidative stress, specially when high concentration of estradiol is used [9,42]. A recent study suggests that the anti-apoptotic neuroprotective effects of estrogen may be mediated by transcription through the activator protein-1 site downstream from c-Jun NH(2)-terminal kinase and

caspase-3 activation [43]. These mechanisms may collectively account for the different intercellular localization of C-3AP immunoreactivity and differential live cell counts in the hippocampal CA1 neurons between ovariectomized, ovary-intact, and estradiol treated rats.

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Legends for figures

Fig.1. Schematic illustration showing the positions for electrode insertion and hippocampal live cell and immunofluorescent positive cell counts. Two electrodes were simultaneously inserted into the left parietal cortex and right hippocampus for determining local cerebral blood flow. The live cell and caspase-3 positive peptide positive cell counts were performed in the middle hippocampal CA1 sector.

Fig.2. Ending serum 17β-estradiol concentrations. Eighteen female rats were divided into ovary intact (I), vehicle (V) and estradiol (E) treated groups (n=6 each, see Materials and Methods for details). Blood samples were obtained just before sacrifice to correlate the serum 17β-estradiol concentrations with histological and immunofluorescent outcome. **p< 0.01 vs. the V and the I groups.

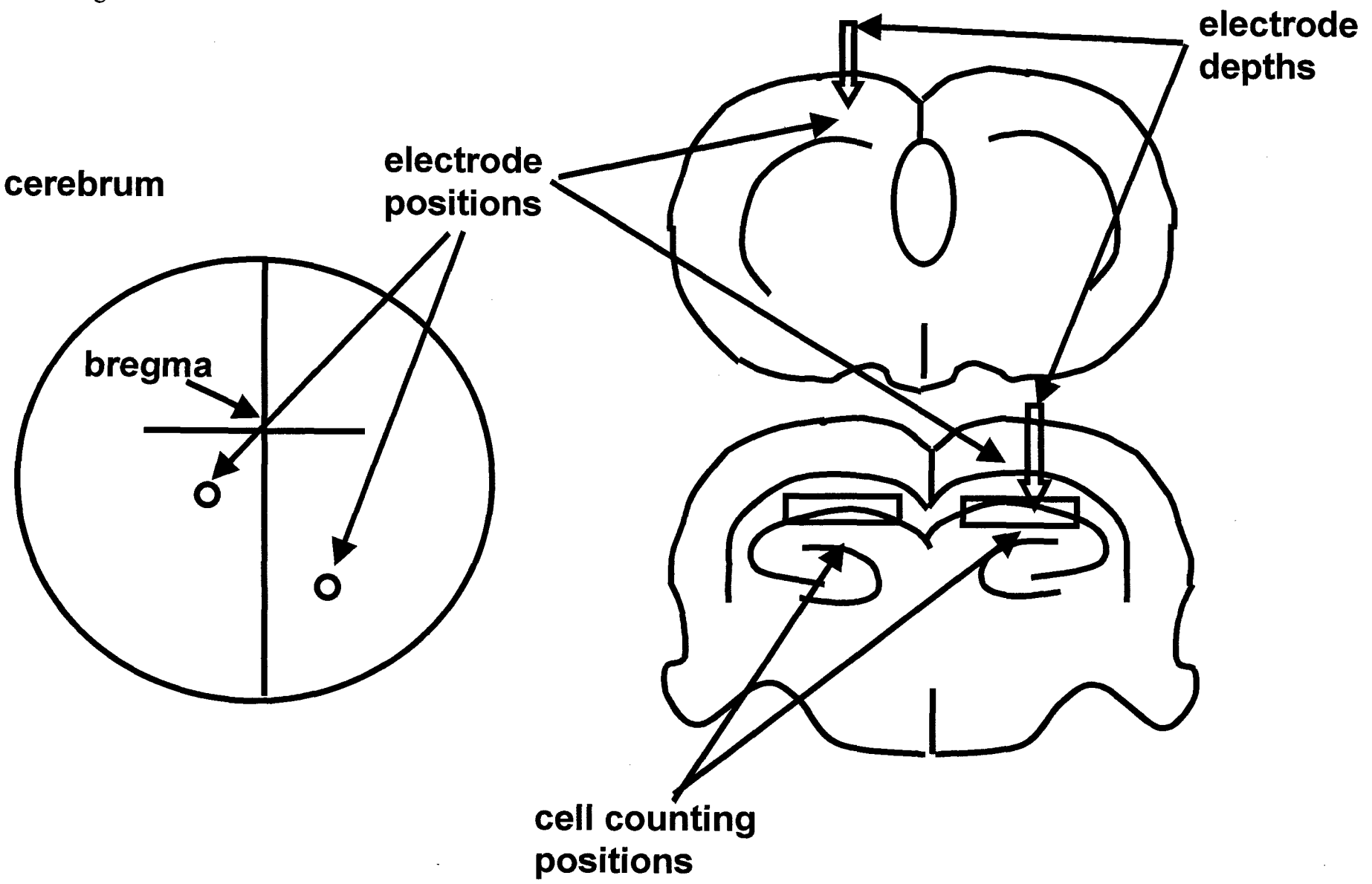
Fig.3. Changes in cerebral blood flow in the parietal cortex (a) and hippocampus (b) following transient global ischemia. The animals were divided into ovary intact (I), vehicle (V) and estradiol (E) treated groups. Local cerebral blood flow was determined before, during (10 minutes into the ischemic interval), and after transient global cerebral ischemia (10 minutes and 30 minutes after perfusion was reestablished) using hydrogen clearance method. **p< 0.01 vs. the V and the I groups; ¶p<0.05 vs. the I group.

Fig.4. Representative histology of the middle hippocampal CA1 sector following transient global ischemia. The images were obtained from 2 vehicle treated (A-D), 2 ovary intact (E-H) and 2 estradiol treated (I-L) rats. The slices were stained using hematoxylin and eosin. Original magnification is 100x for A, C, E, G, I, and K. Images B, D, F, H, J, and L show those noted in the squares of A, C, E, G, I, and K, respectively at 400x magnification.

Fig.5. Representative images of caspase-3 active peptide (C-3AP) positive cells in the middle hippocampal CA1 sector. (A) Immunofluorescent staining in a no-ischemic rat. Little C-3AP positive cells were observed. (B) The staining using a rabbit isotype control IgG to replace the first antibody against C-3AP processed in a vehicle treated rat. Little specific staining was observed. (C) The staining using a brain slice adjacent to the slice shown on (B). C-3AP positive immunoreactivity stains green, while the nuclei double-stained with DAPI appear blue. (D) The staining in an estradiol treated rat. Some cells exhibited mixed C-3AP nuclear-cytoplasmic compartment immunostaining (arrow-pointed). The nucleic deformation labeled with C-3AP immunoreactivity was rather mild. (E) The staining in an ovary-intact rat. The immunoreactivity was often observed in both the disfigured nuclei and the cytoplasmic compartment (arrow-pointed). (F) A high magnificence of figure (C). The C-3AP immunoreactivity was primarily localized in the deformed nuclei (arrow pointed). Original magnification is 200x for A, C, and E, and 630x for B, D, and F.

Fig.6. Comparison of live cell and caspase-3 active peptide positive cell counts between vehicle treated (V), ovary intact (I), and estradiol treated (E) rats. The live cell ratio was produced by the mean live cell number in V, I and E groups divided by that in the no-ischemic group, respectively (see text for details). *$p<0.05$ vs. the V group, **$p< 0.01$ vs. the V group; ¶$p<0.05$ vs. the I group.

Fig. 1.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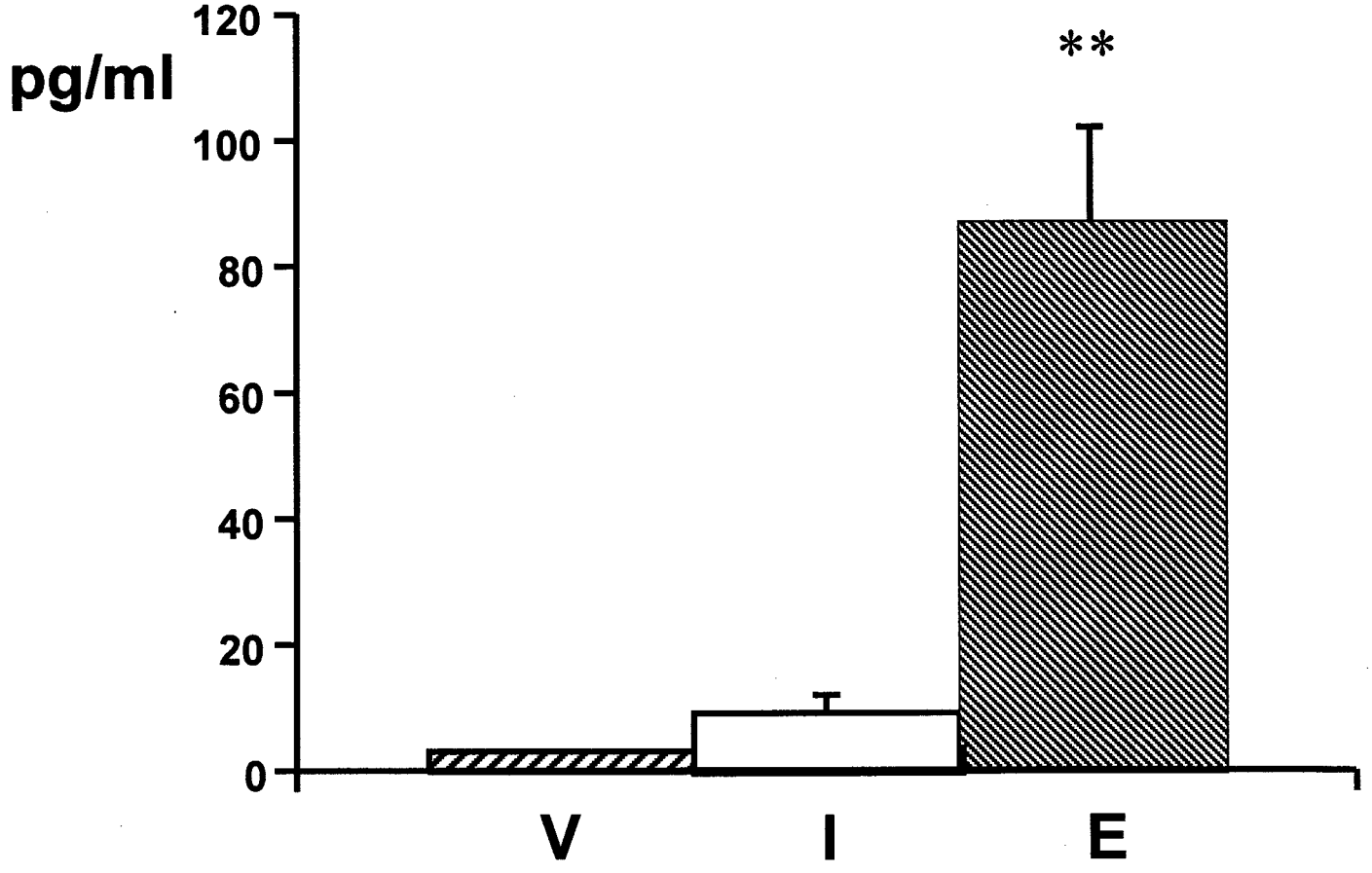

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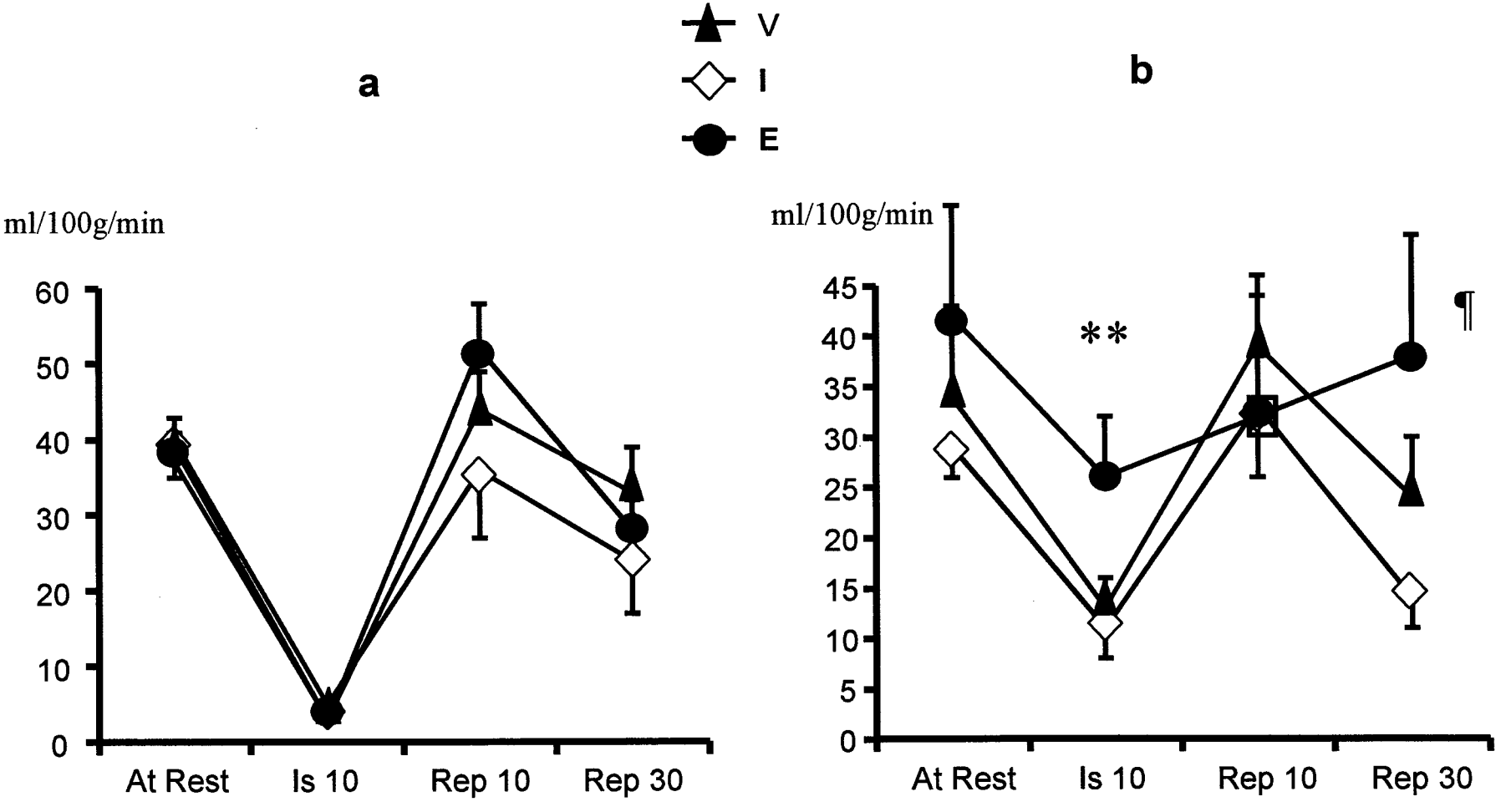
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Fig. 6.

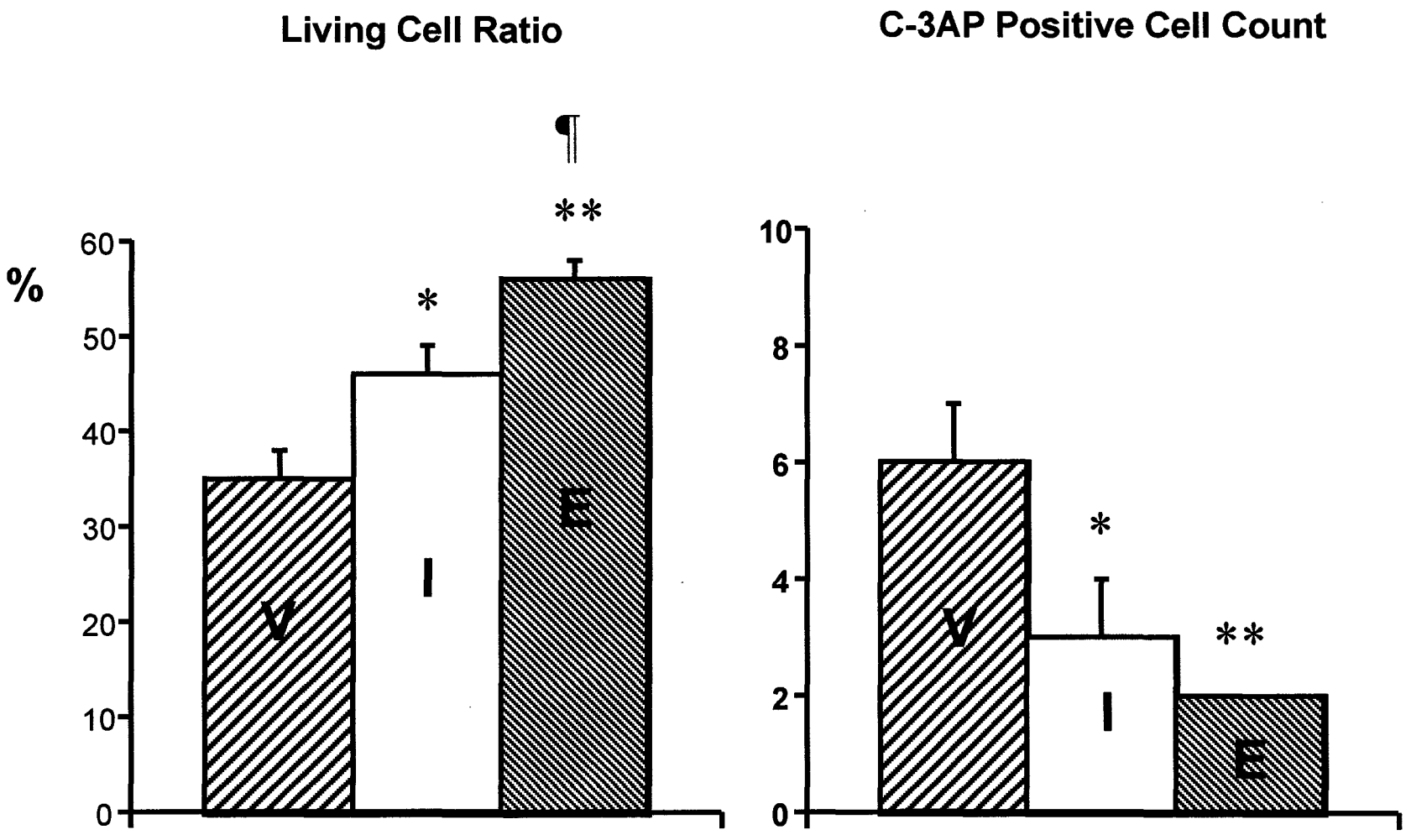
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Table 1. Experimental protocol and It's complement

Group	Experimental rat number	Anesthetic agents	Ovariectomy	BVAO	BCAO	Serum estradiol	H&E staining	C3AP staining
For pathology (n=21)		Halothane						
Estradiol	6		+	+	+	6	6	3
Vehicle	6		+	+	+	6	6	3
Intact	6		Sham-operation	+	+	6	6	3
No ischemia	3		+	+	Sham-operation	3	3	3
For physiological parameters (n=10)		Halothane						
Estradiol	4		+	+	+	-	-	-
Vehicle	3		+	+	+	-	-	-
Intact	3		Sham-operation	+	+	-	-	-
For cerebral blood flow measurement (n=22*)		Ketamine+xylazine						
Estradiol	8		+	+	+	-	-	-
Vehicle	7		+	+	+	-	-	-
Intact	7		Sham-operation	+	+	-	-	-

BVAO, bilateral vertebral artery occlusion; BCAO, bilateral carotid artery occlusion; H&E, hematoxylin and eosin; C3AP, caspase-3 active peptide. *10 rats, 4 in estradiol group and 3 in each of vehicle and intact groups, respectively, were excluded for analysis of cerebral blood flow because of sudden death or respiration stop occurring 3-6 minutes following BCAO.

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See list of themes and topics, pp. 17–18. Indicate below a first and second choice appropriate for programming and publishing your paper.

1st theme title: Disorders of the Nervous System **theme letter:** J
1st topic title: Degenerative disease: Alz **topic number:** 129

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2nd topic title: Ischemia: neuroportection **topic number:** 134

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Nitric Oxide May Mediate Estrogen's Neuroprotection Through a Receptor-Independent Mechanism.

Y. Wen*, E.J. Perez, P. Green and J.W. Simpkins, Department of Pharmacodynamics and Center for Neurobiology of Aging, University of, Florida Gainesville, FL 32610

Estrogens are known to have neuroprotective activity in both *in vivo* and *in vitro* models. Our observation of the lack of correlation between structure-activity relationships for neuroprotection and estrogenicity and potent neuroprotection by estrogens in cell types which lack known estrogen receptors indicate that this activity of estrogens is not mediated by classical estrogen receptor transcriptional activity. In the present study we use H_2O_2 as an insult to measure cytotoxicity using assays for both cell viability and Caspase3-like activity. We observed that both 17β-estradiol (E2) and NO are protective against H2O2 toxicity in SK-N-SH cells. We use DAF2-DA, a cell permeable NO specific dye to examine the effects of E2 on NO production. We observed that E2 induces NO within 10 min, with an EC50 of 8 nM. The protection of E2 can be blocked by a partial nNOS inhibitor L-NNA. We also examined cGMP which is a major mediator of NO's biological functions. We observed that cGMP also protects against H_2O_2 toxicity and NO's protection can be attenuated by ODQ, a cGMP inhibitor. The above data indicate NO may mediate estrogen's potent neuroprotection in a manner that is independent of classical estrogen receptors. (Supported by AG 10485, Apollo BioPharmaceutics, Inc. and U.S. Army Grant, DAMS 17-99-1-9473).

Key Words: (see instructions p. 4)

1. Estrogen
2. NO
3. cGMP
4. Neuroprotection

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2nd theme title: Disorders of NS & Aging **theme letter:** J
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STRUCTURE-ACTIVITY RELATIONSHIP OF ESTRATRIENES AGAINST GLUTAMATE TOXICITY IN A MOUSE HIPPOCAMPAL CELL LINE.

E.J. Perez*, K.L. Eberst, S.M. Oon, Z.Y.Cai, L. Prokai, D. Covey, and J. W. Simpkins. Dept. of Pharmacodynamics, College of Pharmacy, University of Florida, Gainesville, FL, College of Medicine, University of Washington, St. Louis, MO.

Estrogens are neuroprotective against a variety of in vitro and in vivo insults that include peroxide, serum deprivation, Aβ peptides, anoxia, and glutamate toxicity. In several of these cases, estrogen protection is mediated by its intrinsic antioxidant properties. The present study was performed to determine the structure-activity relationship (SAR) for the neuroprotective activities of estratrienes. HT-22 (murine hippocampal) cells that lack estrogen receptors and are sensitive to glutamate were used as our model for this study. Glutamate (10 and 20 mM) for ~16 hours caused a 50-80% decrease in cell viability, as assessed by calcein AM. Each estratriene-derivatized compound was tested for neuroprotection at concentrations that ranged from 0.01 to 10μM. 17β estradiol was used to compare potency and efficacy of each novel compound. Hydroxyl additions to the B- and C-rings of estratrienes eliminated protection of HT-22 cells against glutamate, and opening the B-ring reduced neuroprotection. The addition of alkyl chains to the 17-oxygen or pentanyl groups to the 16 carbon of the D-ring enhanced the neuroprotection potency of the estratrienes. In constrast, addition of alkyl chains to the 3-oxygen of the A-ring completely eliminated neuroprotective activity. These data indicate that modification of estratrienes that are expected to substantially reduce estrogenicity can be achieved with equivalent or enhanced neuroprotection activity, even in a cell type which lacks estrogen receptors. These data support an estrogen receptor-independent mechanism for the neuroprotective effects of estratrienes. The compounds may be useful drug candidates for the treatment of neurodegenerative diseases.

(This work was supported by AG 10485, Apollo BioPharmaceutics, Inc. and U.S. Army Grant, DAMD 17-99-1-9473).

Key Words: (see instructions p. 4)

1. Estrogen
2. Neuroprotection
3. Structure-Activity Relationships
4. Neurotoxicity

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1st topic title: Expression and regulation **topic number:** 11

2nd theme title: Development and Regeneration **theme letter:** A
2nd topic title: Neuronal Death **topic number:** 17

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ESTROGEN MODULATION OF BCL-2 FAMILY PROTEIN EXPRESSION.

P.S. Green*, C.T. Fulp, and J.W. Simpkins. Center for the Neurobiology of Aging and Department of Pharmacodynamics, University of Florida, Gainesville, FL 32610

Estrogens are potent neuroprotective agents in a variety of animal and cell culture models. The purpose of this study was to explore the possible role for modulation of bcl-2 family protein expression in βE2-mediated neuroprotection. We examined βE2-induced changes in expression of bcl-2, bcl-x_L, and bax by Western blot analysis in three different neuronal types. SK-N-SH neuroblastoma cells, HT-22 transformed neuronal cells, and primary neocortical neurons were chosen as βE2 attenuated Aβ-induced toxicity at a 2 nM concentration in all three neuronal types. All three neuronal types showed significant bax immunoreactivity. Bcl-x_L immunoreactivity was readily detectible in both SK-N-SH cells and primary neuronal cells but not HT-22 cells. Bcl-2 was detected by our Western analysis only in the SK-N-SH cells. Exposure of SK-N-SH and primary neuronal cultures to βE2 for 24 h caused a dose-dependent increase in bcl-x_L immunoreactivity. The maximal effect was seen at a 10 nM βE2 concentration and resulted in about a 2.5-fold increase in bcl-x_L band density. No significant effect of βE2 on either bcl-2 or bax immunoreactivity was seen. These data suggest that modulation of bcl-2 family proteins, specifically increased bcl-x_L expression, could contribute to βE2-mediated neuroprotection in SK-N-SH cells and rat primary neocortical neurons.

(Supported by NIH AG 10485, US Army DAMD 17-99-1-9473, and Apollo BioPharmaceutics, Inc.)

Key Words: (see instructions p. 4)

1. Estradiol
2. Neuroprotection
3. BCL-XL
4. A Beta

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1st topic title: Ischemina: Neuro-protection and and tolerance **topic number:** 134

2nd theme title: Disorder of the nervous system **theme letter:** J
2nd topic title: Ischemia: Cellular and molecular **topic number:** 133

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Improvement In Cerebral Ischemia Outcome With Non-Surgical Methods of Reducing Testosterone In Male Rats. J. Cutright, S.Yang, Z. He, A. L. Day, J. W. Simpkins*. Center for The Neurobiology of Aging, Department of Pharmacodynamics, and Neurological Surgery, University of Florida, Gainesville, FL 32610

Cerebral ischemia is less damaging in females than in males. While estrogen's neuroprotective effects have been established, the role of androgens in this sex difference is less clear. We have reported that surgical castration reduces cerebral damage following ischemia in male rats (Brain Research 796: 296-298, 1998). The present study further assesses the role of testosterone in cerebral ischemia using a variety of non-surgical methods to reduce serum testosterone. Male SD rats were treated with leuprolide (0.1 mg/kg every 12 h for 7 d) or saline vehicle, which produced serum testosterone of 0.4 ng/ml and 7 ng/ml, respectively. Anesthetic stress (ketamine 60 mg/kg and xylazine 10 mg/kg) 6 h prior to cerebral infarct reduced serum testosterone to 0.5 ng/ml from 2.2 ng/ml in unstressed controls. All animals were then exposed to cerebral ischemia using 1 h middle cerebral artery occlusion followed by 23 h of reperfusion. Chronic treatment with leuprolide reduced cerebral infarct volume from 16±1% to 8±1%. Additionally, the stress-induced reduction in testosterone was associated with a reduction in infarct volume from 18±3% to 8.5±1.5%. Collectively these data indicate that non-surgical methods of reducing serum testosterone limit cerebral damage following an infarct and suggest that testosterone reduction could be useful in limiting cerebral damage associated with planned ischemic events.

(Supported by NIH AG 10485, Apollo BioPharmaceutics, Inc. and U.S. Army Grant DAMD 17-19-1-9473.)

Key Words: (see instructions p. 4)

1. Androgens
2. Leuprolide
3. Stroke
4. Stress

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1st topic title: Ischemia: Neuroprotection and Tolerance **topic number:** 134

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2nd topic title: Ischemia: Cerebral Blood Flow **topic number:** 135

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PHYSIOLOGICAL ESTRADIOL DELAYS NEURON DEATH FOLLOWING TRANSIENT FOREBRAIN ISCHEMIA IN RATS. Z. He*, S.H. Yang, Y.J. He, J. Cutright, A.L. Day, J.W. Simpkins. Dept. Pharmacodynamics and Neurosurgery, Center of the Neurobiology of Aging, Univ. of Florida, Gainesville, FL 32610

Hippocampal neurons undergo a delayed death over days following transient global ischemia; and understanding the time-course of this phenomenon is necessary to determine whether a therapeutic intervention provides any effective protection. We conducted a study to see whether physiological levels of estradiol would permanently salvage hippocampal neurons in rats subjected to a global ischemic insult. Ovariectomized female rats were subjected to 20 minutes of ischemia using a four-vessel occlusion technique, and then allowed to survive for 48 or 168 hours. Estradiol was administered subcutaneously by implanting a Silastic® pellet containing the steroid (4mg/ml) 24 hours before the ischemia onset. In both groups, hippocampal blood flow was decreased 54-62% from resting levels (41-48 ml/min/100g) at 10 minutes after ischemia, and recovered to 77-91% and 95-126% of resting levels at 10 and 30 minutes after reperfusion, respectively. Serum 17β-estradiol levels in estradiol-treated rats were 92±12 pg/ml at 48 hours and 37±7 pg/ml at 168 hours ($p<0.05$ vs. 48-hour group) after ischemia, and were significantly higher than their corresponding controls. Live cell counts in the hippocampal CA1 subregion were significantly different between the estradiol- and the vehicle-treated groups at 48 hours (25±1% vs. 44±3% cell number reduction, $p = 0.0019$) but not at 168 hours (47±5% vs. 58±4% cell number reduction) following ischemia. Sustained physiological levels of estradiol appear to delay but not prevent hippocampal damage following transient global ischemia. The significant difference between the 48-hour and 168-hour live cell counts may be dependent on serum estradiol concentration, and supra-physiologic levels may be required to produce any sustained neuronal protective effects.

(Supported by NIH grant AG 10485, Apollo BioPharmaceutics, Inc, and U.S. army grant DAMD 17-99-1-9473)

Key Words: (see instructions p. 4)

1. Ischemia
2. CA1
3. Estradiol
4. Neuron Death

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ESTRADIOL PROTECTS AGAINST ATP DEPLETION AND MITOCHONDRIAL MEMBRANE POTENTIAL DECLINE INDUCED BY 3-NITROPROPRIONIC ACID IN SK-N-SH HUMAN NEUROBLASTOMA CELLS.

J. WANG*, E. J. PEREZ, P. S. GREEN, J. W. SIMPKINS. Dept. of Pharmacodynamics, Center for the Neurobiology of Aging, University of Florida, Gainesville, FL 32610

Mitochondria are increasingly recognized as the important target of toxicity during ischemia, hypoxia and toxic chemical exposures. Mitochondria dysfunction leading to ATP depletion may be a common pathway of cell death in these cases. Estrogens have been reported to be neuroprotective against excitotoxicity, oxidative injury and chemical toxicity in experiment models and been proposed to play a role in the modulation of cerebral energy/glucose metabolism. To address whether estrogens are involved in the regulation of mitochondrial function, we chose 3-nitroproprionic acid (3-NPA, a succinate dehydrogenase inhibitor) to uncouple oxidative phosphorylation. ATP levels in SK-N-SH cells were measured after exposed to 3-NPA. The result showed that 3-NPA (10 mM) initially increased ATP levels with peak maxima occuring at 2 h then caused a 40% and a 50%-80% decrease in ATP levels when treated for 12 h and 24 h, respectively. The ATP depletion induced by 3-NPA was attenuated by pretreating cells for 4-5 h with 17β-estradiol. For 12 h and 24 h 10 mM 3-NPA exposures, 17β-estradiol pretreatment caused a dose-dependent increase in ATP level back to 80% and 50%, respectively, of that in control cells. The function of mitochondria following exposures to 3-NPA were also assessed by evaluating the mitochondrial transmembrane potential ($\Delta\psi_m$) using the probe rhodamine 123. 3-NPA induced a 60% decrease in fluorescence intensity at 12 h exposure, an effect that presisted for 24 h. 17β-estradiol pretreatment eliminated this effect of 3-NPA on mitochondria transmembrane potential. This study provides evidence that these mitochondrial actions of 17β-estradiol may contribute to the neuroprotective effect of estrogens. (Supported by AG 10485, U.S. Army Grant DAMD 17-99-1-9473 and Apollo BioPharmaceutics, Inc.)

Key Words: (see instructions p. 4)

1. 17-beta-Estradiol
2. 3-nitroproprionic acid
3. ATP
4. mitochondrial transmembrane potential

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Themes and Topics

See list of themes and topics, pp. 17–18. Indicate below a first and second choice appropriate for programming and publishing your paper.

1st theme title: Disorders of the Nervous System **theme letter:** J
1st topic title: Neurotoxicity **topic number:** 141

2nd theme title: Disorders of the Nervous System **theme letter:** J
2nd topic title: Degenerative disease: other **topic number:** 130

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INHIBITION OR DOWN-REGULATION OF PROTEIN KINASE C ENHANCES ESTROGEN-INDUCED NEUROPROTECTION IN AN *IN VITRO* MODEL.

D.G. Watson*, C. Fiola, and J.W. Simpkins. Department of Pharmacodynamics and Center for the Neurobiology of Aging, University of Florida College of Pharmacy, Gainesville, FL 32610.

The neuroprotective effects of estrogen compounds have been reported in a wide variety of animal and cell culture models. However, the mechanism(s) responsible for estrogen's neuroprotective effects are currently not well understood. In the present studies, the role of protein kinase C (PKC) in mediating estradiol-induced neuroprotection was examined in an immortalized hippocampal cell line, HT-22 cells lack classical nuclear estrogen receptors (ER), eliminating ER-mediated effects as a potential site of neuroprotective action. The neuroprotection model utilized calcein fluorescence to quantitate cell viability following an 18-24 h exposure to glutamate (5-10 mM). In this model, estradiol-induced neuroprotection was observed at concentrations above 1 μM. Inhibition of PKC by bis-indolylmaleimide (BIM, 1 μM) or LY333531 (1 μM) significantly enhanced estradiol-induced neuroprotection when administered prior to the glutamate insult. Similarly, down-regulation of PKC by exposure of HT-22 cells to the phorbol ester PMA also enhanced estrogen-induced neuroprotection. Furthermore, we demonstrated that PKC inhibition and down-regulation were also neuroprotective in a dose-dependent manner in the absence of estradiol. In this model, neuroprotection was also observed following inhibition of MAPK signaling pathway by the MEK inhibitor PD98059. Since we observed that exposure of HT-22 cells to estradiol alters the expression and intracellular distribution of at least one PKC isozyme (epsilon), these data suggest a potential role for PKC and the MAPK signaling pathway in mediating the neuroprotective effects of estrogens. These data support the hypothesis that PKC inhibition or down-regulation mediates a neuroprotective signal from estrogen, which is then propagated through the MAPK pathway and ultimately results in altered gene expression beneficial to cell survival.

(Supported by NIH AG10485, U.S. Army grant DAMD 17-99-1-9473, and Apollo BioPharmaceutics, Inc.)

Key Words: (see instructions p. 4)

1. 17 beta estradiol
2. PKC isozymes
3. Neurotoxicity
4. MAPK signaling pathway

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1st topic title: Ischemia: Neuroprotection and **topic number:** 134

2nd theme title: Disorders of the Nervous System **theme letter:** J
2nd topic title: Ischemia: Cerebral Blood Flow **topic number:** 135

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17-β ESTRADIOL CAN REDUCE SECONDARY ISCHEMIC DAMAGE AND MORTALITY OF SUBARACHNOID HEMORRHAGE

S-H. Yang*, Z. He, S. S. Wu, Y-J. He, J. Cutright, W. J. Millard, A. L. Day, J. W. Simpkins.
Departments of Neurosurgery and Pharmacodynamics, Center of the Neurobiology of Aging, Colleges of Medicine and Pharmacy, Department of Statistics, College of Liberal Arts and Sciences, University of Florida, Gainesville, Florida, 32610.

Subarachnoid hemorrhage (SAH) is a unique disorder commonly occurring when an aneurysm ruptures, leading to bleeding and clot formation, with higher incidence in female. To evaluate the influence of 17-β estradiol (E2) in the outcome of SAH, SAH was induced by endovascular puncture of the intracranial segment of internal carotid artery in 15 intact female (INT), 19 ovariectomized female (OVX) and 13 ovariecomized female rats with E2 replacement (OVX+E2). Cerebral blood flow was recorded before and after SAH. All the animals were decapitated immediately after death or 24 hours after SAH for clot volume analysis. The brains were sliced and stained with 2,3,5-triphenyltetrazolium chloride (TTC) for secondary ischemic lesion analysis. The CBF decreased to 29.6±17.7%, 22.8±8.3% and 43.5±22.9% on the ipsilateral side ($p=0.01$) and to 63.4±14.1%, 57.4±11.0% and 66.6±17.9% on the contralateral side ($p=0.26$) in INT, OVX and OVX+E2, respectively. The mortality was 53.3%, 68.4% and 15.4% in INT, OVX and OVX+E2, respectively ($p=0.01$), while no significant difference in clot volume was noted among groups. The secondary ischemic lesion volume was 9.3±8.4%, 24.3±16.3% and 7.0±6.4% in INT, OVX and OVX+E2, respectively ($p<0.01$). This study demonstrated that E2 can reduce the mortality and secondary ischemic damage in a SAH model without affecting the clot volume.

(Supported by NIH grant AG 10485, Apollo BioPharmaceutics, Inc, and U.S. Army grant DAMD 17-99-1-9473)

Key Words: (see instructions p. 4)

1. Estradiol
2. Stroke
3. Neuroprotection
4. Cerebral Blood Flow

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27th American Heart Association International Stroke Conference
ABSTRACT SUBMISSION FOR 2002

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Category: 0008 Experimental Ischemia (experimental aspects of ischemic and hemorrhagic stroke, including cellular and molecular mechanisms affecting neurons, glia and inflammatory cells)
Keyword 1: Estrogen
Keyword 2: Neuroprotection
Presentation Preference: Poster Only
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Mordecai Y.T. Globus New Investigator Award: No
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Presenter #: 1

Title:

Neuroprotective effects of a novel non-receptor binding estrogen analogue during stroke

Abstract:

Background and Purpose: Estrogens have been shown to be potently neuroprotective. Estrogen analogues with neuroprotective activities but lacking the hormonal properties have advantages from a clinical perspective. The present study was undertaken to determine the neuroprotective effects of a novel non-estrogenic estrogen analogue, ZYC3. **Methods:** Cytotoxicity was induced in HT-22 cells by 10mM glutamate. 17β-estradiol (E2) or ZYC3 was added immediately before the glutamate exposure. Cell viability was determined by a calcein assay. The ability of E2 and ZYC3 to bind to human α (ERα) and β (ERβ) estrogen receptors was determined by a ligand competition binding assay. Ischemia-reperfusion injury was induced by temporary middle cerebral artery occlusion (MCAO). E2 or ZYC3 (100μg/kg) was administered 2 hours or immediately before MCAO, respectively. Infarct volume was determined by 2,3,5-triphenyltetrazolium chloride staining. CBF was recorded during and within 30 minutes after MCAO by the hydrogen clearance method. **Results:** ZYC3 significantly decreased toxicity of glutamate with an effectiveness more than 2 times that of E2, while it did not bind to either ERα or ERβ. Infarct volume was significantly reduced to 83 ± 19 and 122 ± 18 mm^3 in the ZYC3 and E2 group, respectively, compared to 253 ± 16 mm^3 in the ovariectomized (OVX) group. During MCAO, both E2 and ZYC3 significantly increases CBF in the non-ischemic side, which was 92 ± 6 and 83 ± 16 ml/min/100g tissue, respectively, compared to 51 ± 8 ml/min/100g tissue in the OVX group. No significant differences were found in CBF in the ischemic side during MCAO, which was 7 ± 1, 8 ± 2, and 6 ± 1 ml/min/100g tissue in the OVX, ZYC3 and E2 group, respectively. However, E2 and ZYC3 significantly increase CBF in both sides within 30 minutes after reperfusion. **Conclusion:** Our study shows that ZYC3, a novel non-receptor binding estrogen analogue, possesses both neuroprotective and vasoactive effects, which offer the attractive possibility of clinical application for stroke without the hormonal stimulation of estrogens. It also suggests that both the neuroprotective and vasoactive effects of estrogen are receptor-independent.