

Award Number: W81XWH-09-2-0122

TITLE: Amelioration of Ischemia/Reperfusion Injury During Resuscitation from Hemorrhage by Induction of Heme Oxygenase-1 (HO-1) in a Conscious Mouse Model of Uncontrolled Hemorrhage

PRINCIPAL INVESTIGATOR: Phillip Bowman

CONTRACTING ORGANIZATION: The Geneva Foundation
Tacoma, WA 98402

REPORT DATE: October 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE October 201H		2. REPORT TYPE Final		3. DATES COVERED 1 September 20FG- 3F August 201H	
4. TITLE AND SUBTITLE Amelioration of Ischemia/Reperfusion Injury During Resuscitation from Hemorrhage by Induction of Heme Oxygenase-1 (HO-1) in a Conscious Mouse Model of Uncontrolled Hemorrhage				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-09-2-0122	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Phillip Bowman				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Geneva Foundation Tacoma, WA 98402				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Ischemia occurs whenever there is interruption of the flow of blood to tissues or organs. It is the most common cause of death in heart disease and stroke as well as traumatic injury. Survival of the initial insult is followed by further injury that occurs during the reintroduction of oxygen with the restoration of blood flow. This injury occurs following hemorrhage because some tissues are deprived of blood to protect others as part of the fight or flight response. Heme oxygenase-1 (HO-1) induction is correlated with a significant reduction in ischemic injury and 1-[2cyano-3, 12-dioxoleana-1, 9(11)-dien 28-oy]limidazole (CDDO-lm) a new synthetic triterpenoid that has been shown to possess potent anti-inflammatory and antioxidant properties, and is a potent inducer of HO-1. The hypothesis to be tested is that a significant reduction in indices of I/R injury will be obtained by induction of HO-1 during resuscitation with CDDO-IM following hemorrhage.					
15. SUBJECT TERMS Hemorrhage shock, cytoprotection, ischemia/reperfusion injury, drugs					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	Page
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	6
Conclusion.....	7
References.....	8
Appendices.....	8

INTRODUCTION: HIF1 α , a master regulator of the hypoxic response has been implicated in ischemic preconditioning. Ischemic preconditioning has been shown to provide significant protection from a subsequent lethal ischemic event. Additionally, Heme oxygenase-1 (HO-1) is an inducible Phase 2 enzyme that degrades toxic heme. Heme contains an iron and when released under pathological conditions such as cellular stresses and ischemia, free heme may act as a source of free radicals. Cells have therefore evolved a system to degrade heme, a system composed of inducible heme oxygenases 1 (HO-1) and constitutive HO-2. The end products of the degradation include cytoprotective biliverdin and carbon monoxide; as a result, heme oxygenases are potentially cytoprotective. Ischemia occurs whenever there is interruption of the flow of blood to tissues or organs. It is the most common cause of death in heart disease and stroke as well as traumatic injury. Survival of the initial insult is followed by further injury that occurs during the reintroduction of oxygen with the restoration of blood flow. This injury occurs following hemorrhage because some tissues are deprived of blood to protect others as part of the fight or flight response. Recent investigations have shown 2-cyano-3,12 dioxooleana-1,9 dien-28-oyl imidazoline (CDDO-Im) a new synthetic triterpenoid to possess potent anti-inflammatory and antioxidant properties, and is a potent inducer of HO-1. We hypothesized that chemically induced HO-1 upregulation with the novel triterpenoid CDDO-Im (2-cyano-3,12 dioxooleana-1,9 dien-28-oyl imidazoline), a robust inducer of Phase 2 genes, protects against ischemic injury. To measure cytoprotection in terms of luminescence, we also screened genetically engineered mouse cells that express luciferase when HIF1 α accumulates.

BODY: CDDO-Im is a synthetic triterpenoid recently shown to induce cytoprotective genes through the Nrf2-Keap1 pathway, an important mechanism for the induction of cytoprotective genes in response to oxidative stress. Heme oxygenase-1 is highly inducible and its induction is correlated with significant protection from the deleterious effects of ischemia. CDDO-Im (2-cyano-3,12 dioxooleana-1,9 dien-28-oyl imidazoline), a new synthetic triterpenoid has been shown to possess potent anti-inflammatory and antioxidant properties, and is a potent inducer of HO-1 and is being investigated as an additive to a new resuscitation fluid that might be counteracting the deleterious effects of the ischemia of hemorrhage shortly after injury during the initial resuscitation. One of the most critical components of developing a new drug of treatment of a specific disease state is determination of an appropriate dose of the drug with maximum benefit and minimum off-target effects. We employed a new technique, termed snapshot pharmacokinetics, to hone in on an appropriate dose of CDDO-Im for use in the mouse model of hemorrhage. Following determination of an appropriate dose we determined the timing of resuscitation for maximum benefit of the drug.

We also tested and screened other drugs such as CAPE, CAPA for their ability to induce HO-1 and HIF1 α and produce a cytoprotective effect. We screened genetically engineered mouse cells that express luciferase when HIF1 α accumulates. Deferoxamine induces HIF1 α by inhibiting the activity of Fe⁺⁺ dependent prolyl hydroxylase which is required for activation of the oxygen dependent domain of HIF1 α and was used as a positive control. Caffeic acid phenethyl ester (CAPE) has been previously suggested to inhibit HIF1 α prolyl hydroxylase. We have found that Caffeic acid phenethyl amide (CAPA) and CAPE along with CDDO-Im, which induced HO-1 mediated cytoprotection against menadione-induced-oxidative stress, also induces HIF1 α and this may explain their cytoprotective effect.

KEY RESEARCH ACCOMPLISHMENTS:

Due to Base Realignment and Closing (BRAC) issues, significant delays in research occurred at two times during the study. Firstly, the USAISR vivarium was closed for about two years in order to renovate it and merge it with the new vivarium in the newly constructed Battlefield Health and Trauma Center for Excellence. During this period animals were housed at the animal facility at Brook City Base, San Antonio, TX. However, in vivo imaging still had to be performed at the USAISR where the Xenogen in vivo imaging system resided. This required transporting the animals from Brooks City Base to USAISR (about 15 miles away). Also, the rules during this period were that no animal bought from Brooks City Base could be in the Institute for more than 8 hr. Consequently, the imaging data obtained during this period could not be used as the behavior of the mice was significantly when this additional stress of transportation was factored in. Secondly, the entire DCR (Damage Control, Resuscitation) group at US Army Institute of Surgical Research (USAISR) was moved to a new facility in Oct. of 2011. This delayed analysis mouse tissues obtained from repeats of the work done at Brooks City Base an additional 6 months. In addition, a postdoctoral fellow working on an MRMC project left in August 2010 for a teaching job and the postdoctoral fellow working under the Geneva Foundation project had to fill the MRMC postdoctoral fellow position as MRMC projects pays for 90% of the research budget and takes precedence over congressional projects. I was finally able to hire a post doctoral fellow in August 2012 to work full time on this project.

Jan-Dec 2009: Preliminary Studies -> Studied Structure activity relationship of Caffeic Acid Phenethyl Ester (CAPE) and its amide derivative CAPA against oxidant stress in human endothelial cells; Used non-invasive imaging techniques as a tool to demonstrate hemorrhage-induced global ischemia with a transgenic mouse expressing luciferase coupled to hypoxia-inducible factor (HIF1 α).

Jan-Mar 2010: Established role for hypoxia in some organs of the mouse following hemorrhage of the FVB.1 29S6-Gt(ROSA)26Sortml (HIF1 α luc)Kael/J (HIF1 α Luc) inbred

strain using luminometry analysis. The intestine, spleen and liver were effected organ while brain, lung, skeletal muscle and heart were not much affected.

Jan-June 2010: Determined that 100 nm CDDO was optimal in vitro for induction of HO-1 in the skin fibroblasts of the HIF1 α Luc strain of mouse.

Mar-Aug 2010: Determined that 100 nM CDDO was also effective in inducing HO-1 in human umbilical vein endothelial (HUVEC) cells indicating a cross species benefit.

August 2010 – October 2011: Compared CDDO-Im, CAPE, CAPA induced HO-1 mediated cytoprotection against menadione-induced-oxidative stress in HUVEC cells.

November 2011 – December 2012: Determined appropriate dose of CDDO-Im (50-100 nm) in a mouse model.

January 2012 –August 2013: Evaluated cytoprotective effects of CDDO-Im in a mouse model. Analyzed various organs, using Western Blot and other biochemical assays, for proof of cytoprotection.

REPORTABLE OUTCOMES:

List of Presentations and Manuscripts. The presentations and manuscript contains all the relevant data (figures, tables, conclusions) pertaining to this research. Copies of manuscript and presentations are attached with this report.

PRESENTATIONS:

1. Experimental Biology 2009: Cytoprotective effect of a synthetic triterpenoid against oxidative stress in human umbilical vein endothelial cells (HUVEC). *FASEB J.* April 2009 23 (Meeting Abstract Supplement) 937.7
2. ATACC 2009: Noninvasive imaging of hemorrhage-induced global ischemia with a transgenic mouse expressing luciferase coupled to hypoxia-inducible factor (HIF1 α).
3. Experimental Biology 2009: Structure activity relationship of Caffeic Acid Phenethyl Ester (CAPE) and its amide derivative CAPA against oxidant stress in human endothelial cells. *FASEB J.* April 2009 23 (Meeting Abstract Supplement) 937.8.
4. Experimental Biology 2010: Cytoprotection of Human Endothelial Cells from Oxidative Stress by Polyphenols: the Role of Gene Expression versus Direct Antioxidant Effect.
5. Experimental Biology 2010: Induction of Hypoxia Inducible Factor 1 Alpha (HIF1 α) by Caffeic Acid Phenethyl Ester (CAPE) and Caffeic Acid Phenethyl Amide (CAPA) in Mouse Skin Fibroblasts. *FASEB J.* April 2010 24 (Meeting Abstract Supplement) 760.2.

6. ATACC 2011: Poor Correlation between In Vivo Imaging and Production of Light by Organs in Transgenic Mouse Engineered to Express Luciferase in Response to Hypoxia.
7. Experimental Biology 2011: Time Course and Network Analysis of 1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) Induction of Cytoprotective Genes in Human Umbilical Vein Endothelial Cells (HUVEC) Against Oxidant Stress. *FASEB J.* April 2011 25 (Meeting Abstract Supplement) 1090.3.
8. American Association of Pharmaceutical Scientists (AAPS) 2012: Determination of the Minimum Exposure Time for Effecting Cytoprotection in Human Umbilical Vein Endothelial Cells (HUVEC) for Caffeic Acid Phenylethyl Ester (CAPE) and Amide (CAPA).
9. American Association of Pharmaceutical Scientists (AAPS) 2013: Comparison of atmospheric oxygen versus physiological levels on cytotoxicity of menadione and cytoprotection by antioxidants in human endothelial cells.
10. American Association of Pharmaceutical Scientists (AAPS) 2013: Pharmacokinetic Profiles of Caffeic Acid Phenethyl Amide (CAPA) and Caffeic Acid Phenethyl Ester (CAPE) in Male Sprague-Dawley Rats.
11. American Association of Pharmaceutical Scientists (AAPS) 2013: Comparison of caffeic acid phenylester (CAPE), caffeic acid phenylamide (CAPA) and 2-cyano-3,12 dioxooleana-1,9 dien-28-imidazolide (CDDO-Im) in protecting human endothelial cells from oxidative stress: The Role of Heme Oxygenase.
12. American Association of Pharmaceutical Scientists (AAPS) 2013: Network Analysis of the Cytoprotective Effect of CDDO-Im against Oxidant Stress in Human Umbilical Vein Endothelial Cells (HUVEC).

MANUSCRIPTS:

1. Comparison of Bioluminescence Imaging and Luminometry for Detection of Luciferase Activity in Transgenic Mice Engineered to Express Luciferase in Response to Hypoxia. (submitted)
2. Cytoprotection of human endothelial cells against menadione-induced oxidative stress by 2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im): a more potent cytoprotectant than caffeic acid phenethyl ester (CAPE). (In preparation)
3. Gene expression of the cytoprotective response of human endothelial cells to 2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) and methyl ester (CDDO-Me). (In preparation)
4. Pharmacodynamics of HO-1 induction in mice pretreated with CDDO-Me and subjected to hemorrhagic shock. (In preparation)

CONCLUSION: One of the current requirements for development of drugs for treatment hemorrhagic shock is that a candidate drugs be FDA approved for some use or close to approval. CDDO-Me and CDDO-Im are in phase 3 clinical trials for chronic kidney disease and diabetes and as a chemopreventative for cancer development. Both CDDO-Me and CDDO-Im have been demonstrated to potently upregulate HO-1 in vitro. Synthetic oleanolic acid derivatives may become important contributors to devising polypharmacological approaches to reducing the impact of hemorrhagic shock.

REFERENCES: See presentations and manuscripts in preparation

APPENDICES: Please refer to **REPORTABLE OUTCOMES** section.

SUPPORTING DATA: Please refer to **REPORTABLE OUTCOMES** section.



Cytoprotective effect of a synthetic triterpenoid against oxidative Stress in human umbilical vein endothelial cells (HUVEC)

²Phillip Bowman, ^{1,2}Xinyu Wang, ²James Bynum, ^{1,2}John Yang, and ¹Salomon Stavchansky,

¹Pharmaceutics Division, College of Pharmacy, University of Texas, Austin TX and

²United States Army Institute of Surgical Research, San Antonio, TX 78234



Abstract

Abstract Number: 2128

Induction of phase II enzymes, in particular the 32 kd stress protein heme oxygenase-1 (HO-1), is cytoprotective in human endothelial cells. We previously demonstrated that caffeic acid phenethyl ester (CAPE) was cytoprotective against menadione-induced oxidative stress in HUVEC largely via the induction of HO-1. * Here, we tested the cytoprotective activity of 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), a new synthetic triterpenoid (Dr. Michael Sporn, Dartmouth University) against oxidative stress. Dose response studies indicated that CDDO-Im at 200 nM was more cytoprotective against menadione toxicity than an optimal dose of CAPE (20 μM), resulting in endothelial cell survival of 80% compared to 60% for CAPE. Messenger RNA for HO-1 was increased 90-fold in the presence of CDDO-Im, while only 13-fold by CAPE. Western blot analysis of HO-1 protein product indicated that by 6 h, CDDO-Im induced an 8-fold higher level of HO-1 while CAPE induced a 2-fold increase. The results indicate that CDDO-Im is a much more potent cytoprotectant than CAPE, and this beneficial effect correlates well with the induction of HO-1.

*Wang X, et al. Eur J Pharmacol. 2008 Sep 4;591(1-3):28-35.

Introduction

The induction of phase II gene products has been proposed to protect cells from oxidative stress (1). We have previously shown that heme oxygenase-1 (HO-1), a phase II enzyme, protected HUVEC from menadione (MD)-induced oxidative injury following treatment by caffeic acid phenethyl ester (CAPE), a polyphenolic antioxidant (2). To further improve cytoprotection, we investigated 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), a new synthetic triterpenoid (from Dr. Michael Sporn, Dartmouth University) and potent inducer of phase II enzymes (3).

Methods

Cell culture:

HUVEC (LifeLine Cell Technology, Walkersville, MD) were cultivated on 1% gelatin-coated 48-well multiplates (Corning Incorporated, Corning, NY) in Vasculife[®] Medium. Only the second through fifth population doublings of cells were used.

Methods

In vitro assay:

Cell viability was assessed at 24 hours after initiation of treatment using Alamar Blue[™] (Biosource International, Camarillo, CA). CAPE and CDDO-Im were assayed for cytotoxic effects in HUVEC. Doses of CAPE and CDDO-Im causing less than 90% cell viability (compare to control group) were considered toxic and not applied in cytoprotection assay. Confluent HUVEC were pretreated with either various concentrations of CDDO-Im and CAPE or 0.1% DMSO (control) for 6 hrs, then exposed to a toxic dose of MD for additional 24 hrs. Cell viability was assessed compared to the vehicle controls. At least three independent experiments were performed and each was done in triplicate.

HO-1 induction confirmation:

HUVEC were pretreated with 100 nM CDDO-Im and 20 μM CAPE or 0.1% DMSO (control) for 6 hrs (RNA) and 24 hrs (Protein), respectively. HO-1 induction was confirmed using RT-PCR and western blot. For RT-PCR, the cDNA was obtained by reverse transcription RNA obtained directly from the treated cells using the Cells-to-cDNA[™] II kit (Applied Biosystems/Ambion, Austin, TX) and Real-time PCR was performed on a LightCycler[™] thermal cycler (Idaho Technology, Salt Lake City, UT). HO-1 gene was normalized to the expression level of 18S for each sample. Relative quantification was performed with the comparative C_T method. For western blot, the protein was obtained by direct lysis of the treated cells and directly run on Invitrogen E-page gels, which were transferred to nitrocellulose membrane using the iBlot system (Invitrogen Corporation, Carlsbad, CA). Prior to HO-1 antibody application, the blots were stained with SyPro Ruby blot stain (Invitrogen) to normalize the amount of protein.

Results

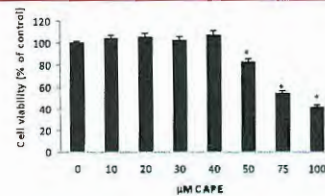


Figure 1. Cytotoxicity of CAPE in HUVEC. *: p < 0.05 versus control (0 μM CAPE). CAPE at doses of 50, 75, and 100 μM were cytotoxic and less than 40 μM were used for cytoprotection assay.

Results

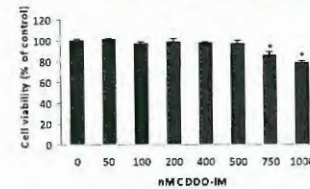


Figure 2. Cytotoxicity of CDDO-IM in HUVEC. *: p < 0.05 versus control (0 nM CDDO-IM). CDDO-IM at doses of 750 and 1000 nM were cytotoxic and less than 500 nM were used for cytoprotection assay.

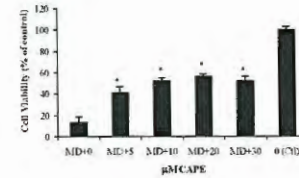


Figure 3. Cytoprotection of CAPE against 70 μM MD toxicity in HUVEC. *: p < 0.05 versus MD alone (MD+0 μM CAPE). The cytoprotective effect of CAPE was dose dependent. CAPE at 20 μM protected HUVEC against MD-induced toxicity (~10% cell survival) resulting in around 60% cell survival.

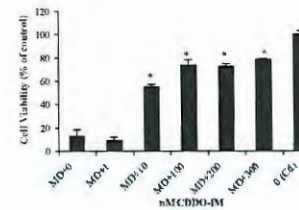


Figure 4. Cytoprotection of CDDO-IM against 70 μM MD toxicity in HUVEC. *: p < 0.05 versus MD alone (MD+0 nM CDDO-IM). The cytoprotective effect of CDDO-IM was dose dependent. CDDO-IM at 100 nM protected HUVEC against MD-induced toxicity (~10% cell survival) resulting in around 80% cell survival, which is much more potent than CAPE in cytoprotection.

Results

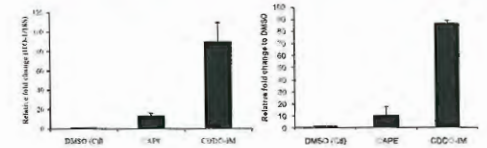


Figure 5. HO-1 mRNA induction in HUVEC by 6 hr treatment of 20 μM CAPE and 100 nM CDDO-Im. HO-1 RNA was induced up to 90 fold by CDDO-Im compared to a 13-fold increase following CAPE treatment.

Figure 6. HO-1 protein expression in HUVEC by 24 hr treatment of 20 μM CAPE and 100 nM CDDO-Im. HO-1 protein was induced up to 87 fold by CDDO-Im compared to a 10-fold increase following CAPE treatment.

Conclusions

1. Cytotoxicity profiles of CAPE and CDDO-Im were established in HUVEC. CAPE above 40 μM and CDDO-Im over 500 nM were cytotoxic.
2. The cytoprotective effect of CAPE and CDDO-Im were dose dependent. The cytoprotection of CDDO-Im are much more potent than that of CAPE.
3. The induction of HO-1 by CAPE and CDDO-Im correlated well with their cytoprotection.
4. Since CDDO-Im appears to provide improved cytoprotection against oxidative stress it is a good candidate for testing in animal models of ischemia reperfusion injury.

References

1. Holtzclaw WD, Dinkova-Kostova AT, Talalay P. Protection against electrophile and oxidative stress by induction of phase 2 genes: the quest for the elusive sensor that responds to inducers. *Adv Enzyme Regul.* 2004;44:339-67. Review.
2. Wang X, Stavchansky S, Zhao B, Bynum JA, Kervin SM, Bowman PD. Cytoprotection of human endothelial cells from menadione cytotoxicity by caffeic acid phenethyl ester: the role of heme oxygenase-1. *Eur J Pharmacol.* 2008 Sep 4;591(1-3):28-35.
3. Liby K, Hoek T, Yore MM, Suh N, Place AE, Rinkingsong R, Williams CR, Royce DB, Honda T, Honda Y, Grizzle GW, Hill-Kapturczak N, Agrawal A, Sporn MB. The synthetic triterpenoids, CDDO and CDDO-imidazole, are potent inducers of heme oxygenase-1 and Nrf2/ARE signaling. *Cancer Res.* 2005 Jun 1;65(11):4789-98.



Structure activity relationship of Caffeic Acid Phenethyl Ester (CAPE) and its amide derivative CAPA against oxidant stress in human endothelial cells



^{1,2}J. Yang, ³S.M. Kerwin, ^{1,2}X. Wang, ¹S. Stavchansky ²J.A. Bynum and ²P.D. Bowman

¹Division of Pharmaceutics and ³Division of Medicinal Chemistry, College of Pharmacy, University of Texas, Austin TX ²US Army Institute of Surgical Research, San Antonio TX

Abstract

Numerous reports have described the amelioration of ischemia/reperfusion injury by CAPE post-injury¹, and we have recently shown that induction of heme oxygenase (HMOX1) in vitro is highly correlated with this cyto-protection². In vivo, the presence of esterase which are abundant in blood and tissues, would severely limit the effectiveness of CAPE by degrading it to caffeic acid and phenethyl alcohol, neither of which is cyto-protective in vitro. Therefore an amide derivative of CAPE, Caffeic Acid Phenethyl Amide (CAPA), was synthesized and screened for cyto-protection by examining its ability to induce HMOX1 mRNA in human endothelial cells. CAPA was produced by a classic Wittig reaction and was shown to be 90% pure by ¹H nuclear magnetic resonance spectroscopy. CAPA was as effective as CAPE in inducing HO-1 mRNA (9-fold over vehicle control) as determined by RT-PCR. Assays utilizing CAPA have also shown that it is as effective as CAPE in protecting endothelial cells against menadione induced oxidant stress. It remains to be determined if it exhibits greater stability than CAPE in vivo.

Introduction

Interruptions to the flow of blood to an organ or tissue results in ischemic injury that is exacerbated by the restoration of flow and reintroduction of oxygen, leading to Ischemia/reperfusion (I/R) injury. Caffeic Acid Phenethyl Ester (CAPE) has been found to ameliorate I/R injury¹ and protects cells from oxidant stress in vitro². This cytoprotective effect is highly correlated with the expression of the heme oxygenase (decycling) 1 gene (HMOX1)^{2,3}. As an ester, however, CAPE is subject to esterases that readily hydrolyze CAPE in plasma and in cells, eliminating the cytoprotective effect. We hypothesized that an amide derivative of CAPE, Caffeic Acid Phenethyl Amide (CAPA), would be able to avoid esterase hydrolysis and be active for a longer period of time, while maintaining CAPE's cytoprotective ability.

Materials and Methods

- Human Umbilical Vein Endothelial Cells (HUVEC) used in both gene expression and cyto-protection assays
- Cell RNA treated with reverse transcriptase using the method described by Ambion's "Cells to cDNA II"
- Roche Light Cycler 480 RT-PCR used to quantify the induction of HMOX1 gene expression, with the 18S ribosome gene used as a control
- Menadione used in the cyto-protection assay as the inducer of oxidant damage
- Cell viability in the cyto-protection assay was assessed with Alamar Blue

Synthesis

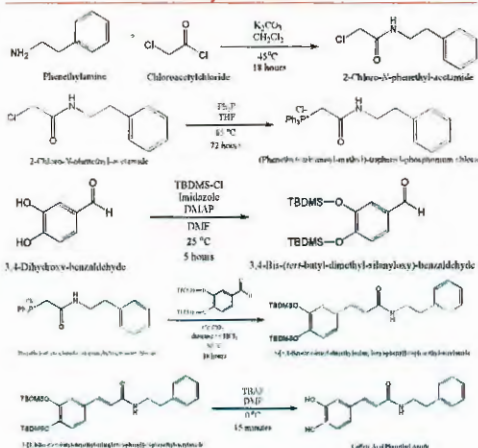


Figure 2 – Stepwise synthesis of Caffeic Acid Phenethyl Amide (CAPA). Final compound was purified by flash column chromatography and re-crystallization

Additional Derivatives

Previous studies have shown that fluorination of the catechol ring in CAPE improves the stability of the compound while maintaining its cyto-protective ability. A similar approach was taken here. The following CAPA fluorinated derivatives are currently being synthesized and tested for stability and cyto-protective properties.

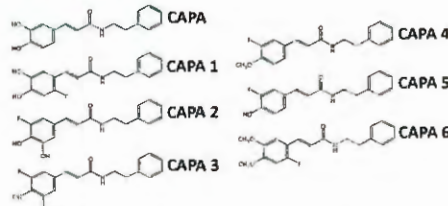


Figure 3 – CAPA fluorinated derivatives. Synthesis pathways similar to CAPA

Cyto-Protection

Cytoprotection against MD by CAPE and CAPA

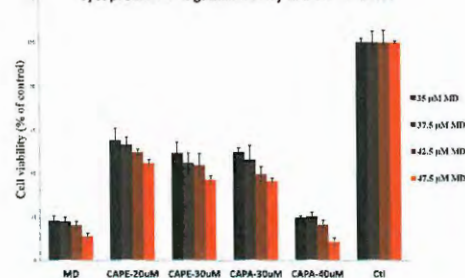


Figure 4 – Cyto-protection of CAPE and CAPA against menadione (MD) toxicity in HUVEC

Gene Expression

HMOX1 gene expression by CAPE and CAPA

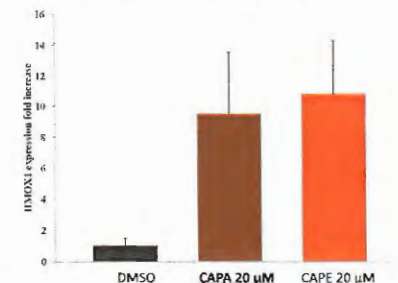


Figure 5 – RT-PCR Gene expression assay quantifying the expression of HMOX1

Conclusions

- There is no significant difference in cytoprotection of HUVEC against menadione induced oxidant stress between CAPE and CAPA
- There is no significant difference in the expression of the HMOX1 gene between CAPE and CAPA

References

1. Tan et al., *Am J Physiol Heart Circ Physiol.* 289. H2265-H2271. 2005
2. Wang X., *Euro J Pharmacol.*, 591, 28-35, 2008
3. Katori et al., *Transplant Immunology.* 9. 227-233. 2002

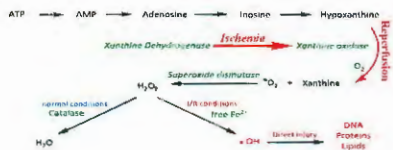


Figure 1 – Proposed mechanism of ischemia/reperfusion injury modified from Granger et al., *Acta Physiol Scand Suppl.* 548. 1986

Noninvasive imaging of hemorrhage-induced global ischemia with a transgenic mouse expressing luciferase coupled to hypoxia-inducible factor (HIF1 α)



¹P.D. Bowman, ¹J.A. Bynum, ²J.A. Kiang, ¹D.G. Baer, ¹J. Johnson, and ¹M.A. Dubick,
¹US Army Institute of Surgical Research, Fort Sam Houston, TX 78234-6315
 and ²Armed Forces Radiobiology Research Institute, Bethesda, Maryland

ABSTRACT #2487

ABSTRACT

Hemorrhagic shock leads to global ischemia, but available blood is distributed unevenly to the body's organs and it is generally accepted that blood is shunted to those organs that maintain critical functions. The organs that become most ischemic and their role in hemorrhagic shock, however, have not been determined. We used the recently described, genetically engineered mouse FVB.129S6-Gt(ROSA)26Sortm1(HIF1 α /luc)Kael/J⁺ (Jackson Laboratories) which has a luciferase gene fused to the region of HIF1 α that binds to von Hippel-Lindau protein in an oxygen-dependent manner generating a reporter that can be used to monitor oxygen availability in intact tissues. Thus more light would be emitted from ischemic tissue. To determine if this mouse could be used to identify organs affected by hemorrhagic shock, 40 percent of the calculated blood volume was removed via the submaxillary vein, and the mice were injected with luciferin, anesthetized with isoflurane, and imaged in the Xenogen IVIS 100 Imaging system as a function of time after hemorrhage. The ventral surface of these mice exhibited increased light emitted from the region of the intestine that was most prominent at 3-6 h after hemorrhage but still evident at 72 h. This technique should allow more detailed studies of those tissues most affected by hemorrhagic shock.

INTRODUCTION

Following hemorrhage, delivery of blood to organs and tissue is compromised, resulting in hypoxia to some organs but those tissues most effected by hypoxia are not known. Safran et al. have recently described a transgenic mouse that has been engineered to express the firefly luciferase bioluminescent reporter fused to a region of HIF that is sufficient for oxygen-dependent degradation. This mouse is designed for use in monitoring hypoxic tissues and evaluating therapeutic agents that stabilize HIF1 α . We asked if it could be used to monitor hypoxia induced by hemorrhage

MATERIALS AND METHODS

Male mice (25-30 g) of the FVB.129S6-Gt(ROSA)26Sortm1(HIF1 α /luc)Kael/J strain (Catalog #006206) were obtained from the Jackson Laboratories, Bar Harbor, ME. Mice were anesthetized with 2% isoflurane and about forty percent of the calculated blood volume was withdrawn over a 30 second period by inserting the Medipoint Lancet (Medipoint, Mineola, NY) into the submaxillary vein. Submaxillary veins of sham animals were punctured with the lancet but bleeding stopped by the application of pressure from sterile gauze. For bioluminescence imaging, mice were anesthetized with isoflurane/air and injected with 100 μ l of luciferin (dissolved in phosphate buffered saline into the peritoneal cavity). Five min after luciferin injection, mice were imaged for 1-5 minutes. Photons emitted from specific regions will be quantified using the LivingImage software (Xenogen) and luciferase activity acquired as photons emitted per second. Organs (liver, lung, kidney, spleen, duodenum, jejunum, ileum, stomach, brain, salivary gland, skeletal muscle, and testes) were removed 5 min following injection of luciferin to produce a higher resolution view of the light coming from organs as a function of luciferase production concomitant with HIF α induction.

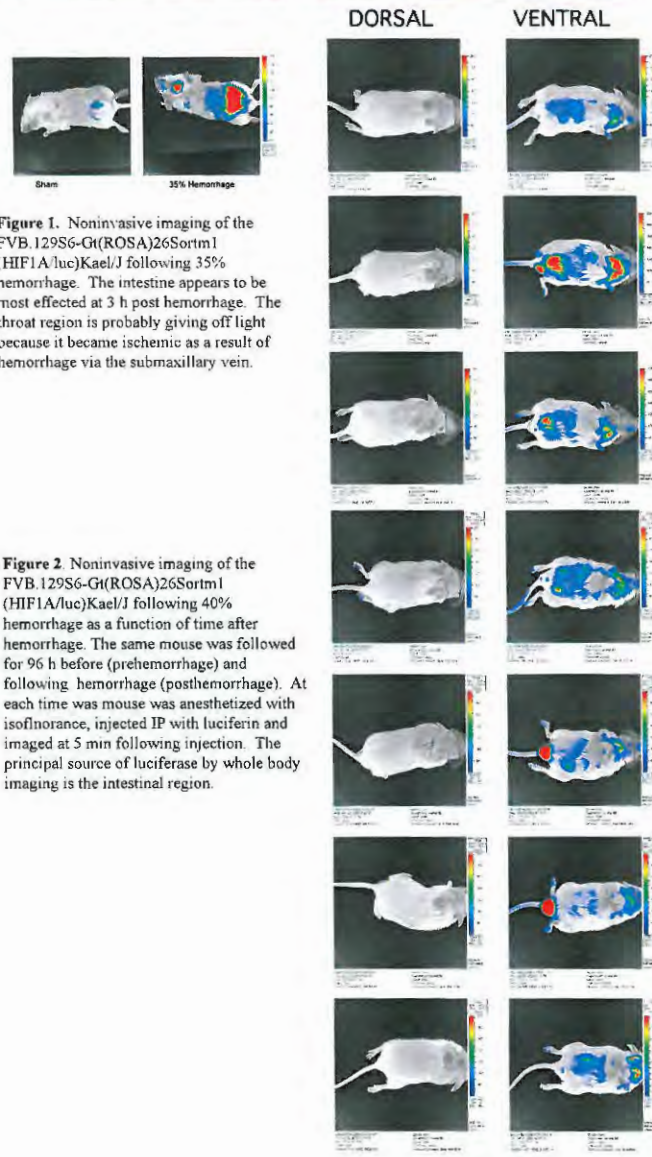


Figure 1. Noninvasive imaging of the FVB.129S6-Gt(ROSA)26Sortm1(HIF1 α /luc)Kael/J following 35% hemorrhage. The intestine appears to be most effected at 3 h post hemorrhage. The throat region is probably giving off light because it became ischemic as a result of hemorrhage via the submaxillary vein.

Figure 2. Noninvasive imaging of the FVB.129S6-Gt(ROSA)26Sortm1(HIF1 α /luc)Kael/J following 40% hemorrhage as a function of time after hemorrhage. The same mouse was followed for 96 h before (prehemorrhage) and following hemorrhage (posthemorrhage). At each time was mouse was anesthetized with isoflurane, injected IP with luciferin and imaged at 5 min following injection. The principal source of luciferase by whole body imaging is the intestinal region.

Figure 2

Prehemorrhage

3 h Post Hemorrhage

6 h Post Hemorrhage

20 h Post Hemorrhage

48 h Post Hemorrhage

72 h Post Hemorrhage

96 h Post Hemorrhage

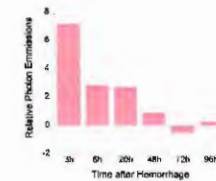


Figure 3. Quantification of light emanating from the intestinal region by luminescent imaging. The region of interest (ROI, intestine) was demarcated and applied to each time point in Figure 2. Bars represent average number of photons/ROI at each time.

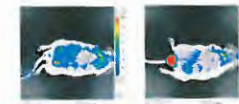
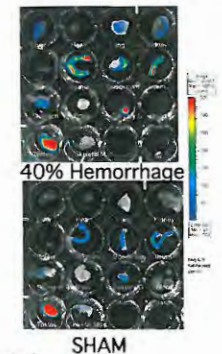


Figure 4. The testes as a site of HIF1 α /luciferase activity in mice. The same mouse imaged on different days. On the left, the testes are retracted into the body. On the right the testes have descended and express high levels of luciferase activity. This has recently been reported by Lysiak et al. Hypoxia Inducible Factor-1 α is Constitutively Expressed in Murine Leydig Cells and Regulates 3 β -Hydroxysteroid Dehydrogenase Type I Promoter Activity. *J Androl*. 2009 Mar-Apr;30(2):146-56.

Figure 5. Evaluation of light emanating from isolated organs of hemorrhaged and sham HIF1 α /luciferase mice. Organs were immediately removed from mice following in vivo imaging and placed in wells of a 24-well multiplate and reimaged. In addition to high photo emissions from components of the intestine, testes and salivary gland, liver, lung, kidney, and stomach also produced light indicating some hypoxia in these organs.



Summary and Conclusion

1. The HIF1 α /luciferase mouse provides a useful model for studying hypoxia associated with ischemia of hemorrhage. The high signal/noise allows detection of HIF1 α activity at very low levels.
2. In vivo imaging is problematic as many effected organs are not evident from in vivo imaging.
3. Even imaging of organs may not accurately reflect the degree of hypoxia as photons emitted from highly pigmented organs may be reduced requiring homogenization of tissue prior to luciferase determination.

REFERENCES

1. Safran, M., W. Y. Kim, F. O'Connell, L. Flippin, V. Gunzler, J. W. Heimer, R. A. Depinto, and W. G. Kaelin, Jr. Mouse model for noninvasive imaging of HIF1 α hydroxylase activity: assessment of an oral agent that stimulates erythropoietin production. *Proc Natl Acad Sci U S A* 2006;103:105-110.
2. Zhang, N., A. Weber, B. Li, R. Lyons, P. R. Contag, A. J. Puchio, and D. B. West. An inducible nitric oxide synthase-luciferase reporter system for in vivo testing of anti-inflammatory compounds in transgenic mice. *J Immunol* 2003;170:6307-6319.



Cytoprotection of Human Endothelial Cells from Oxidative Stress by Polyphenols: the Role of Gene Expression versus Direct Antioxidant Effect

^{1,2}Xinyu Wang, ²James Bynum, ¹Salomon Stavchansky, ²Michael Dubick, ³Robert Hackman, ³Carl Keen, and ²Phillip Bowman
¹Pharmaceutics Division, College of Pharmacy, University of Texas, Austin TX; ²United State Army Institute of Surgical Research, San Antonio, TX 78234; and ³Department of Nutrition, University of California at Davis, Davis, CA, 95616.



Abstract

Abstract Number: 4941

Polyphenols have been implicated in protecting cells against oxidative stress. Several polyphenols including caffeic acid phenethyl ester (CAPE), curcumin, resveratrol, caffeic acid, catechin, and Oligonol™, a commercial source of a mixture of polyphenolics were investigated for their cytoprotective effects and effects on the transcriptional activity in an *in vitro* model of menadione-induced oxidative stress in human umbilical vein endothelial cells. CAPE, curcumin, and resveratrol showed dose-dependent cytoprotection against menadione-induced cytotoxicity, whereas Oligonol™, (+)-catechin, and caffeic acid did not. The results of 'direct' antioxidant capacities of those compounds by 2, 7-dichlorofluorescein assay indicated that most compounds tested showed good free radical scavenging abilities except resveratrol. However, 'direct' antioxidant activity did not correlate well with their cytoprotective effects. Gene expression analysis with whole genome microarrays and submission of statistically significant results to Ingenuity Pathway Analysis showed that a number of genes were up- or down-regulated by these compounds effecting common molecular networks and compound-specific molecular networks, which may account for their beneficial effects, in particular the heat shock protein family and IL-8 signaling pathway.

Introduction

Polyphenols have been reported to provide beneficial effects, including anticancer, antibacterial, anti-inflammatory, and antioxidant activities. To better understand the purported protective properties of several polyphenols, including caffeic acid phenethyl ester (CAPE), curcumin (CUC), resveratrol (RES), caffeic acid (CA), catechin (CAT), and Oligonol™, a mixture of polyphenols derived from lychee fruit, an *in vitro* model using menadione (MD)-induced oxidative stress in human umbilical vein endothelial cells (HUVEC) was investigated [1].

Methods

In vitro assay:

Confluent HUVEC were pretreated with test compounds or control (0.1% DMSO) for 6 hrs, then exposed to MD for an additional 24 hrs. N-acetyl cysteine (NAC) served as a positive control, providing complete protection against this stress. Cell viability was assessed using Alamar Blue. The experiments were done in triplicate. Intracellular production of reactive oxygen species was evaluated using fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA).

Gene expression analysis:

Total RNAs from 6h-pretreated or control HUVECs were isolated and labeled for microarray analysis using agilent whole-genome microarrays. Microarray data analysis and statistical comparison were performed using BRB Array Tools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>). Genes were considered statistically significant with *P* value < 0.001 and FDR (false discovery rate) value < 10%, and genes significantly altered in their expression were submitted to Ingenuity Pathway Analysis (IPA) for further pathway investigation

Methods

(www.ingenuity.com). IPA used Fischer's exact test to calculate a *p*-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. Significant biological networks with a score greater than 30 (*P* < 10⁻³⁰) were merged. Genes are represented as a single node in the network. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation.

Results

1. Cytoprotection assay:

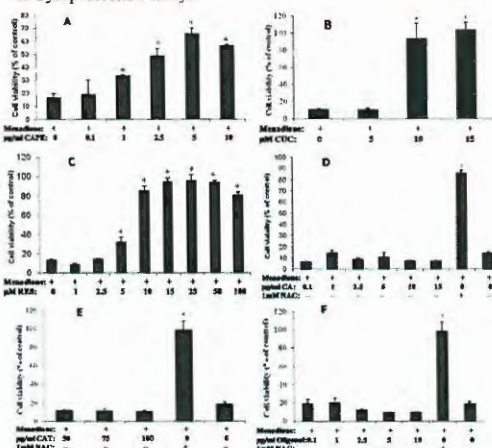


Figure 1: Cytoprotection assay of various polyphenols against MD-induced oxidative stress in HUVEC. CAPE (A), CUC (B), and RES (C) showed dose-dependent cytoprotection, while CA (D), CAT (E), and Oligonol (F) showed no cytoprotective effect at the non-toxic doses tested. *, *P* < 0.05 versus MD alone.

2. Cell-based antioxidant assay:

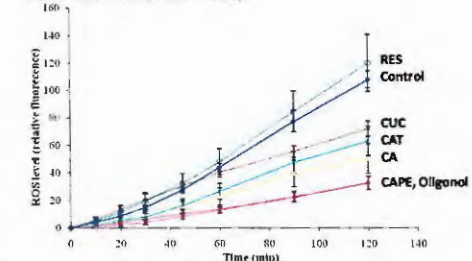


Figure 2: Direct antioxidant activities of polyphenols in HUVEC demonstrated by measuring the alteration of intracellular reactive oxygen species (ROS) level. After treatment with CAPE, CUC, CA, CAT, or Oligonol, ROS was decreased compared to that of control, while incubation with RES generated ROS similar to control.

Results

3. Gene expression analysis:

Table 1: Number of genes significantly changed by test compounds (*P* < 0.001, FDR < 0.1, * FDR < 0.34).

	CAPE	CUC	RES	CAT	CA	Oligonol™
total	208	1940	176	202	44	43*
UP-regulation	132	377	71	44	6	10*
Down-regulation	76	1563	105	158	38	33*

4. Ingenuity Pathway Analysis (IPA):

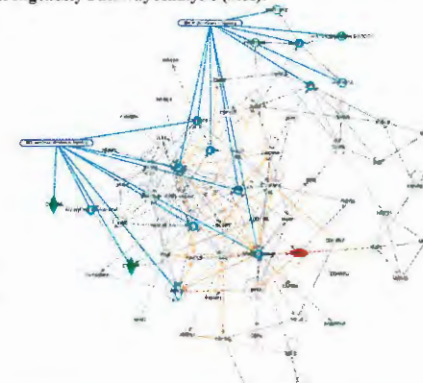


Figure 3: Network analysis of CAPE. The most significant biological networks were merged with functions involving cell cycle, DNA replication, metabolism, and canonical pathways associated with protein kinase A and xenobiotic metabolism signaling.

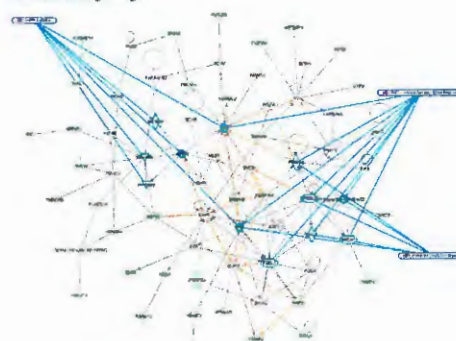


Figure 4: Network analysis of CUC. The most significant biological networks were merged with functions involving RNA post-transcriptional modification, gene expression, metabolism, and canonical pathways associated with AMPK, NRF2-mediated oxidative stress response, and xenobiotic metabolism signaling.

Results

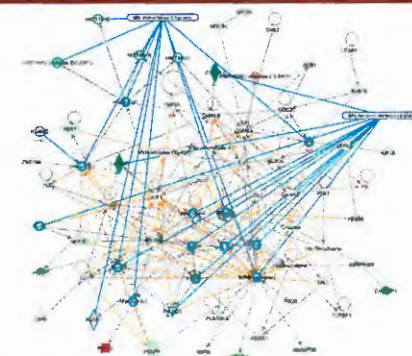


Figure 5: Network analysis of RES. The most significant biological networks were merged with functions involving cell cycle, DNA replication, metabolism, and canonical pathways associated with protein kinase A and xenobiotic metabolism signaling.

Conclusions

1. CAPE, CUC, and RES showed dose-dependent cytoprotection against MD toxicity in HUVEC, while CA, CAT, and Oligonol did not. Cytoprotection did not correlate well with antioxidant activity as determined by the CM-H₂DCFDA assay, as almost all tested polyphenols showed similar free radical scavenging activity except RES.
2. Gene expression profiling and submission of genes significantly altered in expression to IPA identified common pathways among the cytoprotective polyphenols CAPE, CUC, and RES such as protein kinase A signaling for CAPE and RES and xenobiotic metabolism signaling for all three compounds. In addition, CUC induced different pathways including AMPK and NRF2-mediated oxidative stress response signaling.
3. CA and CAT did not induce pathways similar to the cytoprotectants, suggesting that these pathways may provide an understanding of the cytoprotection mechanism. While Oligonol showed good free radical scavenging activity, it did not activate HUVEC transcription event significantly, which may account for its lack of protection in this HUVEC-MD model.

Reference

1. Wang X, Stavchansky S, Zhao B, Bynum JA, Kerwin SM, Bowman PD. Cytoprotection of human endothelial cells from menadione cytotoxicity by caffeic acid phenethyl ester: The role of heme oxygenase-1. *European Journal of Pharmacology* 2008; 591(1-3): 28-35.



Induction of Hypoxia Inducible Factor 1 Alpha (HIF1 α) by Caffeic Acid Phenethyl Ester (CAPE) and Caffeic Acid Phenethyl Amide (CAPA) in Mouse Skin Fibroblasts

Ashish Rastogi^{1,2}, John Yang^{1,2}, Xinyu Wang^{1,2}, James Bynum¹, Salomon Stavchansky² and Phillip Bowman¹

¹US Army Institute of Surgical Research, Brooke Army Medical Center, San Antonio, Texas 78234.

²Division of Pharmaceutics, College of Pharmacy, The University of Texas at Austin, Texas 78712.



Abstract

Abstract # 5877

Esters and amides of caffeic acid (CA) such as CAPE and CAPA exhibit significant cytoprotection of a variety of cell types from oxidative stress while CA does not. A role for HIF1 α in cytoprotection has been described and to better understand the mechanism, we investigated skin fibroblasts from the FVB.1 29S6-Gt(ROSA)26Sortm1 (HIF1 α Luc)Kael/J (HIF1 α Luc) inbred strain (Jackson Laboratory, Bar Harbor, ME). This mouse (RosaLuc) has been genetically engineered to express luciferase in conjunction with accumulation of HIF1 α . RosaLuc mice skin fibroblasts (MSF) were obtained from skin specimens dissociated in 0.1% collagenase for 3 h and cultivated in 10% fetal bovine serum in alpha Minimal Essential Medium. Passages 1-5 were used for these studies and subcultivated into 96-well multiplates. CAPA was synthesized in the lab and CAPE (Cayman Labs, Ann Arbor, MI) and deferoxamine (DEF), a well known inducer of HIF1 α and an iron chelator, were dissolved in DMSO. MSF cells were treated for 5 h with these drugs and assayed for luciferase activity (Promega, Madison, WI). CA was inactive but CAPE and CAPA were significantly better than DEF in producing a luminescent signal from MSF. These results indicate that CAPE and CAPA may directly activate HIF1 α by inhibiting prolyl hydroxylase thereby preventing the degradation of nascent HIF1 α .

Introduction

Ischemic preconditioning has been shown to provide significant protection from a subsequent lethal ischemic event¹. HIF1 α , a master regulator of the hypoxic response has been implicated in ischemic preconditioning. To develop drugs that produce a similar cytoprotective effect we are screening genetically engineered mouse cells that express luciferase when HIF1 α accumulates. Deferoxamine induces HIF1 α by inhibiting the activity of Fe²⁺ dependent prolyl hydroxylase which is required for activation of the oxygen dependent domain of HIF1 α and was used as a positive control. CAPE has been previously suggested to inhibit HIF1 α prolyl hydroxylase². We have found that CAPA and CAPE, which induced cytoprotection against menadione-induced oxidative stress, also induces HIF1 α and this may explain their cytoprotective effect.

Methods

1. Cell Culture: Mouse skin fibroblasts (MSF) were obtained from RosaLuc mice dissociated in 0.1% collagenase for 3 h, and cultivated in 10% fetal bovine serum in Minimal Essential Medium. Passages 1-5 were used for these studies and were subcultivated into 96-well multiplates.

2. Dose and time-dependence of induction: Stock solutions of CAPA (synthesized in the lab), CAPE (Cayman Labs, Ann Arbor, MI), and deferoxamine mesylate (Sigma Aldrich, St. Louis, MO) were prepared in DMSO. MSF cells were treated as a function of concentration and time and assayed for luciferase activity (Promega, Madison, WI). Induction of HIF1 α was estimated by luminescent intensity.

3. Cytoprotection: MSF cells were treated with 0.1% DMSO, 1.0 μ M CA, CAPE, and CAPA for 5 h. Following dosing, the cells were treated with 30-50 μ M menadione to induce an oxidative stress and after 2-4 h viability was determined.

Results

1. Dose and time-dependence of induction: Deferoxamine was used as a positive control in this study and the effect of deferoxamine on accumulation of HIF1 α was initially studied. The MSF cells were dosed with various concentrations of deferoxamine for 5 h and HIF1 α activity determined. Fig. 1 shows that the luminescence intensity decreased with decreasing concentration of deferoxamine. A median concentration of 100 μ M was selected for the time course study. Fig.2 shows incubation of MSF cells with deferoxamine at different time points. An incubation of 3-5 h showed significant luminescence indicating significant induction of HIF1 α .

Fig. 3. CA was found to be inactive but CAPE and CAPA showed luminescence as a function of concentration gradient. In Fig. 4, the MSF cells were dosed with the highest dose of 2.5 μ M CA, CAPE, and CAPA. As earlier observed with deferoxamine, both CAPE and CAPA also showed time dependent luminescence corresponding to HIF1 α induction. These results indicate that CAPE and CAPA may also induce hypoxia by activating HIF1 α through inhibition of prolyl hydroxylase.

2. Cytoprotection: CAPE and CAPA were found to be cytoprotective against menadione (Fig. 5).

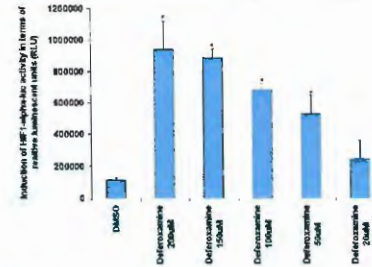


Fig. 1. MSF cells were dosed with deferoxamine solutions (20-200 μ M) for 5 h. A 0.1% DMSO was used as a negative control. Data is represented as mean+standard deviation. *; P<0.05 versus 0.1% DMSO alone.

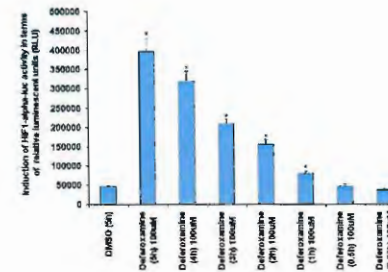


Fig. 2. MSF cells were dosed with 100 μ M deferoxamine for up to 5 h. A 0.1% DMSO was used as a negative control. Data is represented as mean+standard deviation. *; P<0.05 versus 0.1% DMSO alone.

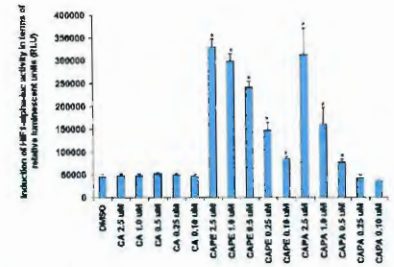


Fig. 3. MSF cells were dosed with solutions of CA, CAPE, and CAPA (0.10-2.50 μ M) for 5 h. A 0.1% DMSO was used as a negative control. Data is represented as mean+standard deviation. *; P<0.05 versus 0.1% DMSO alone.

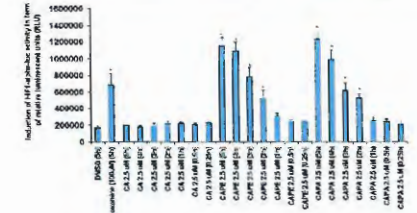


Fig. 4. MSF cells were dosed with 0.1% DMSO (negative control), 100 μ M deferoxamine (positive control), and 2.5 μ M CA, CAPE, CAPA for up to 5 h. Data is represented as mean+standard deviation. *; P<0.05 versus 0.1% DMSO alone.

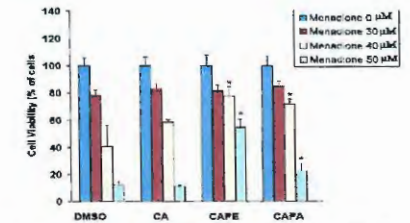


Fig. 5. MSF cells were dosed with 1.0 μ M CA, CAPE, CAPA and cell viability measured after dosing with 30-50 μ M menadione. A 0.1% DMSO was used as a negative control. Data is represented as mean+standard deviation. *; P<0.05 versus 0.1% DMSO alone in individual groups.

Conclusions

- Both CAPE and CAPA induced a dose dependent increase in HIF1 α in the range of 0.1- 2.5- μ M and 0.5-2.5- μ M, respectively. CA was found to be inactive.
- By 2 h of treatment with CAPE and CAPA, a significant induction of HIF1 α was observed.
- A 1.0 μ M dose of CAPA and CAPE that induced significant HIF1 α were found to be cytoprotective at 5 h after the beginning of treatment. CA did not show any significant cytoprotection.
- The accumulation of HIF1 α may play a role in the cytoprotective effect of these compounds. The MSF provide a simple method for screening other drugs for cytoprotection against oxidant stress.

References

- Murry CE, Jennings RB, Reimer KA (1986). "Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium". *Circulation* 74 (5): 1124-36.
- Choi D, Han J, Lee Y, Choi J, Han S, Hong S, Jeon H, Kim YM, Jung Y (2009). "Caffeic acid phenethyl ester is a potent inhibitor of HIF prolyl hydroxylase: structural analysis and pharmacological implication". *J Nutr Biochem*. Sep 7. [Epub ahead of print]



Poor Correlation between In Vivo Imaging and Production of Light by Organs in Transgenic Mouse Engineered to Express Luciferase in Response to Hypoxia

Ashish Rastogi^{1,2}, James Bynum¹, Salomon Stavchansky² and Phillip Bowman¹

¹US Army Institute of Surgical Research, San Antonio, Texas 78234.

²Division of Pharmaceutics, College of Pharmacy, The University of Texas at Austin, Texas 78712.



Abstract

The FVB.129S6-*Gt(ROSA)26Sox²m1(HIP1a)luc/Kael1* (Jackson Labs) mouse strain (Safran M. et al. PNAS, 2006, 103(1), pp 105-110), genetically engineered to express luciferase when the hypoxia-inducible factor 1α (HIF-1α) accumulates, was used to identify organs most affected by the ischemia of hemorrhage. Luciferin (50mg/ml) was administered subcutaneously by osmotic pump (Alzet) implanted 24 h prior to hemorrhage. Forty percent of the calculated blood volume was removed with a lancet through the submaxillary vein. In vivo imaging at 4 h indicated affected organs. Mice were then euthanized and portions of each organ reimaged or frozen for homogenization of tissues followed by luciferase quantification using a luminometer. The ratio of relative luminescence units/mg of protein for hemorrhage versus sham groups were 3.0, 1.8, 3.2, and 1.2 for lung, liver, kidney, and spleen, respectively. The hemorrhaged mice showed differential expression compared to sham indicating upregulation of HIF-1α. The luminometer method was found to be more precise than the in vivo imaging for the determination of effect of hypoxia in different organs. Comparison between in vivo imaging of the whole animal, imaging of isolated organs, and luminometer readings showed a poor correlation between these methods.

Introduction

A central problem of devising treatments for hemorrhagic shock is identifying organs that are affected and determining how they respond to the global ischemia of hemorrhage. As blood flow is redistributed following hemorrhage, some organs are more affected than others. To address the question of most affected organs we studied HIF-1α, the master sensor of hypoxia as a guide and investigated the use of a transgenic mouse engineered to express the luciferase gene in tandem with HIF-1α for identifying affected tissues. In vivo imaging that uses bioluminescence provides a non-invasive and real time method to acquire longitudinal information from the same live animal making it attractive as a high throughput technique. In vivo imaging was first used followed by imaging of isolated organs. Homogenization of organs followed by quantification of luminescence by luminometer proved to be the most sensitive and reliable technique.

Materials and Methods

- 1. Mice and Hemorrhage** - The FVB.129S6-*Gt(ROSA)26-Sox²m1(HIP1a)luc/Kael1* (Jackson Labs, Bar Harbor, Maine) mouse strain [1] is genetically engineered to express luciferase when the hypoxia-inducible factor 1α (HIF-1α) accumulates. Hemorrhage is accomplished by removing 40 % of the calculated blood volume with a lancet via submaxillary vein.
- 2. Imaging** - A 50 mg/ml solution of potassium salt of D-Luciferin (Caliper Life Sciences, Hopkinton, MA) was prepared with phosphate buffered saline, pH 7.4. Continuous delivery of luciferin was achieved using osmotic pumps as described by Gross et al [2]. The osmotic pumps are filled with luciferin solution and implanted on the dorsal side of the mouse. Four hours after hemorrhage, mice from both groups were anesthetized and imaged using IVIS to reveal light coming from organs as a function of luciferase production concomitant with HIF-1α induction. Mice were then euthanized and portions of each organ reimaged or frozen for in vitro luciferase quantification using a luminometer.
- 3. Statistical Analysis** - Levene's test was used to access the homogeneity of variance. Student's t-test was used to analyse differences between sham and hemorrhage groups in Tables 1 and 2. A difference of p value < 0.05 was considered significant.

IV. In vitro analysis of homogenized organs using a luminometer

	Lung	Liver	Kidney	Spleen
Hemorrhage	3.2 ± 0.9	13.7 ± 3.2	5.4 ± 1.1	4.1 ± 1.2
Sham	1.1 ± 0.4	7.6 ± 2.3	1.7 ± 0.3	3.4 ± 1.7
Ratio (H/S)	3.0*	1.8*	3.2*	1.2

Table 2: Average luminescence (in millions relative luminescence units) of organs isolated from hemorrhage and sham groups are shown. The luminometer analysis shows hemorrhage groups have higher luminescence values than the sham group indicating hypoxia in the hemorrhage group. (N=6; *p > 0.05)

Results

I. In vivo imaging for determination of organs affected by hypoxia -

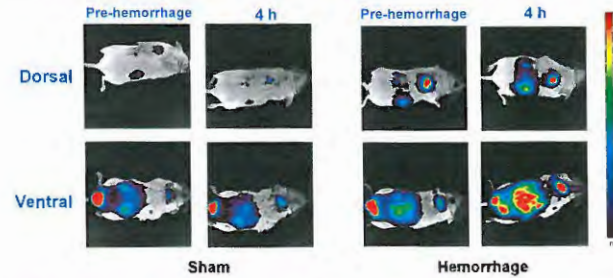


Fig. 1: Non-invasive imaging of FVB.129S6-*Gt(ROSA)26-Sox²m1(HIP1a)luc/Kael1* mice are shown. Images are of a representative mouse from each group. A reference intensity scale with units in counts or photons emitted is also shown. Although bioluminescence in the hemorrhage group is higher than the sham, a quantitative estimate cannot be made with the images.

II. Quantitative analysis of organs from in vivo imaging

	Testes	Liver	Kidney
Hemorrhage	165.4 ± 60.2	117.8 ± 62.9	54.8 ± 45.5
Sham	359.6 ± 53.6	74.6 ± 32.4	47.0 ± 23.5
Ratio (H/S)	0.5*	1.6*	1.2

Table 1: Average bioluminescence intensity (in counts) of organs as observed from the in vivo images of hemorrhage and sham groups are shown. Not all organs can be identified in the images and hence they cannot be quantified. Kidneys on dorsal images and testes and liver on ventral images were easily quantified than the other organs. The testes from the hemorrhage group exhibited lesser bioluminescence than the sham. Liver showed higher bioluminescence whereas, kidney showed no significant difference between the groups. (N=3; *p > 0.05)

III. Imaging of isolated organs at 4 h

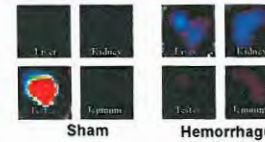


Fig.2: Imaging of various organs after removal from a sham or hemorrhage animal. Images are from a representative mouse from each group. Intensity of bioluminescence exhibited from an organ from animals belonging to the same group was variable. Not all organs exhibited bioluminescence in each animal.

Conclusions

1. The data shown in Tables 1 and 2 indicate a poor correlation between organs by in vivo imaging and in vitro luminometer analysis for the HIF-1α transgenic mouse. Luminometer data rather than in vivo imaging analysis confirm our original hypothesis that hemorrhage animals will show higher luminescence due to accumulation of HIF-1α.
2. Some affected (hypoxic) organs did not yield significant amounts of light by in vivo imaging.
3. The luminometer was found to be more reliable than optical imaging in evaluation of hypoxic organs.
4. The transgenic mouse produces useable information if organs are isolated, homogenized and HIF-1α activity determined in a luminometer.

References

1. Safran M. et al. Mouse model for noninvasive imaging of HIF prolyl hydroxylase activity: Assessment of an oral agent that stimulates erythropoietin production. PNAS. 2006, 103(1), pp 105-110
2. Gross S. et al. Continuous delivery of D-luciferin by implanted micro-osmotic pumps enables true real-time bioluminescence imaging of luciferase activity in vivo. Mol imaging . 2007, 6(2), pp 121-30



Determination of the Minimum Exposure Time for Effecting Cytoprotection in Human Umbilical Vein Endothelial Cells (HUVEC) for Caffeic Acid Phenylethyl Ester (CAPE) and Amide (CAPA)



J. Yang¹, P.D. Bowman², and S. Stavchansky¹

¹Division of Pharmaceutics, College of Pharmacy, The University of Texas, Austin TX ²US Army Institute of Surgical Research, San Antonio TX

Abstract

We have previously shown that CAPE and CAPA protect HUVEC from oxidant stress.¹ This activity was correlated with the production of heme oxygenase-1 (HO-1). The objective of this study was to determine the minimum exposure time necessary to provide cytoprotection and HO-1 production.

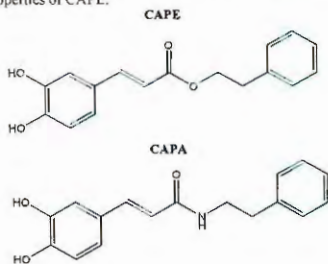
HUVEC were exposed to cytoprotective doses of CAPE and CAPA for 0.5, 1, 2, 3, 4 and 6 hrs. The compounds were removed at the end of each time period and placed in fresh media. Levels of HO-1 were then evaluated by gel electrophoresis and immunoblotting, and cytoprotection against H₂O₂ toxicity was measured. CAPE and CAPA both showed a significant increase in HO-1 expression over vehicle control within 30 min of exposure. HO-1 levels for both CAPE and CAPA peaked with a 4 h incubation time with no significant differences beyond 4 hours. Significant cytoprotective effects against hydrogen peroxide for both CAPE and CAPA were found with as little as 1 h exposure time.

CAPE and CAPA both showed significant effects on HUVEC HO-1 expression and cytoprotection with only a brief exposure. It appears that the compounds do not need to be continually exposed to the cells in order for the beneficial properties to be expressed. Whether this is due to cell loading of the drug or that minimum exposure to drug triggers a switch that leads to the effect is under investigation. Funding provided by the US Army Medical and Materiel Command

¹ Yang J, Marmor GA, Wang X, Bowman PD, Kerwin SM, Stavchansky S. Synthesis of a series of caffeic acid phenylethyl amide (CAPA) fluorinated derivatives: comparison of cytoprotective effects to caffeic acid phenylethyl ester (CAPE). *Biorg Med Chem* 2010; 18(14):5032-4.

Introduction

Interruptions to the flow of blood to an organ or tissue result in ischemic injury that is exacerbated by the restoration of flow and reintroduction of oxygen, leading to Ischemia/reperfusion (I/R) injury. Caffeic Acid Phenethyl Ester (CAPE) has been found to ameliorate I/R injury and protects cells from oxidant stress in vitro.² This cytoprotective effect is highly correlated with the activity of the Heme Oxygenase-1 (HO-1) enzyme. It has been shown that CAPE is rapidly decomposed and exhibits a short half life in both plasma and in circulation.³ Caffeic Acid Phenethyl Amide (CAPA) was synthesized to improve the stability and activity properties of CAPE.



² Wang X, Stavchansky S, Bowman PD, Kerwin SM. Cytoprotective effects of caffeic acid phenylethyl ester (CAPE) and catechol ring-fluorinated CAPA derivatives on against monofluorine-induced oxidative stress in human endothelial cells. *Biorg Med Chem* 2006; 14(14):4873-82.

³ Wang X, Pang J, Mallick J, et al. Pharmacokinetics of caffeic acid phenylethyl ester and its cytoprotective fluorinated derivatives following intravenous administration in rats. *Pharmazie (Jg)* 2009; 34(5):121-4.

Materials and Methods

• CAPE was obtained from Cayman Chemical (Ann Arbor, MI), and CAPA was synthesized previously in our laboratory. HUVEC were obtained from Lifeline Technologies (Walkersville, MD) and grown to confluence at 37°C in humidified atmosphere with 5% CO₂.

• HUVEC were treated with 5 µg/ml of CAPE and CAPA. The compounds were removed and the cells washed with PBS at 30 minutes, 1 hr, 2 hrs, 3 hrs, 4 hrs, and 6 hrs post treatment. After removal of the compounds the cells were incubated with fresh media. At the 6 hour time point, HUVEC were lysed and a protein polyacrylamide gel electrophoresis and western blots prepared. HO-1 was quantified using rabbit anti HO-1 and mouse anti-β-actin, using Licor Odyssey system. HO-1 levels normalized against β-Actin.

• In the cytoprotection assay, HUVEC were treated similarly as described above. At 6 hours, media was removed and replaced by MCDB 131 salts containing hydrogen peroxide for one hour. Hydrogen peroxide solution was then removed and fresh media reintroduced to the cells. Viability was assessed 18 hours following this using the Cell Titer Blue assay.

Objectives

1. To determine the relationship between exposure time of CAPE and CAPA to HUVEC and cytoprotective activity.
2. To determine the amount of CAPE and CAPA exposure time necessary to significantly induce HO-1 in HUVEC.

Results

Temperature	CAPE t _{1/2} (hours)	CAPA t _{1/2} (hours)
4 °C	5.36	N/A
25 °C	1.39	63
37 °C	0.30	14.2
60 °C	N/A	0.92

Table 1 – Plasma stability of CAPE and CAPA. Male Sprague-Dawley rat plasma was spiked with CAPE and CAPA to final concentrations of 100 µg/ml. Decomposition observed at 3 temperatures per compound for a minimum of 3 half lives. Concentrations determined by HPLC-UV at 320 nm.

³ Yang J, Kerwin SM, Bowman PD, Stavchansky S. Stability of Caffeic Acid Phenethyl Amide (CAPA) in Rat Plasma. *Bioind Chem* 2011.

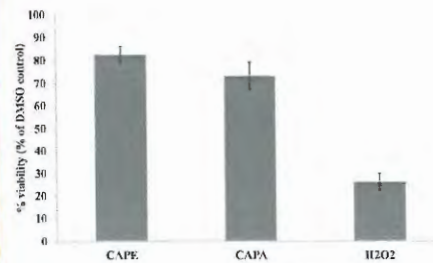


Figure 1 – Cytoprotection of HUVEC. CAPE and CAPA (20 µM) incubated for 6 hours prior to a 1 hour exposure to hydrogen peroxide (2 mM). Cell Titer Blue assay for quantification of cell viability.¹

¹ Yang J, Marmor GA, Wang X, Bowman PD, Kerwin SM, Stavchansky S. Synthesis of a series of caffeic acid phenylethyl amide (CAPA) fluorinated derivatives: comparison of cytoprotective effects to caffeic acid phenylethyl ester (CAPE). *Biorg Med Chem* 2010; 18(14):5032-4.

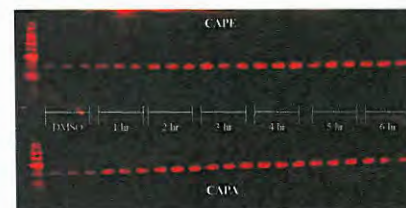


Figure 2 – HO-1 induction of CAPE and CAPA as a function of exposure time in HUVEC.

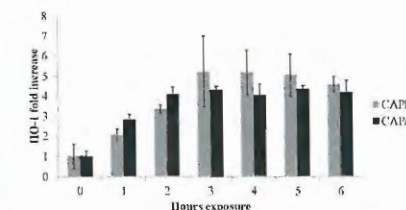


Figure 3 – HO-1 induction of CAPE and CAPA as a function of exposure time in HUVEC. Values normalized against β-Actin q

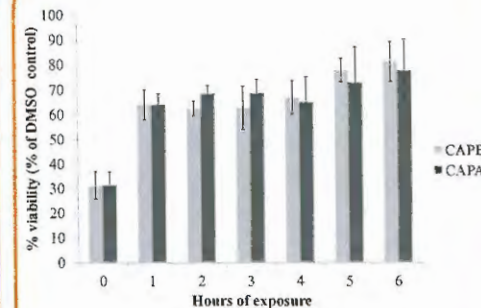


Figure 4 – Cytoprotection of HUVEC. CAPE and CAPA (5 µg/ml) incubated prior to a 1 hour exposure to hydrogen peroxide (2 mM).

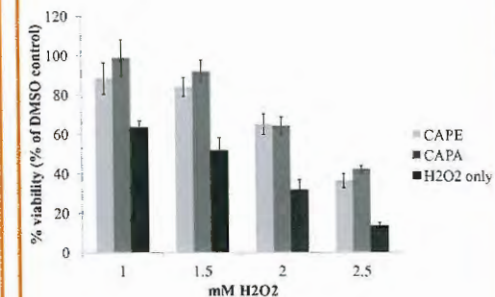


Figure 5 – Cytoprotection of HUVEC. CAPE and CAPA (5 µg/ml) incubated for 1 hour prior to a 1 hour exposure to hydrogen peroxide.

Conclusions

1. A hour to both CAPE and CAPA was sufficient to effect significantly increase HO-1 and to affording significant cytoprotection against oxidative stress 6 hours later in HUVEC.
2. CAPE and CAPA do not need to be continually exposed to HUVEC to induce protective effects.
3. Achievement of a cytoprotective effect in vivo may only require achievement of a dose necessary to induce HO-1 and may not require repeated dosing.



Comparison of atmospheric oxygen versus physiological levels on cytotoxicity of menadione and cytoprotection by antioxidants in human endothelial cells

James Bynum^{1,2}, Ashish Rastogi^{1,2}, Salomon Stavchansky² and Phillip Bowman¹

¹US Army Institute of Surgical Research, Brooke Army Medical Center, San Antonio, Texas 78234; ²College of Pharmacy, The University of Texas at Austin, Texas 78712;



Abstract

Abstract # 1120

The goal of *in vitro* screens of potential cytoprotective agents is to identify drugs that will be beneficial *in vivo*. However, *in vitro* screens are usually performed on cells cultured in atmospheric oxygen (AtmO₂; 20.9%), which is much higher than the levels found in tissues *in vivo* (3-5%). The aim of this study was to evaluate the cytotoxicity and cytoprotective effects of an oxidant generating injurant (50, 60, and 70µM menadione; MD) and several antioxidants on human umbilical vein endothelial cells (HUVEC). Dose response studies demonstrated that 50 µM MD reduced viability by 80% at AtmO₂ but only by 40% at 3% O₂. Antioxidants (N-acetyl cysteine, gamma-glutamyl cysteine, and glutathione) were more effective cytoprotectants at 3% O₂ than AtmO₂, and lower doses (5 and 50µM) were more effective at 3% O₂ than at 20.9% AtmO₂. However, all three compounds proved to be cytoprotective to HUVEC against MD-induced oxidant stress at their highest dose of 500µM. These preliminary results may suggest that the relatively high amount of oxygen in room air is a substrate for generating oxidants, and screens for cytoprotective antioxidants might be more predictive of *in vivo* performance if done at more physiologic levels.

Introduction

While most cell culture is performed at 20.9% oxygen, several studies have indicated that cells perform better at lower, more physiological levels of oxygen¹⁻⁵. Low levels of reactive oxygen species (ROS) can function as redox-active signaling messengers, whereas high levels of ROS induce cellular damage. Menadione generates ROS through redox cycling, and high concentrations trigger cell death⁶. N-Acetyl cysteine (NAC), Glutathione (gamma-glutamyl-cysteinyl-glycine; GSH), and Gamma-glutamyl cysteine (GGC). GSH and NAC are popular antioxidants known for their ability to minimize oxidative stress and the downstream negative effects thought to be associated with oxidative stress. GSH is largely known to minimize the lipid peroxidation of cellular membranes and other such targets that is known to occur with oxidative stress⁷. NAC is a by-product of GSH and is popular due to its cysteine residues and the role it has on glutathione maintenance and metabolism. GGC is a precursor of GSH and is used by glutathione synthetase to form GSH in cells.

In this study, we aim to evaluate the cytotoxicity and cytoprotective effects of an oxidant generating injurant (50, 60, and 70µM menadione; MD) and antioxidants on human umbilical vein endothelial cells (HUVEC) at oxygen concentrations of 3% and 20.9%, respectively.

Methods

- 1. Cell Culture:** Pooled HUVEC (Lonza, Walkerville MD) were cultivated on 75-cm² culture flasks (Corning Incorporated, Corning, NY, USA) in Medium 131 (Life Technologies, Carlsbad, CA) supplemented with 5% fetal calf serum, penicillin (100 units/ml), streptomycin (100 units/ml), and Fungizone (0.25 µg/ml) and endothelial supplements supplied by ATCC. Stock cultures were cultivated at 37°C in a humidified atmosphere of 3% oxygen and 5% CO₂ with medium changes every 2 days until confluent. Prior to an experiment, HUVEC were subcultivated with Trypsin/EDTA onto Costar® 96 well multiplates (Corning Incorporated, Corning, NY, USA) and used when confluent.
- 2. Oxygen Measurement:** Forma incubator equipped with Fyrite Analyzer Kit was used to non-invasively monitor oxygen levels. A Sensor Dish Reader (SDR) were used within a controlled oxygen environment provided by microprocessor controlled chambers (Coy, Laboratories, Grass Lake, MI).
- 3. Dose Response Studies:** Stock solutions of NAC (Sigma), GSH (Sigma), and GGC (United Peptides) were prepared in media. HUVEC cells were concurrently dosed with 0, 5, 50, and 500 µM of antioxidants and with 50-70 µM of menadione for 24 h.
- 4. Cytoprotection:** Following the duration of treatment, cell titre blue was added to the cells and cell viability was determined as the function of fluorescence at Ex 560 nm and Em 590 nm.
- 5. Western blotting:** Following appropriate culturing, cells were lysed in buffer and run on an 8% polyacrylamide gel (ePAGE) and transferred to a nitrocellulose membrane (iBlot: Life Technologies). Primary HIF-1A antibody was obtained from Novus (Littleton, CO) and secondary antibody was an from Li-Cor, Lincoln, Ne) and blots were scanned on an Odyssey. Western blots were quantified by ImageJ.

Results

Comparison of cells grown under 3% O₂ versus those grown at Atm O₂ demonstrated that the former were more effective against the menadione induced cytotoxicity even in the absence of any anti-oxidants (Fig. 1). Dose response studies demonstrated that 50 µM MD reduced viability by 80% at AtmO₂ but only by 40% at 3% O₂. This data suggests that the high oxygen levels in atmosphere may actually be causing oxidative injury to the cells and are thus cytotoxic.

Antioxidants (N-acetyl cysteine, gamma-glutamyl cysteine, and glutathione) were more effective cytoprotectants at 3% O₂ than AtmO₂, and lower doses (5 and 50µM) were more effective at 3% O₂ than AtmO₂ (Figs 2 and 3). However, all three compounds proved to be cytoprotective to HUVEC against MD-induced oxidant stress at their highest dose of 500µM. (Fig.4).

Additionally, western blot analysis revealed higher intensity of HIF-1 alpha induction in cells grown at 3% O₂ than those grown in 20.9% O₂ (Fig. 5).

0 uM Compounds at 50 uM Menadione

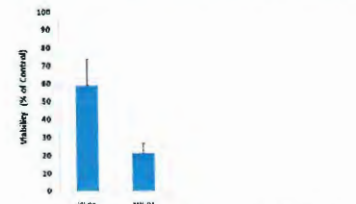


Fig. 1: Comparison of cells grown under 3% O₂ versus those grown at Atm O₂. A 50 µM MD reduced viability by 80% at AtmO₂ but only by 40% at 3% O₂. (n=3)

50 uM Compounds at 50 uM Menadione

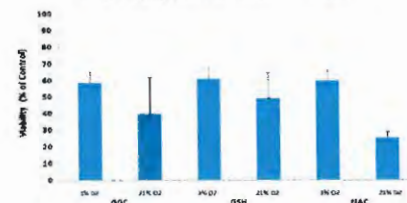


Fig. 3: Antioxidants: Gamma-Glutamyl Cysteine (GGC), Glutathione (GSH), and N-Acetyl Cysteine (NAC) were more effective cytoprotectants at 3% O₂ than AtmO₂. (n=3)

5 uM Compounds at 50 uM Menadione

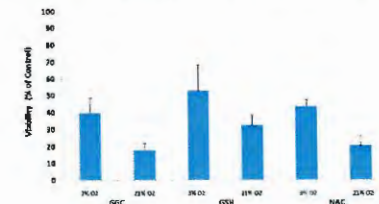


Fig. 2: Antioxidants: Gamma-Glutamyl Cysteine (GGC), Glutathione (GSH), and N-Acetyl Cysteine (NAC) were more effective cytoprotectants at 3% O₂ than AtmO₂. (n=3)

500 uM Compounds at 50 uM Menadione

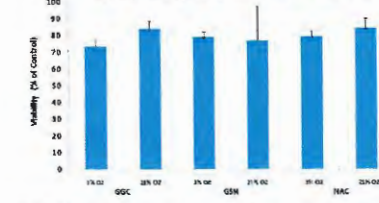


Fig. 4: All three Antioxidants: Gamma-Glutamyl Cysteine (GGC), Glutathione (GSH), and N-Acetyl Cysteine (NAC) were effective cytoprotectants at both 3% O₂ than AtmO₂. (n=3)

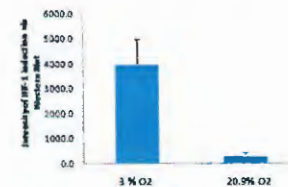


Fig. 5: Intensity of HIF-1 alpha induction was observed higher in cells grown in 3% oxygen as compared to those grown in 20.9% oxygen.

Conclusions

1. Physiological levels of oxygen, that is, 3% O₂ was found to be more cytoprotective than Atm O₂ against the menadione induced cytotoxicity.
2. Antioxidants were more effective cytoprotectants at 3% O₂ than Atm O₂.
3. The data suggests that the relatively high amount of oxygen in room air may be a substrate for generating oxidants, and screens for cytoprotective antioxidants might be more predictive of *in vivo* performance if done at more physiologic levels.

References

1. Packer L, Fuchs K. Low oxygen concentration extends the lifespan of cultured human diploid cells. Nature. 1977;267:423-425.
2. Chen Q, Fischer A, Reagan JD, Yan LJ, Ames BN. Oxidative DNA damage and senescence of human diploid fibroblast cells. Proceedings of the National Academy of Sciences of the United States of America. 1995;92:4337-4341.
3. Saito H, Hamanaka AT, Moses RE. The effect of low oxygen tension on the *in vitro* replicative life span of human diploid fibroblast cells and their transformed derivatives. Experimental Cell Research. 1995;217:272-279.
4. Akutsu KR, Hazenberg LA, Niemi AK, Cowan T, Hazenberg LA. Importance of culturing primary lymphocytes at physiological oxygen levels. Proc Natl Acad Sci U S A. 2007;104:4547-4552.
5. Parrinello S, Samperi T, Krolicka A, Goldstein J, Melov S, Campisi J. Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. Nature Cell Biology. 2003;5(5):741-747.
6. Lior G, Kowalczuk J, Schriber JM, Chandel NS, Vanden Hoek TL, Schumacker PT. Menadione triggers cell death through ROS-dependent mechanisms involving PARP activation without requiring apoptosis. Free Radic Biol Med. 2010 Dec 15;49(12):1925-36.
7. Kerkwick C, Willoughby D. The antioxidant role of glutathione and N-acetylcysteine supplements and exercise-induced oxidative stress. J Int Soc Sports Nutr. 2005 Dec 9;2:38-44. doi: 10.1186/1545-2703-2-2-38.



Pharmacokinetic Profiles of Caffeic Acid Phenethyl Amide (CAPA) and Caffeic Acid Phenethyl Ester (CAPE) in Male Sprague-Dawley Rats



¹J. Yang, ^{1,2}J. Bynum, ²P.D. Bowman, and ¹S. Stavchansky

¹Division of Pharmaceutics, College of Pharmacy, The University of Texas, Austin TX ²US Army Institute of Surgical Research, San Antonio TX

Abstract

Purpose
The pharmacokinetic profile of CAPA was investigated compared to CAPE in male Sprague-Dawley rats with the purpose of determining whether CAPA, an amide derivative of CAPE, resulted in prolongation of the elimination half-life from blood plasma. CAPA and CAPE have been shown to exhibit significant cytoprotective properties *in vitro*. CAPE has also been previously found to be significantly protective against ischemia/reperfusion injury *in vivo*.

Methods
Male Sprague-Dawley rats were administered CAPA at 5, 10 and 20 mg/kg doses and CAPE at 20 mg/kg (n=5) via intravenous bolus through a surgically implanted jugular vein catheter. Blood samples were collected at 8 time points up to 8 hours for CAPA and 3 hours for CAPE. Compounds were extracted with ethyl acetate, concentrated with a rotary evaporator, reconstituted with methanol and quantitatively determined using a validated LCMS method with electrospray ionization. Separation was performed with a Phenomenex MAX-RP (150x2.00 mm, 5 µm) column. Mass spectrometry analysis was performed with an Agilent 1100 series single quadrupole MSD with multimode spray chamber run in ESI-negative mode. Plasma concentration time data of CAPA and CAPE were analyzed by WinNonlin Professional (Pharsight). Pharmacokinetic parameters were analyzed by non-compartmental analysis (NCA) as well as through non-linear fit to a biexponential equation.

Results
There was a statistical significant difference found in the elimination half-life between CAPA and CAPE at 20 mg/kg (255.1 minutes versus 92.3 minutes, P < 0.05). Clearance was found to be 156.1, 102.6 and 45.1 ml/min, and volume of distribution was found to be 52.4, 39.2 and 17.8 l for the 5, 10 and 20 mg/kg CAPA dose groups from NCA. Pharmacokinetic parameters were analyzed by non-compartmental analysis (NCA) as well as through non-linear fit to a two compartment model.

Conclusions
Intravenous bolus administration of 20 mg/kg of CAPA, the amide derivative of CAPE, to male Sprague Dawley rats resulted in approximately a 170% increase in the elimination half-life when compared to CAPE. CAPA's clearance and volume of distribution were dose dependent suggesting non-linear pharmacokinetics.

Introduction

CAPE, a natural plant product and component of honeybee propolis has been found to exhibit a large variety of beneficial effects such as anti-inflammatory, anti-cancer, anti-viral and immunomodulatory activities. It was previously reported that CAPE is cytoprotective against menadione induced oxidative stress *in vitro*, and this effect has been correlated to the ability of CAPE to induce hemeoxygenase-1 (HO-1) rather than to direct antioxidant activity. It was found however that CAPE is hydrolyzed rapidly *in vitro* and after intravenous administration of 5, 10, and 20 mg/kg to catheterized male Sprague Dawley rats, CAPE exhibited a very rapid elimination. Efforts to improve the *in vitro* and *in vivo* stability of CAPE led to the synthesis of CAPA. The structures of CAPA and CAPE are shown in Figure 1. This compound was shown to be as cytoprotective as CAPE against hydrogen peroxide induced oxidative stress in human umbilical vein endothelial cells (HUVEC). CAPA has also been shown to have anti-oxidant and radical scavenging activities. The elimination half-life of CAPA was also found to be significantly longer than CAPE in male Sprague Dawley rat plasma, as CAPA exhibited a half-life of 10 hours at 37 °C compared to 0.13 hours for CAPE.

The objective of the present study was to develop and validate a LCMS method with electrospray ionization for the quantitative determination of CAPA and CAPE following extraction from rat plasma. This method was then used to conduct an exploratory study of the pharmacokinetic profiles of CAPA following intravenous bolus administration of 5, 10 and 20 mg/kg doses to male Sprague Dawley rats and compared to CAPE administered at 20 mg/kg.

Materials and Methods

•CAPE and *trans*-resveratrol (internal standard) were commercially available from Cayman Chemical (Ann Arbor, MI), and CAPA was synthesized previously in our laboratories

•Agilent 1100 series single quadrupole MSD was used for the quantitative determination of CAPE, CAPA and resveratrol

•Separation was achieved using Phenomenex MAX-RP (150x2.00 mm, 5 µm) column, water acetonitrile mobile phase in gradient elution

•Jugular vein catheterized male Sprague-Dawley rats were obtained from Charles River Laboratories

•CAPE and CAPA were administered via intravenous bolus via jugular vein catheter. Injection solution comprised of 45% propylene glycol, 40% sterile saline and 15% ethanol

•Pharmacokinetic parameters calculated using WinNonlin in both model independent and model dependent (2 compartment) analysis

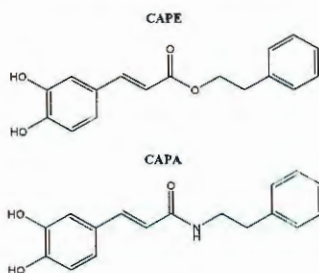


Figure 1 – Structures of CAPE and CAPA

Results

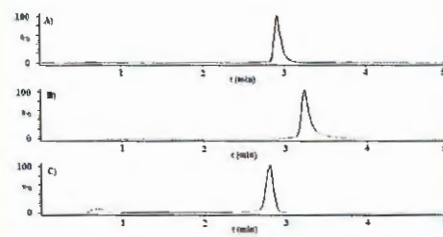


Figure 2 – LCMS chromatograms of A) CAPA B) CAPE and C) Resveratrol

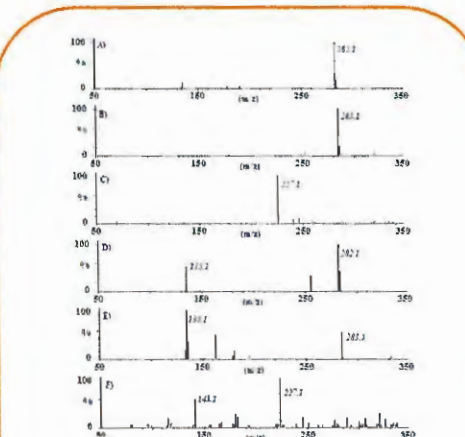


Figure 3 – Full mass scan spectra of A) CAPA at 70V fragmentor, B) CAPE at 70V fragmentor, C) Resveratrol at 70V fragmentor, D) CAPA at 280V fragmentor, E) CAPE at 280V fragmentor, F) Resveratrol at 280V fragmentor

Nominal Concentration (ng/ml)	Observed Concentration (ng/ml ± SD)	Intra-day precision (%RSD)	Inter-day precision (%RSD)	Accuracy (% deviation)
CAPA				
20	17.02 ± 1.51	6.17 – 8.86	10.74	14.9
500	510.4 ± 10.1	1.96 – 2.02	2.13	2.08
2000	1991 ± 61	1.98 – 4.20	3.63	0.47
CAPE				
20	26.37 ± 0.89	1.89 – 6.64	9.08	1.83
500	490.3 ± 19.3	2.45 – 5.26	4.01	1.94
2000	1965 ± 51	2.42 – 2.95	2.78	1.76

Table 1 – Assay validation parameters

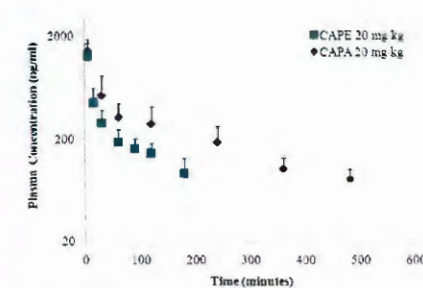


Figure 4 – Plasma concentration-time profiles for CAPA and CAPE

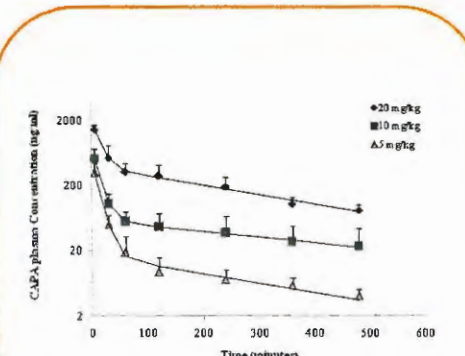


Figure 5 – Plasma concentration-time profiles for CAPA (20, 10 and 5 mg/kg)

	CAPE 20 mg/kg (N=5 ± SD)	CAPA 20 mg/kg (N=5 ± SD)	CAPA 10 mg/kg (N=3 ± SD)	CAPA 5 mg/kg (N=1 ± SD)	P value
NCA					
$t_{1/2}$ (min)	92.3 ± 19.5	255.1 ± 94.2	295.8 ± 61.4	243.1 ± 51.2	< 0.05
Cl_T (ml/min)	119.5 ± 50.5	45.0 ± 11.8	102.6 ± 54.8	116.1 ± 57.5	< 0.05*
V_d (l)	15.3 ± 5.1	17.8 ± 10.7	40.4 ± 13.5	52.4 ± 14.0	< 0.05*
$AUC_{0-\infty}$ (µg·min/ml)	59.2 ± 19.3	148 ± 32	39.2 ± 23.0	11.7 ± 4.0	0.01*
Biexponential fit					
A (ng/ml)	2249 ± 1268	1674 ± 436	716.2 ± 416.3	417.0 ± 195.2	
B (ng/ml)	249 ± 71	364 ± 117	56.58 ± 27.81	116.1 ± 6.41	
α (min ⁻¹)	0.17 ± 0.05	0.087 ± 0.037	0.086 ± 0.019	0.068 ± 0.019	
β (min ⁻¹) ^{10⁻³}	7.12 ± 0.97	2.94 ± 0.55	2.55 ± 0.69	3.35 ± 1.04	
$t_{1/2}$ (min)	98.9 ± 14.1	257.3 ± 82.1	286.6 ± 73.9	238.5 ± 51.9	< 0.02
Cl_T (ml/min)	124.6 ± 51.1	45.83 ± 12.53	115.2 ± 51.9	184.1 ± 97.4	< 0.05*
V_d (l)	17.2 ± 5.5	14.9 ± 10.5	43.6 ± 10.5	55.7 ± 15.5	< 0.05*
$AUC_{0-\infty}$ (µg·min/ml)	16.34 ± 17.42	146 ± 30	33.35 ± 18.29	10.66 ± 4.35	< 0.05*

Table 2 – Pharmacokinetic parameters for CAPE and CAPA. Non-compartmental analysis (NCA) and bi-exponential fit to a 2 compartment model are shown

Conclusions

- An HPLC-MS method for the quantitative determination of CAPE and CAPA from rat plasma was developed and validated

- The validated method was used to determine levels of CAPE and CAPA following intravenous administration to male Sprague-Dawley rats in a pharmacokinetic study

- CAPA exhibits a significantly longer elimination half life from the systemic circulation than CAPE

- CAPA appears to exhibit non linear pharmacokinetics in the dose range of 5 – 20 mg/kg



Network Analysis of the Cytoprotective Effect of CDDO-IM against Oxidant Stress in Human Umbilical Vein Endothelial Cells (HUVEC)



¹J. Bynum, ²P.D. Bowman, and ¹S. Stavchansky

¹Division of Pharmaceutics, College of Pharmacy, The University of Texas, Austin TX ²US Army Institute of Surgical Research, San Antonio TX

Abstract

[12-cyano-3,12-dioxoleano-1,9(11)-dien-28-oyl]imidazole (CDDO-IM), a synthetic derivative of oleanolic acid, has been demonstrated to possess anti-inflammatory activity. CDDO-IM (gift from Dr. Michael Sporn, Dartmouth University) was compared to the phenolic cytoprotectants caffeic acid phenethyl ester (CAPE) and caffeic acid phenethyl amide (CAPA). CDDO-IM at 0.20 μM was more effective than CAPE or CAPA at 500 and 5000 nM in protecting HUVEC from oxidant stress produced by menadione. Since CDDO-IM exhibits no direct antioxidant activity we tested it for transcriptional activation with whole genome microarrays and found that about 250 genes were up- or down-regulated by CDDO-IM. In addition to up-regulating heme oxygenase-1, a well-known cytoprotective gene, it also induced members of the heat shock protein family. Submission of genes statistically altered in their expression by greater than two-fold up-regulation to Ingenuity Pathway Analysis (IPA) produced networks known to be related to cellular development, growth and proliferation, cell signaling, and canonical pathways including NRF2-mediated oxidative stress response and PPAR signaling indicating that cytoprotection involves multiple pathways in addition to the well described phase II enzyme induction.

Introduction

Oxidative stress is commonly encountered in neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's, vascular disorders including strokes and heart attacks as well as traumatic injuries. We previously reported that CAPE and CAPA displayed cytoprotective activity against menadione (MD)-induced oxidative stress in human umbilical vein endothelial cells (HUVECs). The induction of heme oxygenase-1 (HO-1), a phase II enzyme, in HUVEC played an important role for CAPE and CAPA cytoprotection (1,2). To improve the beneficial effect, a more potent phase II enzyme inducer, [12-cyano-3,12-dioxoleano-1,9(11)-dien-28-oyl]imidazole (CDDO-IM), was examined. The cytoprotection mechanism was investigated through gene expression and signaling pathway analysis.

Materials and Methods

In vitro assay:

Confluent HUVEC were pretreated with 0.5 μM or 5 μM CAPE or CAPA: 0.20 μM CDDO-IM; or control (0.1% DMSO) for 6 hrs, then exposed to MD for an additional 24 hrs. Cell viability was assessed using CellTiter Blue. Each experiment was performed in quadruplicate.

Gene expression analysis:

BRB Array Tools

Total RNAs from 6h-CDDO-IM (0.20 μM) pretreated or vehicle control HUVECs were isolated and labeled for microarray analysis using Agilent whole-genome microarrays. The experiments were done in quadruplicate. Microarray data analysis and statistical comparison were performed using BRB Array Tools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>). Genes were considered statistically significant with P value < 0.001 and FDR (false discovery rate) value < 10%.

Ingenuity Pathway Analysis

Genes significantly altered in their expression were submitted to Ingenuity Pathway Analysis (IPA) for further network analysis (www.ingenuity.com). IPA used Fischer's exact test to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. The score of network represents the negative log of the P value. Therefore, scores of 2 have at least 99% confidence of not being generated by chance alone. Genes are represented as a single node in the network. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation.

Results

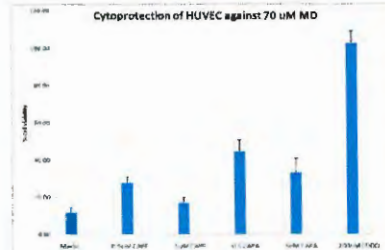


Figure 1. Cytoprotection by CAPA, CAPA, and CDDO-IM against menadione induced oxidative stress. A six hr pretreatment before MD induced injury resulted in significantly better cell survival. CDDO-IM provided 100% cell viability.

Table 1: Most mapped up- and down-regulated genes by CDDO-IM through IPA:

Gene description	Fold change	p-value	FDR
Top 10 mapped up-regulated genes			
zinc finger protein 323 (ZNF323), transcript variant 1, (NM_008899)	33.2	2.62E-05	0.0067
heat shock 70kDa protein 1A (HSPA1A), (NM_003343)	20.8	2.96E-05	0.0067
cellular stress induced growth inhibitor 1 (CSG1), transcript variant 1, (NM_013870)	12.5	6.76E-05	0.0078
home oxygenase 1 (hemoxygen 1) (HMOX1), (NM_002133)	9.3	3.96E-05	0.0017
vasostatin A (VASA), (NM_138410)	7.7	0.00647	0.0177
nuclear receptor subfamily 0, group B, member 1 (NR0B1), (NM_000475)	7.5	4.70E-05	0.0020
DnaI (Hsp40) homolog, subfamily A, member 4 (DNAI4), (NM_019622)	6.6	0.00613	0.0175
glutamate-cysteine ligase, modifier subunit (GCLM), (NM_007061)	6.0	0.01017	0.0208
nitrohydroxy-sulfhydryl oxidase 2 (NHSOX2), (NM_138340)	5.8	0.00247	0.0150
thioredoxin inducible 1 (TXNRI1), transcript variant 1, (NM_037390)	5.5	0.000114	0.0298
Top 10 mapped down-regulated genes			
vascular cell adhesion molecule 1 (VCAM1), transcript variant 1, (NM_001078)	-14.3	9.70E-06	0.0056
baculoviral IAP repeat-containing 3 (BIRC3), transcript variant 1, (NM_001165)	-11.7	0.00625	0.0177
selectin E (endothelial adhesion molecule 5) (SELE), (NM_000456)	-11.1	0.000313	0.0160
secreted phosphoprotein 1 (SPPL), transcript variant 1, (NM_001057)	-8.4	0.000323	0.0160
nitrohydroxy-sulfhydryl oxidase 1 (NHSOX1), (NM_004753)	-5.6	8.31E-05	0.0045
cytochrome P450, family 26, subfamily B, polypeptide 1 (CYP2A6), (NM_019885)	-5.5	0.00013	0.0098
N-acetyltransferase 8 (NAT8), (NM_003960)	-5.1	0.000133	0.0098
thioredoxin interacting protein (TXNIP), (NM_006477)	-4.5	0.000238	0.0220
insulin-like growth factor binding protein 1 (IGFBP1), transcript variant 1, (NM_000596)	-4.2	0.00549	0.0164
leucine rich repeat containing 32 (LRRC32), (NM_025312)	-4.2	0.000270	0.0132

Table 2: Top regulated biological networks by CDDO-IM through IPA (scores ≥ 14):

ID	Network Name	Score	Top 5 Affected
1	Cell Proliferation, Cell Cycle, and Cell Growth	25	Cell cycle, Cell cycle, Cell cycle, Cell cycle, Cell cycle
2	Cellular Stress Response	20	Cellular stress response, Cellular stress response, Cellular stress response, Cellular stress response, Cellular stress response
3	Cellular Signaling	18	Cellular signaling, Cellular signaling, Cellular signaling, Cellular signaling, Cellular signaling
4	Cellular Homeostasis	17	Cellular homeostasis, Cellular homeostasis, Cellular homeostasis, Cellular homeostasis, Cellular homeostasis
5	Cellular Differentiation	16	Cellular differentiation, Cellular differentiation, Cellular differentiation, Cellular differentiation, Cellular differentiation

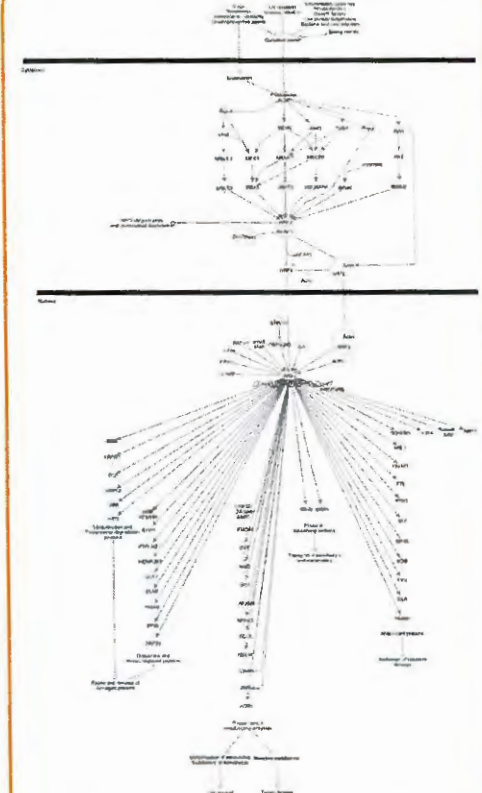


Figure 2: NRF2-mediated oxidative stress signaling pathway activated by CDDO-IM treatment (P value = 1.5×10^{-5}). The red color of the node represents up-regulation.

Conclusions

- CDDO-IM is a more potent cytoprotectant than CAPE or CAPA against MD-induced oxidative stress in HUVEC in the menadione model.
- Gene expression profiling and signaling pathway analysis indicated that NRF2 mediated pathway is activated by CDDO-IM, which confirmed previous finding in the literature (3). Other signaling pathways associated with CDDO-IM treatment include PPAR and xenobiotic metabolism. These results indicate additional targets that may explain its better cytoprotection.

References

- Wang X, Stavchansky S, Zhao B, Bynum JA, Kerrin SA, Bowman PD. Cytoprotection of human endothelial cells from menadione cytotoxicity by caffeic acid phenethyl ester: the role of heme oxygenase-1. *Eur J Pharmacol*. 2004 Sep 4;491(1-3):23-35.
- Yang J, et al. Synthesis of a series of caffeic acid phenethyl amide (CAPA) derivatives: comparison of cytoprotective effects to caffeic acid phenethyl ester (CAPE). *Phy Med Clin Res*. 2010;5(3):107-116.
- Lilly, K, et al. The synthetic tricyclic, CDDO and CDDO-methylamide, are potent inducers of heme oxygenase-1 and Nrf2 ARE signaling. *Cancer Res*. 65, 4759-4768. doi:10.1158/0008-7472.CCR-04-4537 (2005).

Comparison of Bioluminescence Imaging and Luminometry for Detection of Luciferase Activity in Transgenic Mice Engineered to Express Luciferase in Response to Hypoxia

Ashish Rastogi^{1,2}, James Bynum^{1,2}, Salomon Stavchansky², and Phillip Bowman¹

¹US Army Institute of Surgical Research, Fort Sam Houston, TX 78234

²Division of Pharmaceutics, College of Pharmacy, The University of Texas at Austin, TX 78712

Abstract: Bioluminescence imaging was compared with luminometry for quantitative determination of luciferase activity using transgenic mice as a model that is engineered to accumulate luciferase in response to hypoxia. Mice were hemorrhaged and *in vivo* imaging was performed at 4 h. Mice were then euthanized and organ reimaged *ex vivo* or frozen prior to luminometry. Luminometry rather than imaging showed differential expression of luciferase in hemorrhaged mice compared to sham indicating upregulation of HIF-1 α . Luminometry was found to be more precise than *in vivo* or *ex vivo* imaging for determining the effect of hypoxia for this particular mouse strain.

©2010 Optical Society of America

OCIS codes: (170.0110) Imaging Systems; (170.3880) Medical and biological imaging.

References and links

1. A. Sato, B. Klaunberg, and R. Tolwani, "In vivo bioluminescence imaging," *Comp Med* **54**, 631-634 (2004).
2. Y. Kong, Y. Shi, M. Chang, A. R. Akin, K. P. Francis, N. Zhang, T. L. Troy, H. Yao, J. Rao, S. L. Cirillo, and J. D. Cirillo, "Whole-body imaging of infection using bioluminescence," *Curr Protoc Microbiol* Chapter 2, Unit2C 4 (2011).
3. R. T. Sadikot and T. S. Blackwell, "Bioluminescence: imaging modality for in vitro and in vivo gene expression," *Methods Mol Biol* **477**, 383-394 (2008).
4. C. H. Contag and M. H. Bachmann, "Advances in in vivo bioluminescence imaging of gene expression," *Annu Rev Biomed Eng* **4**, 235-260 (2002).
5. P. E. Andreotti, G. Caceres, R. Zamkina, and M. Dauphinee, "Luminescence applications for chemotherapeutic drug development," *Recent Results Cancer Res* **161**, 3-12 (2003).
6. A. Moller and J. K. Jansson, "Detection of firefly luciferase-tagged bacteria in environmental samples," *Methods Mol Biol* **102**, 269-283 (1998).
7. G. R. Rettig, M. McAnuff, D. Liu, J. S. Kim, and K. G. Rice, "Quantitative bioluminescence imaging of transgene expression in vivo," *Anal Biochem* **355**, 90-94 (2006).
8. G. R. Rettig and K. G. Rice, "Quantitative in vivo imaging of non-viral-mediated gene expression and RNAi-mediated knockdown," *Methods Mol Biol* **574**, 155-171 (2009).
9. N. Porakishvili, J. L. Fordham, M. Charrel, P. J. Delves, T. Lund, and J. M. Roitt, "A low budget luminometer for sensitive chemiluminescent immunoassays," *J Immunol Methods* **234**, 35-42 (2000).
10. P. E. Andreotti and F. Berthold, "Application of a new high sensitivity luminometer for industrial microbiology and molecular biology," *Luminescence* **14**, 19-22 (1999).
11. M. Safran, W. Y. Kim, F. O'Connell, L. Flippin, V. Gunzler, J. W. Horner, R. A. Depinho, and W. G. Kaelin, Jr., "Mouse model for noninvasive imaging of HIF prolyl hydroxylase activity: assessment of an oral agent that stimulates erythropoietin production," *Proc Natl Acad Sci U S A* **103**, 105-110 (2006).
12. H. M. Sowter, R. R. Raval, J. W. Moor, P. J. Ratcliffe, and A. L. Harris, "Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia," *Cancer Res* **63**, 6130-6134 (2003).

13. K. K. To and L. E. Huang, "Suppression of hypoxia-inducible factor 1alpha (HIF-1alpha) transcriptional activity by the HIF prolyl hydroxylase EGLN1," *J Biol Chem* **280**, 38102-38107 (2005).
14. H. L. Foster, J. D. Small, and J. G. Fox, *The Mouse in Biomedical Research: Normative biology, immunology, and husbandry. American College of Laboratory. (Academic Press: New York., 1983), Vol. III.*
15. S. Gross, U. Abraham, J. L. Prior, E. D. Herzog, and D. Pivnicka-Worms, "Continuous delivery of D-luciferin by implanted micro-osmotic pumps enables true real-time bioluminescence imaging of luciferase activity in vivo," *Mol Imaging* **6**, 121-130 (2007).
16. J. J. Lysiak, J. L. Kirby, J. J. Tremblay, R. I. Woodson, M. A. Reardon, L. A. Palmer, and T. T. Turner, "Hypoxia-inducible factor-1alpha is constitutively expressed in murine Leydig cells and regulates 3beta-hydroxysteroid dehydrogenase type I promoter activity," *J Androl* **30**, 146-156 (2009).
17. S. Carlin, A. Pugachev, X. Sun, S. Burke, F. Claus, J. O'Donoghue, C. C. Ling, and J. L. Humm, "In vivo characterization of a reporter gene system for imaging hypoxia-induced gene expression," *Nucl Med Biol* **36**, 821-831 (2009).
18. D. A. Chan, P. D. Sutphin, N. C. Denko, and A. J. Giaccia, "Role of prolyl hydroxylation in oncogenically stabilized hypoxia-inducible factor-1alpha," *J Biol Chem* **277**, 40112-40117 (2002).
19. G. F. Lungu, M. L. Li, X. Xie, L. V. Wang, and G. Stoica, "In vivo imaging and characterization of hypoxia-induced neovascularization and tumor invasion," *Int J Oncol* **30**, 45-54 (2007).
20. S. Salceda and J. Caro, "Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes," *J Biol Chem* **272**, 22642-22647 (1997).
21. R. J. Viola, J. M. Provenzale, F. Li, C. Y. Li, H. Yuan, J. Tashjian, and M. W. Dewhirst, "In vivo bioluminescence imaging monitoring of hypoxia-inducible factor 1alpha, a promoter that protects cells, in response to chemotherapy," *AJR Am J Roentgenol* **191**, 1779-1784 (2008).

1. Introduction

Bioluminescence imaging is a simple and sensitive method that is based on detection of light emission from cells or tissues [1]. The luciferase gene is commonly used as a reporter under the control of a promoter of interest. The technique provides a low-cost, non-invasive, and real-time method to perform gene expression assays in living animals [2-3]. The technique greatly reduces the number of animals sacrificed per experiment while providing sufficient information on gene regulation and protein function in the context of functional tissues and organ systems [4]. It has been widely used to track tumor cells, bacterial and viral infections, and gene expression and to develop chemotherapeutic drugs [5-6]. Additionally, quantitative *in vivo* bioluminescence imaging has been used to study the efficiency of gene transfer in the liver and other organs [7-8]. Luminescence from the expression of the luciferase gene can also be measured *in vitro* by using a luminometer, which provides a convenient, rapid, and sensitive method for quantifying gene expression when used with a luciferase reporter assay system [9-10]. However, the two techniques – bioluminescence imaging and luminometry - have not yet been compared to determine which provides a better quantification of luciferase activity. In the present study, bioluminescence imaging and luminometry techniques were compared using a transgenic FVB.129S6-*Gt(ROSA)26Sor^{tm1(HIF1A)luc}Kael/J* mouse strain that has been genetically engineered to express luciferase with accumulation of HIF-1 α [11]. Excessive loss of blood, such as in hemorrhage, causes reduced oxygen supply to some organs leading to accumulation of hypoxia inducible factor (HIF-1 α) [12-13]. However, those organs most effected have not yet been determined. In this study we have attempted to use *in vivo* imaging to determine the luciferase activity as a function of hypoxia in organs most affected by hemorrhage. Organs were removed and imaged again *ex vivo*. Finally, organs were homogenized, and luminescence was measured with a luminometer. The results of the three studies were compared to determine which method provided the most precise way to quantify luminescence from hemorrhaged organs.

2. Materials and Methods

2.1 Mice and Hemorrhage

Experiments were conducted in accordance with the guidelines set forth by both the US Army Institute of Surgical Research (USAISR) and the guidelines of the National Institutes of Health (NIH) for animal care and use. The study was approved by the USAISR's Institutional Animal Care and Use Committee. Male FVB.129S6-*Gt(ROSA)26-Sor^{tm1(HIF1A luc)Kaell3}* mice (Jackson Labs, Bar Harbor, ME) 8-12 weeks old and weighing 25-30 g were used in the study. The mice were allowed food and water ad libitum and provided with environmental enrichment tools. Before the study, they were observed for 1 week to allow for environmental changes and to exclude the possibility of pre-existing disease.

Animals were divided into two experimental groups, sham and hemorrhage. After anesthesia with 2% isoflurane in air, hemorrhage was accomplished by removing 40% of the calculated blood volume with a lancet (Medipoint, Mineola, NY) via the submaxillary vein of the anesthetized mouse, and sterile gauze was applied to the vein to stop further bleeding. The calculated blood volume to be removed for each mouse was based on its weight [14]. For sham controls, lancet was applied but bleeding was immediately stopped by application of sterile gauze.

2.2 Imaging

A 50-mg/ml solution of potassium salt of D-Luciferin (Caliper Life Sciences, Hopkinton, MA) was prepared with phosphate buffered saline, pH 7.4. Continuous delivery of luciferin was achieved by using osmotic pumps (Alzet, Cupertino, CA) as described by Gross et al. [15]. At least 24 h prior to imaging, the osmotic pumps were filled with luciferin solution and implanted on the dorsal side of the mice. Four hours after hemorrhage, mice from both groups were anesthetized with 2% isoflurane-air mixture. The mice were then imaged with the *In Vivo* Imaging System (IVIS[®]) Lumina II bioluminescence system (Caliper Life Sciences, Hopkinton, MA). The light coming from various organs, as a function of luciferase production concomitant with HIF-1 α induction, was quantified from the images with Living Image software 3.0.4. Mice were then euthanized and portions of each organ reimaged or frozen in liquid nitrogen and stored at -80° C for *in vitro* luciferase quantification with a luminometer.

2.3 Luminometer Analysis

The luciferase assay system (Promega, Madison, WI) was used for quantitative analysis of tissues from the sham and hemorrhage groups. The analytical method was developed in accordance with the manufacturer's protocol. Briefly, tissues from both groups were homogenized in 500 μ l of lysis buffer that was supplied as part of the luciferase assay system and previously mixed with 1 \times proteinase inhibitor (Thermo Fisher Scientific, Waltham, MA). The tissue lysates was centrifuged at 14000 rpm for 5 minutes. The supernatant was collected, and an aliquot was assayed for luciferase activity by using the modulus microplate luminometer (Promega, Madison, WI) and luciferin as the substrate. The light intensities were calculated by measuring the relative luminescence unit (RLU) signal. A portion of the supernatant was also used for determining the amount of protein in the tissue lysates with the Pierce 600-nm Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The luminescence emitted from each organ was recorded as RLU per milligram of protein.

2.4 Statistical Analysis

Levene's test was used to assess the homogeneity of variance. Student's t-test was used to analyze differences between the sham and hemorrhage groups. A difference of p value < 0.05 was considered significant.

3. Results

3.1 Quantitative Analysis of Organs Affected by Hypoxia by *in vivo* Bioluminescence Imaging

In vivo bioluminescence imaging has the potential to detect and quantify the expression of HIF-1 α . The results of imaging FVB.129S6-*Gt(ROSA)26-Sor^{tm1(HIF1 α :luc)Kael1}* mice are shown in Fig. 1. The images indicate that overall the bioluminescence in the hemorrhage group is higher than in the sham group. However, in most cases, it was difficult to determine luminescence from a specific organ and to study luminescence from the images themselves. For example, kidneys on dorsal images and testes and liver on ventral images were more easily quantified than the other organs. In Table 1, average bioluminescence intensity (in counts) of these organs was estimated from the *in vivo* images of the hemorrhage and sham groups. Theoretically, organs from the hemorrhage group should depict more luminescence as a function of HIF-1 α activity due to hypoxia. The testes from the hemorrhage group exhibited lesser bioluminescence than from the sham group, the liver showed higher bioluminescence, and the kidney showed no significant difference between the groups (p > 0.05).

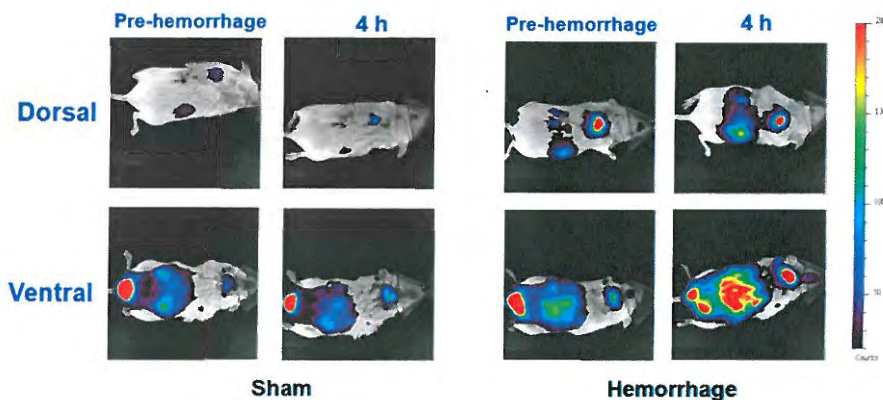


Fig. 1. Non-invasive images of FVB.129S6-*Gt(ROSA)26-Sor^{tm1(HIF1 α :luc)Kael1}* mice (a representative mouse from each group) are shown. A reference intensity scale with units in counts or photons emitted is also included.

Table 1: Average bioluminescence intensity (in counts) of organs as observed from the *in vivo* images of the hemorrhage and sham groups are shown. Quantitative analysis of kidney, liver, and testes is shown as accurate positioning of other organs was not visible. The data is represented as mean with standard deviation (N = 3; *p < 0.05).

Group	Testes	Liver	Kidney
Hemorrhage	165.4 ± 60.2	117.8 ± 62.9	54.8 ± 45.5
Sham	359.6 ± 53.6	74.6 ± 32.4	47.0 ± 23.5
Ratio (H/S)	0.5*	1.6*	1.2

3.2 *Ex vivo* Imaging of Organs

Bioluminescence imaging of the whole animal may be affected by various factors such as blood flow and scattering of light photons. Hence, *ex vivo* imaging of isolated organs was performed to observe the luminescence in individual organs. Fig. 2 shows *ex vivo* images of various organs isolated from both the sham and hemorrhage groups. It is our observation that the intensity of bioluminescence exhibited from an organ from animals belonging to the same group was variable. In addition, not all organs exhibited bioluminescence in each animal.

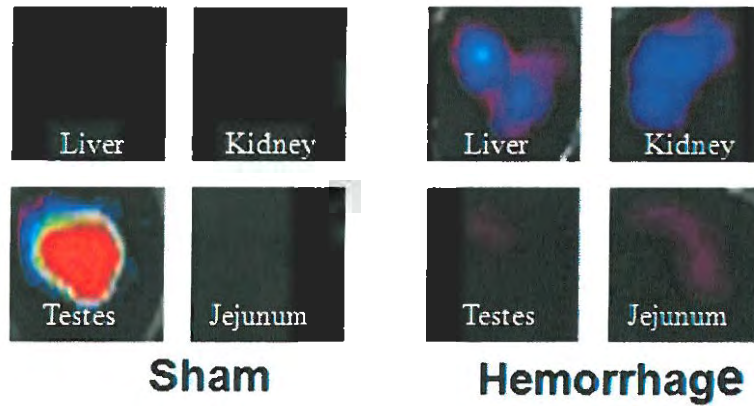


Fig. 2. Images of various organs after removal from a sham or hemorrhage animal. Images are from a representative mouse from each group.

3.3 Quantitative Luminometer Analysis of Homogenized Organs

After homogenization, isolated organs were quantified with the luminometer. Table 2 shows the average luminescence/mg of protein of organs isolated from the hemorrhage and sham groups. The luminometer analysis shows that the hemorrhage group has higher luminescence values than the sham group in all sets of organs ($p < 0.05$). The results obtained with the luminometer are more in accordance with the theory that animals with hemorrhage due to hypoxia will exhibit higher luminescence than the sham group because of upregulation of HIF-1 α .

Western blot analysis for HIF-1 α in proteins from nuclear extracts of various organs such as lung, liver, kidney, and spleen failed to yield any significant results. Our results were in accordance with a previous study by Lysiak et al., who were only able to detect HIF-1 α in testes [16]. One reason could be the low density of HIF-1 α in the organs. In such a scenario, the *in vitro* luminometer provides a reliable and sensitive tool to detect HIF-1 α in hypoxic organs.

Table 2: Average luminescence (in millions relative luminescence units/ mg of protein) of organs isolated from hemorrhage and sham groups are shown. The luminometer analysis shows hemorrhage groups have higher luminescence values than the sham group indicating hypoxia in the hemorrhage group. The results are shown as mean with standard deviation (N = 4; * $p < 0.05$).

Group	Lung	Liver	Kidney	Spleen
Hemorrhage	3.2 \pm 0.9	13.7 \pm 3.2	5.4 \pm 1.1	4.1 \pm 1.2
Sham	1.1 \pm 0.4	7.6 \pm 2.3	1.7 \pm 0.3	3.4 \pm 1.7
Ratio (H/S)^a	3.0*	1.8*	3.2*	1.2

^aH/S = hemorrhage/sham group.

4 Discussion

Although *in vivo* bioluminescence imaging and *in vitro* luminometer analysis have both been previously used to study hypoxia-related luminescence [17-21], to our knowledge, there hasn't been a single report that discusses the correlation between the two techniques. In the present study, we employed a transgenic mice model to compare the two techniques for their ability to quantitatively determine the degree of luciferase activity in response to hypoxia. Hypoxia was induced by subjecting the Rosa-Luc mice to 40% hemorrhage. The resulting induction of HIF-1 α in various organs was studied in correlation to the amount of luminescence emitted. *In vivo* bioluminescence images demonstrated that HIF-1 α induction was greater in the hemorrhage group than in the sham group. However, bioluminescence imaging showed poor quantitative ability, possibly because quantitative differentiation of

different organs using *in vivo* bioluminescence imaging is difficult in conditions such as hemorrhagic shock, which affects various regions in a hemorrhaged animal. Different organs may also exhibit luminescence differently due to their positioning and distance from the light source. On the other hand, the luminometer provided a simple and reliable tool to quantify the amount of luminescence emitted from several organs. Nevertheless, one clear advantage of bioluminescence imaging over luminometer analysis is that the former is a total noninvasive technique, whereas the latter requires euthanization of the animal. Bioluminescence imaging is an attractive method for qualitative analysis as it enables serial and rapid collection of data.. However, for quantitative determination of luciferase activity, luminometry provides a more precise method.

5 Conclusion

Luminometer data rather than *in vivo* or *ex vivo* imaging analysis confirmed that hemorrhaged animals show higher luminescence due to accumulation of HIF-1 α in specific organs. Some affected (hypoxic) organs did not yield significant amounts of light by *in vivo* imaging. The luminometer was found to be more reliable than bioluminescence imaging methods in the evaluation of hypoxic organs. The Rosa-Luc transgenic mice provided a useful model to determine luciferase activity in organs affected by hypoxia.