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The formation of sphingosine, a potent inhibitor of protein kinase C, has been characterized in cell models of interest to studies of hypoxic and ischemic injury. With J774 macrophages, we find that sphingosine is released in an acidic compartment and is inhibitable by ammonium ion, an agent that is well known to accumulate during ischemia. The increase in sphingosine is correlated with inhibition of protein kinase C; therefore, these findings may provide a link between changes in ammonia in vivo and alteration in cell function.

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INTRODUCTION

Hypoxia and ischemia are major causes of human morbidity and mortality. In most traumatic injuries and surgical procedures, a major complicating factor is interruption of normal blood supply and oxygenation of tissues. In addition, over half of the deaths in the United States are due to respiratory and circulatory causes and most of these involve tissue hypoxia. Despite decades of intensive research, the molecular basis for such irreversible injury is not known.

It is likely that a contributing factor in hypoxic and ischemic injury is the derangement of various cellular signal transduction systems. Furthermore, therapeutic intervention at the level of intracellular second messengers provides a potentially powerful approach to minimize irreversible damage and promote repair of tissues that have undergone traumatic injury and ischemia.

Our laboratory has recently identified what appears to be a new lipid second messenger system that utilizes the long-chain (sphingoid) base backbones of cellular sphingolipids as inhibitors of protein kinase C, activators of growth factor receptor kinases, and modulators of a growing list of other cellular systems. For an overview of the current status of this field, see the enclosed proofs of a review that will be published in early 1991.

Our original proposal had four objectives, to determine:

1) the time course of O_2-dependent changes in concentrations of sphingosine and correlate these with the pathophysiological changes taking place in cells;

2) the molecular basis for any alterations in sphingosine levels by studying the involvement of specific receptors and their respective G proteins, enzymes, and second messengers. This will entail experiments to address integration of signals, and include factors that influence Ca^{2+} homeostasis because of the known effects of anoxia on Ca^{2+} balance;

3) the possible protection of cells against anoxic cell death when optimally balanced against the other signals to which the cells are exposed; and,

4) the effects of different structural analogues of sphingosine, in expectation of

identifying compounds of greater utility in modulating the responses of these systems.

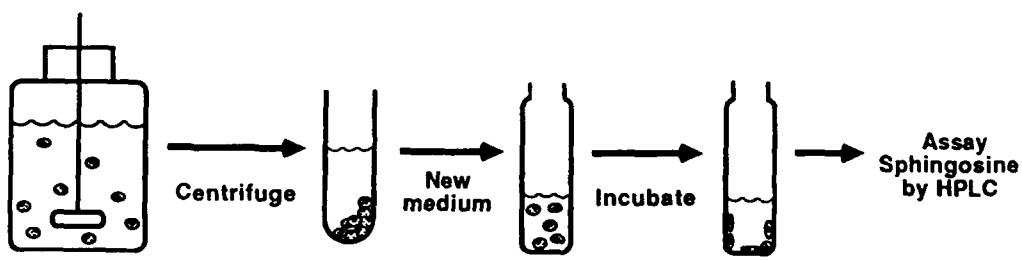
RESULTS

Measurement of free sphingosine in hepatocytes during ischemia.

Our original plan called for analyses of sphingosine metabolism by rat hepatocytes and renal cells because there is a wealth of information about the effects of ischemia on these cell types. We have measured the amounts of sphingosine in isolated hepatocytes, as well as over a time course of incubation of the cells under atmospheric oxygen and hypoxic conditions. To our disappointment, the data have been very confusing because there is considerable variability among different cell preparations. This has not been remedied by use of defined media. Furthermore, attempts to reduce this variability using a liver cell line (HepG2 cells) have met with mixed success. For reasons described below, we have elected to shift our focus to another cell type that is of considerable relevance to hypoxic/ischemic cell injury (i.e., a macrophage cell line), that is easy to handle and obtain reproducible results, and that has already yielded interesting discoveries about sphingosine metabolism and functions.

Measurement of free sphingosine in J774 macrophages under different culture conditions.

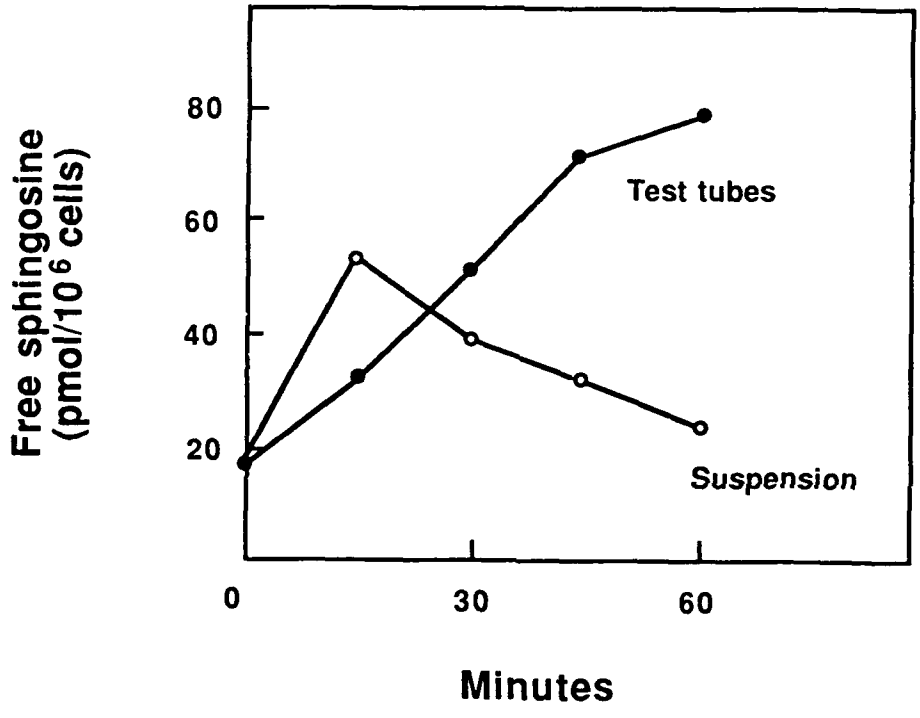
We have hypothesized that sphingolipids may help adjust the behavior of cells to different external environments. J774 cells are a good model for this phenomenon because the cells can be grown in suspension culture then transferred to test tubes or culture dishes, where they bind in minutes. The basic protocol that we use is outlined in this diagram:



Protocol for studying sphingosine formation in J774 cells

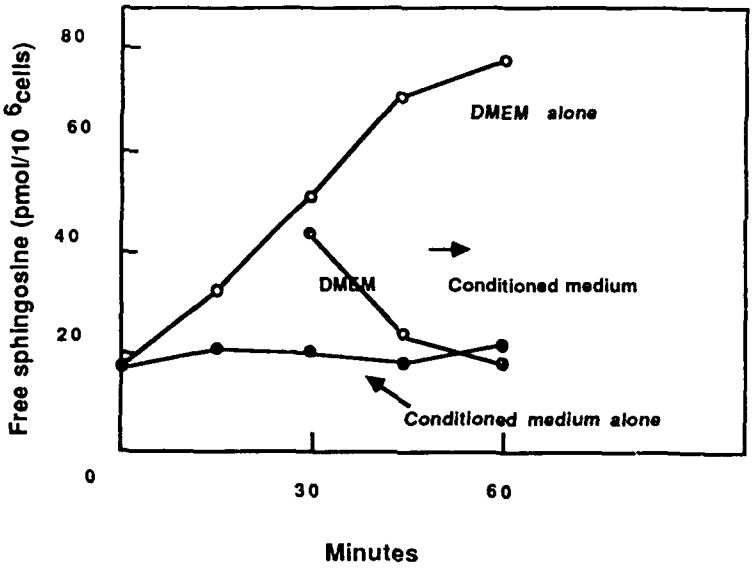
Legend: Protocol for growing J774 cells insuspension culture in DMEM containing 10% fetal bovine serum, followed by centrifugation and transfer to test tubes in new DMEM (without FBS).

We have analysed amounts of free sphingosine in J774 cells using the HPLC assay developed at Emory (Anal. Biochem. 171:373, 1988), and found that transfer of suspended cells to test tubes stimulated up to a 10-fold increase in sphingosine within an hour as shown below and in the accompanying abstracts (Merrill et al., 1990; Warden et al., 1990).



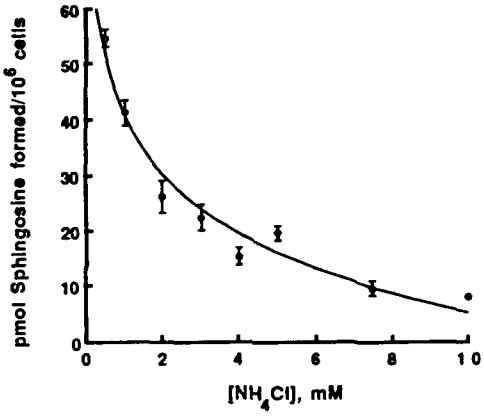
Legend: Time course of increases in sphingosine in J774 cells upon transfer to test tubes or return to a spinner flask. The initial spike in free sphingosine with the suspended cells is highly variable, and may represent the cell-surface contact that takes place during centrifugation of the cells.

One of the more interesting features of the increase is that it is inhibited by conditioned medium. In addition, if new medium is added (resulting in an increase in sphingosine), then the cells are returned to conditioned medium, the sphingosine levels drop rapidly to the initial level (on the next page).



Legend: Effect of adding conditioned medium to cells while allowing them to adhere to glass, or of adding conditioned medium to cells already attached in new DMEM.

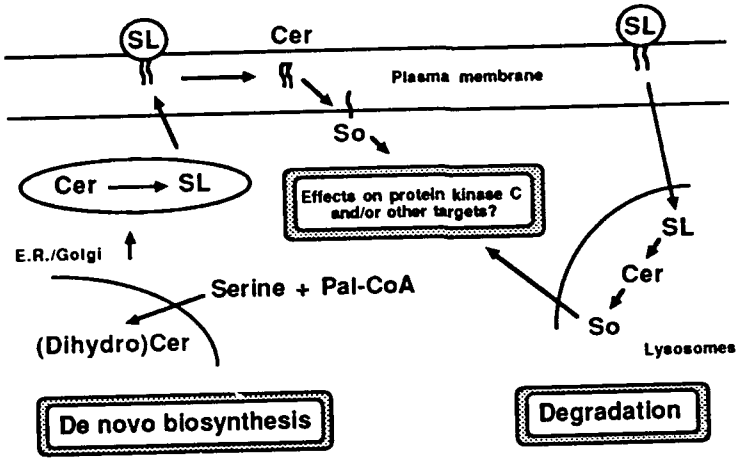
We have concluded that ammonia is one of the components of conditioned medium that suppresses sphingosine formation. Addition of ammonium ion (>1 mM) inhibited the appearance of sphingosine, as shown by the scheme below:



Legend: Reduction of the levels of free sphingosine by ammonium chloride. J774 cells were transferred to new DMEM plus the shown concentrations of ammonium chloride, and the levels of sphingosine were measured after one hour.

Analyses of ammonia in new and conditioned medium revealed that the concentration increased from 0.25 to 3 mM. These findings suggested that adherence triggers turnover of sphingolipid(s) to free sphingosine in an acidic compartment; hence, we have determined the effect of chloroquine (25 uM) and determined that this reagent also blocks the increases in sphingosine.

Based on these results, we have concluded that J774 cells have an endosomal or lysosomal system for forming sphingosine; hence, there may be multiple pathways for forming this compound, as shown below:



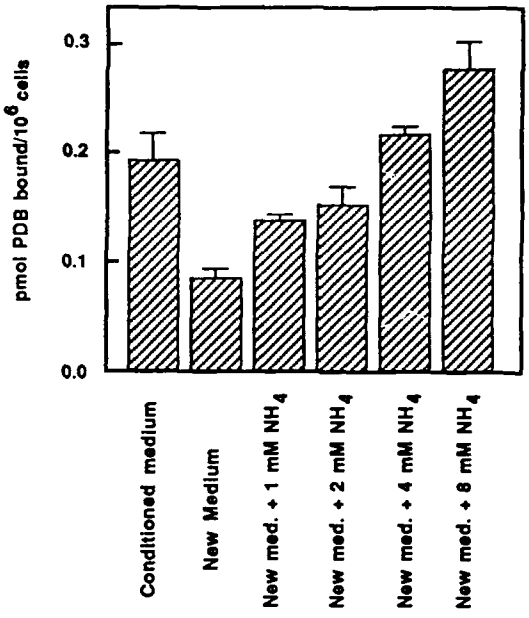
Legend: Possible sources of sphingosine in J774 cells.

We are continuing to study this effect of ammonia because it is well known that cells can be exposed to levels of ammonia as high as 25 to 50 mM in some regions of the bowel, kidney, and liver (in cirrhosis). Furthermore, one expects ammonia concentrations to rise when there is an interruption of blood flow, as occurs in the most commonly encountered causes of ischemia.

Although we have described these findings at two national meetings (see attached abstacts), we have been slow to publish them because the suppression by conditioned medium was consistently greater than the equivalent concentration of NH_4Cl, which suggested that additional factor(s) are present. We now know (Warden et al., 1991), from fractionation of conditioned medium by cation exchange and gel permeation chromatography, that conditioned medium contains an additional low-molecular weight fraction (ca 100-200 daltons) that contains a ninhydrin and TNBS-positive amino group. Based on the amount of the amine, this material suppresses sphingosine formation at micromolar concentrations and is now undergoing structural elucidation. These findings underscore the complexity of sphingosine formation in cells, and may reflect a mechanism to alter macrophage behavior.

One of the questions that we have attempted to answer while elucidating the nature of the "factors" that govern the sphingosine levels of these cells is: "Does an elevation in cellular sphingosine have an effect on protein kinase C or another cell system?" The ability to control this aspect of sphingolipid metabolism using different

media allowed assessment of the effects of endogenous sphingosine on phorbol phorbol dibutyrate binding, a striightforward index of the status of protein kinase C. These investigations found that there is a close correlation in the ability of ammonia to reduct the amounts of sphingosine in J774 cells and an increase in PDB binding (see below). This is the predicted finding because sphingosine is a competitive inhibitor of PDB binding by protein kinase C.



Legend: Phorbol dibutyrate binding by J774 cells upon transfer to new medium, conditioned medium, or new medium plus ammonium chloride. Other experiments did not observe a direct effect of ammonia on PDBu binding.

Hence, persuant to our first and second objectives, we have identified cell conditions where sphingosine is modified under conditions of relevance to ischemia, albeit in an indirect manner, and have established the mechanism of formation of the sphingosine (i.e., as an event in an endosomal or lysosomal compartment). Our hope is that the information that is being gained with this model system may be either directly relevant to this pathological condition, or at least, may help us better design experiments with other systems that would be better models.

Effects of sphingosine and other sphingolipids on programmed cell death.

The goal of specific aims 3 and 4 is to determine if the addition of sphingosine to cells might help protect them from cell injury. This idea has been tested, thus far, with a well characterized model: programmed cell death in thymocytes (see attached abstract). In this study, rat thymocytes were incubated in methylpregnisolone with varying concentrations of sphingosine or gangliosides. Our finding has been that the presence of sphingosine increases DNA strand breakage (one marker of programmed cell death in this model). It is believed that protein kinase C activation helps protect against programmed cell death; hence, this effect of an inhibitor of protein kinase C is not

surprising. Our view is that these results underscore the diverse ways that long-chain bases can affect cell behavior.

Studies of signal transduction processes in mitochondria.

Previous studies by our and other labs have been focussed on identifying whether cytochrome oxidase is the primary sensor for suppression of mitochondrial functions during anoxia. Cytochrome oxidase rapidly becomes reduced when oxygen concentration falls into the micromolar range, such as occurs during ischemia. Cytochrome oxidase contains at least 11 protein subunits, of which only 2 have known functions in the catalytic activity. Mitochondria with mutations in some of the other subunits have altered kinetic properties. Thus, it seems likely that mitochondrial regulation during anoxia may involve sensing of oxidation-reduction changes by these non-catalytic subunits within cytochrome oxidase.

Thus, our working hypothesis is that 1) cytochrome oxidase becomes reduced in the absence of O_2, 2) subunits within cytochrome oxidase sense the change in oxidation-reduction state and signal ion transport systems to slow or stop ion flux, and 3) this inhibition of ion flux allows the mitochondria to extend the duration of anoxia during which mitochondria can preserve structural and functional integrity. We have studied the phenomenon in isolated hepatocytes and in isolated liver mitochondria. Suppression of ion trransport during anoxia is much less effective in the mitochondria than it is in the intact cells. Thus, we believe the second messenger involved in signal transduction is a low molecular weight compound.

Our studies during this year have been primarily focussed on whether responses of cytochrome oxidase to agents that stabilize either the oxidized or reduced form are consistent with cytochrome oxidase functioning as the sensor of anoxia. These studies have been performed primarily by Dr. Youngja Park, a postdoctoral associate with Dr. Jones. Cyanide has been used to stabilize the oxidized form of cytochrome oxidase and carbon monoxide has been used to stabilize the reduced form. The results show 1) that in the presence of cyanide, the mitochondrial membrane potential and pH gradient collapses very rapidly and the cells die much more rapidly than duing simple anoxia, 2) that mitochondrial membrane potential is preserved much better by strict anoxia than by severe hypoxia (5 uM O_2), 3) that carbon monoxide protects against the loss of mitochondrial membrane potential in the presence of 5 uM O_2, and 4) that carbon monoxide protects against post-anoxic lipid peroxidation. Thus, the results are consistent with the working hypothesis and suggest that the post-anoxic changes in lipid metabolism may be related to the signal transduction mechanism that controls mitochondrial function during anoxia.

This latter point is particularly exciting because it provides a framework for investigating the second messengers that function to regulate mitochondrial function during anoxia. Specifically, aberrant lipid metabolism has been known to occur during and subsequent to anoxia. This response has been investigated extensively as a potential mechanism for ischemic and post-ischemic injury. The fact that a compound (CO) that binds to and stabilizes cytochrome oxidase in the reduced form blocks changes in lipid metaboilism suggests that the changes in lipid metabolism could be involved in the signalling response to anoxia. Thus, the lipid changes could reflect changes to generate or remove the second messenger.

During the next year we plan to begin to investigate this possibility. We plan two specific studies, namely, to examine the potential role of the prostaglandin B_1 derivative, calciphor, that Dr. Devlin has found to have potent activities on mitochondrial function

and to examine the effects of sphingosine and derivatives that Dr. Merrill has found to have potent effects on signal transduction and ion transport systems.

We feel that we have made substantive progress during the current year in defining the sensor of a novel process that functions to preserve mitochondrial structure and function during short-term anoxia. The results of these studies have provided evidence that the second messenger may be lipid in nature, and thus, we plan to examine the potential role of two important types of lipid compounds as mediators of ion transport suppression in mitochondria during anoxia.

PUBLICATIONS ARISING FROM THIS GRANT

1. Merrill, A. H., Jr., Warden, L. A., and Smith, E. R., Regulation of free sphingosine levels in J774 macrophages by cell adheremce amd a factor om conditioned medium, FASEB J. 4:(4) Abs. 5051, 1990.

2. Warden, L. A , Smith, E. R., and Merrill, A. H., Jr., Cell adherence triggers free sphingosine formation by a mechanism that is inhibitable by ammonium ion--A suppressive factor that is found in conditioned medium, FASEB J. 4(7):Abs. 1258, 1990.

3. Bai, C., Aw, T. Y., Wang, E., Merrill, A. H., Jr., and Jones, D. P., Effect of sphingosine, gangliosides, cyclic AMP, and interferons on programmed cell death, FASEB J. 4(7): Abs. 477, 1990.

4. Warden, L. A. and A.H. Merrill, Jr., Isolation of a second factor in conditioned medium of J774 cells that suppresses the formation of free sphingosine, FASEB J. (in press), 1991.

PERSPECTIVES FOR THE NEXT YEAR

We think that, although our studies have taken us in an unexpected direction with respect to signal transduction systems in hypoxia/ischemia, our findings offer considerable promise in understanding the metabolic derangements of ischemic injury. In particular, ammonia has long been associated with the pathogenesis of hepatic (e.g., Kaiser et al., Eur. J. Clin. Invest. 18:535-542, 1988), renal (Golchine et al., J. Clin. Invest. 84:1767-1779, 1989), and other diseases. Moreover, accumulation of ammonia has been associated with ischemia (e.g., Fitzpatrick et al., Brit. J. Urol. 54:608-612, 1982; Vicario et al., Biol. Neonate 57: 119-125, 1990). The further characterization of the marked changes in sphingolipid metabolism of J774 cells in the presence and absence of ammonia at levels achieved in ischemia may well lead to the discovery of a hitherto unknown factor in the cell injury during hypoxia/ischemia/reperfusion syndromes.

FASEB J. Vol. 4 (7) 1990

1258

CELL ADHERENCE TRIGGERS F[illegible] SPHINGOSINE FORMATION IN J774 MACROPHAGES BY A MECHANISM THAT IS INHIBITABLE BY AMMONIUM ION--A SUPPRESSIVE FACTOR THAT IS FOUND IN CONDITIONED MEDIUM. L.A. Warden, E.R. Smith, and A.H. Merrill, Jr. Dept. Biochem., Emory Univ. Sch. Med., Atlanta, GA 30322.

Sphingosine has the potential to serve as another "lipid second messenger" since it inhibits protein kinase C, activates the EGF receptor kinase, and affects other cell systems; however, little is known about the formation, removal, and function(s) of free sphingosine in cells. This study determined the amounts of free sphingosine in J774 cells using an HPLC assay (Anal. Biochem. 171:373, 1988), and found that transfer of suspended cells to test tubes (where they adhere) stimulated up to a 10-fold increase within an hour. The increase was inhibited by a factor in conditioned medium. Addition of ammonium ion (>1 mM) inhibited the appearance of sphingosine, and analyses of ammonia in new and conditioned medium revealed that the concentration increased from 0.25 to 3 mM. These findings suggest that adherence triggers turnover of sphingolipid(s) to free sphingosine in an acidic compartment, and that at least one component of conditioned medium that suppresses this change may be ammonia. The ability to control this aspect of sphingolipid metabolism using different media allowed assessment of the effects of endogenous sphingosine on phorbol ester-induced superoxide formation, phorbol dibutyrate binding, and other indices of protein kinase C. Supported by grant 88061709 from the ONR.

1259

PHOSPHATIDATE-DEPE[illegible]DIET[illegible]E ACTIVIT[illegible] IN THE RAT LIVER. S.B. Bocckino and J.H. [illegible], Howard Hughes Med. Inst., Dept. Molecular Physiol. Biophys., Dept. Pharmacol., Vanderbilt Univ. Sch. Med., Nashville, TN 37232 U.S.A.

Exogenous phosphatidate has been shown to elicit varied cellular responses including increased DNA synthesis, induction of c-fos and c-myc, increased superoxide formation in neutrophils, platelet aggregation, inhibition of vasopressin-induced hydraulic conductivity in kidney and many other effects. The mechanism of action of phosphatidate in these responses is largely unknown. We have tested whether rat liver subcellular fractions contain phosphatidate-dependent protein kinase activity by incubating the fractions with [^{32}P]ATP, $MgCl_2$ and various lipids. Protein phosphorylation was assessed by SDS PAGE and autoradiography. When a 37000 x g/20 min supernatant fraction was incubated with phosphatidate, phosphorylation of proteins at 18, 20, 28, 33, 35, 52 and 58 kDa was stimulated. Phosphatidylserine also stimulated phosphorylation of many of these proteins but the bands at 33 and 35 kDa were uniquely phosphorylated in the presence of phosphatidate. PA-dependent phosphorylation was stimulated by Ca^{2+} at high nanomolar concentrations but was inhibited by higher Ca^{2+} concentrations. These data suggest that a PA-dependent kinase may mediate some of the effects of PA, similarly to other second messenger-kinase systems.

1260

CONTROL OF PLATELET-ACTIVATING FACTOR (PAF) PRODUCTION IN HUMAN PROMYELOCYTIC LEUKEMIC HL-60 CELLS. **T-c. Lee, D.S. Vallari, B. Malone and F. Snyder. Med. Sci. Div., Oak Ridge Assoc. Univ., Oak Ridge, TN 37831-0117.**

Ca^{2+} ionophore A23187 increases the production of PAF in differentiated (D), but not undifferentiated (U) HL-60 cells, by stimulating the incorporation of labeled acetate into the mediator. We have now determined the activities of acetyl-CoA:alkyllyso-glycerophosphocholine (-GPC) acetyltransferase and PAF acetylhydrolase in U and D HL-60 cells treated with ionophore A23187. Microsomal acetyltransferase activity of both U and D cells is enhanced severalfold by BSA, with an optimal alkyllyso-GPC/BSA molar ratio of 1.5-2.0. Ionophore treatment or inclusion of protein phosphatase inhibitors in the homogenization media further increases the acetyltransferase activities in both forms of the cells. Neither the degree of differentiation nor ionophore treatment affects the acetylhydrolase activity in HL-60 cells. Also, a variety of PAF receptor antagonists has no effect on the activities of acetyltransferase and acetylhydrolase in U and D cells ± ionophore. However, the amounts of alkylacetyl-GPC versus acyl-acetyl-GPC produced after treatment of intact cells with ionophore differ in the two cell forms. These data document the observed difference in PAF generation by U and D HL-60 cells following ionophore stimulation is not due to acetyltransferase/acetylhydrolase activities; instead it is likely caused by the availability of lyso-PAF in the remodeling pathway of PAF. [DOE DE-AC05-76OR00033, NHLBI 27109-10, ACS BE-26U]

1261

FORMATION OF THE BIOLOGICALLY ACTIVE PLATELET-ACTIVATING FACTOR METABOLITE 1-O-ALKYL-2-ACETYL-SN-GLYCEROL (AAG) IN HL-60 CELLS. L. L. Stoll, N. R. Yerram, M. A. Yorek, and A. A. Spector. Depts. of Biochemistry and Internal Medicine, University of Iowa, Iowa City, IA 52242.

Metabolism of platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PAF) was studied in HL-60 cells to determine whether differentiation may influence this process. HL-60 cells differentiated to macrophages (HL-60/MΦ) with a phorbol ester partially convert added PAF to 1-O-alkyl-2-acetyl-sn-glycerol (AAG). Undifferentiated HL-60 cells (HL-60/U) do not produce AAG from PAF, nor do HL-60 cells differentiated to granulocytes (HL-60/GN) with retinoic acid. However, when HL-60/MΦ are incubated with [^{3}H]-PAF, 22% of the incorporated radioactivity is converted to AAG within 15 s. The HL-60/MΦ rapidly convert radiolabeled AAG to 1-O-alkyl-sn-glycerol and subsequently to two other unidentified metabolites; however, some apparently unmodified AAG persists in cellular lipids for at least 6 h. Cells which do not make AAG can nevertheless also catabolize it efficiently; HL-60/U and HL-60/GN form a similar pattern of AAG metabolites. AAG activates protein kinase C (PKC) in HL-60 cells as effectively as diolein or 1-oleoyl-2-acetyl-sn-glycerol, with maximal activity at 0.25 μg/ml AAG and 300 μM Ca^{2+}. These observations suggest that PAF may produce at least some of its biological effects in macrophages by conversion to AAG and long-term interaction of this metabolite with PKC. (Supported by NIH HL-14,230.)

1262

A MODEL FOR HEPATOCARCINOGENESIS: 1,2-*sn*-DIACYLGLYCEROL AND PROTEIN KINASE C ACTIVITY IN CHOLINE-DEFICIENT LIVER. S.H. Zeisel Boston University School of Medicine, Boston.

Choline deficiency causes fatty liver and is the only nutritional state known to trigger hepatic cancer. Since the tumor-promoting phorbol esters modify the activity of a regulatory enzyme, protein kinase C (PKC), by substituting for the natural activator, 1,2-*sn*-diacylglycerol (DAG), it is possible that the carcinogenic effect of choline-deficiency is mediated by DAG which may accumulate in this state and alter PKC activity. Rats were pair-fed for six weeks with control (0.2% choline), or choline-deficient (0.002% choline) diets. Choline deficiency in CD animals was verified by measuring hepatic choline and phosphocholine content. Hepatic DAG was determined by a radioenzymatic assay in which DAG was phosphorylated by a specific 1,2-*sn* diacylglycerol kinase in the presence of [^{32}P-γ]ATP to [^{32}P]phosphatidic acid (PA). PA was purified and its radioactivity determined. In livers from choline deficient rats DAG content was 2-3-fold higher than in livers from control animals. The subcellular distribution of DAG within livers from both groups is described, as is the DAG content of human liver. Hepatic cytosolic and membrane associated (plasma membrane, nuclear and endoplasmic reticular membranes) PKC activities were determined by a radioenzymatic assay in which the radioactivity transferred from [^{32}P-γ]ATP to histone in the absence of phosphatidylserine and 12-*O*-tetradecanoyl-phorbol-13-acetate was subtracted from that occurring in the presence of those activators.

This work was supported by a grant from the American Institute for Cancer Research.

1263

ROLE OF SPHINGOMYELIN TURNOVER IN 1α,25-dihydroxyvitamin D_3 - induced HL-60 cell differentiation. T. Okazaki, A. Bielawska, R. Bell and Y. Hannun. Duke University Medical Center, Durham, N.C. 27710.

We previously reported that 1α,25-dihydroxyvitamin D_3 (vitamin D_3) induced sphingomyelin turnover due to the activation of a neutral sphingomyelinase in HL-60 cells. We investigated the role of ceramide, the breakdown product of sphingomyelin, in modulating HL-60 cell differentiation by vitamin D_3. The mass of ceramide increased by 41% at 2 hrs after treatment with 100 nM vitamin D_3 compared to the control level. The generated ceramide (13.2 pmol/nmol phospholipid) corresponded to the decrease in mass of sphingomyelin (17.4 pmol/nmol phospholipid). Ceramide levels produced by endogenous sphingomyelinase or by sphingomyelinase added exogenously correlated with the induction of HL-60 cell differentiation along the monocytic lineage. Sphingosine and its analogs did not differentiate HL-60 cells to monocytes. These studies suggest that sphingomyelin turnover and ceramide may play an important role in HL-60 cell differentiation induced by vitamin D_3.

5050

REGULATION OF INTERFERON-γ (IFNγ) AND PLATELET ACTIVATING FACTOR- (PAF) STIMULATED Na^+/H^+ ANTIPORT ACTIVITY AND GENE EXPRESSION BY cAMP IN MURINE PERITONEAL MACROPHAGES. M.S. Cowlen, V. Prpic, F. Figueiredo, K. Okonogi, S.F. Yu, R.J. Uhing, and D.O. Adams. Duke Univ. Med. Center, Durham, NC

Activation of an amiloride-sensitive Na^+/H^+ antiport is an early transductional event involved in IFNγ-mediated transcription of class II MHC (Ia) genes and surface expression of Ia (Science 244:469-471). In this study, we examined the regulation of IFNγ- and PAF-induced responses by prostaglandin E_2 (PGE_2). PGE_2 elevated intracellular cAMP concentration and increased cAMP-dependent protein kinase activity. Pretreatment with PGE_2 inhibited IFNγ-stimulated $^{22}Na^+$ uptake, transcription of Ia genes, and surface expression of Ia. Cholera toxin, forskolin (FSK), dibutyryl-cAMP (Bt_2cAMP), and isobutylmethylxanthine (IBMX) also inhibited IFNγ-induced $^{22}Na^+$ uptake and surface expression of Ia. Treatment with PAF stimulated an amiloride-sensitive $^{22}Na^+$ uptake and cytosolic alkalinization and increased levels of JE mRNA. Pretreatment with PGE_2, FSK, or Bt_2cAMP inhibited the ability of PAF to stimulate $^{22}Na^+$ uptake, and IBMX potentiated the PGE_2-induced inhibition of PAF-stimulated $^{22}Na^+$ uptake. These results suggest that PGE_2 can exert inhibitory effects on macrophage functional responses by cAMP-dependent inhibition of agonist-stimulated Na^+/H^+ antiport activity.

5051

REGULATION OF FREE SPHINGOSINE LEVELS IN J774 MACROPHAGES BY CELL ADHERENCE AND A FACTOR IN CONDITIONED MEDIUM Lisa A. Warden and Alfred H. Merrill, Jr. Dept. Biochem., Emory Univ. Sch. Med., Atlanta, GA 30322.

Sphingosine is a potent inhibitor of protein kinase C and diverse cell functions when added exogenously, and is a natural constituent of cells--although little is known about the factors that regulate its levels. This study determined the amounts of sphingosine in J774 cells under different culture conditions using a sensitive HPLC assay (Merrill et al., Anal. Biochem. 171:373,1988). When grown in suspension culture, J774 cells have 10 to 30 pmol of free sphingosine per 10^6 cells; however, upon transfer to test tubes (where the cells adhere rapidly), the levels increase up to 10-fold within an hour. This increase only occurs if the cells are placed in new medium, and appears to be a consequence of both adherence and removal of a factor in conditioned medium; that is, there is no increase when cells are allowed to adhere in conditioned medium, and there is a smaller, temporary increase when cells are placed in spinner bottles in new medium. These changes in free sphingosine may provide a mechanism to alter macrophage behavior in response to adherence and other cell surface phenomena. Supported by grant 88061709 from the Office of Naval Research.

5052

ACTIVATION OF THE NEUTROPHIL NADPH-OXIDASE BY FATTY ACIDS REQUIRES BOTH DISORGANIZATION OF MEMBRANE LIPID AND AN IONIZED CARBOXYL GROUP M.J. Steinbeck, J.M. Robinson, and M.J. Karnovsky, Harvard Medical School, Boston, MA 02115

This study showed that similar to the cis-unsaturated fatty acids, trans-unsaturated fatty acids disorganized the membrane lipid structure and activated the NADPH-oxidase in intact neutrophils in the presence of 0-0.1 mM calcium and in cell-free O_2^- generating systems containing 2 mM EGTA. In calcium-free buffer, cis-unsaturated, trans-unsaturated, and to a lesser extent saturated fatty acids decreased the emission polarization (%Δp) of the membrane structure probe, 1,6-diphenyl-1,3,5-hexatriene. The percentage decrease in the Δp values correlated with the ability of the fatty acids to increase the liquid crystalline fraction of the membrane lipid and to stimulate the release of O_2^- in the cell-free and intact neutrophil preparations. The addition of 0.1 mM calcium had no effect on the %Δp readings of resting neutrophils, but reduced the increases in the liquid crystalline fraction of the membrane lipid in response to the trans-unsaturated fatty by >60%. This concentration of calcium inhibited the release of O_2^- in response to the trans-unsaturated fatty acids by only 20%. If 0.1 mM calcium was added after trans-unsaturated fatty acid insertion, the decreases in the %Δp were reversed by 65% without affecting O_2^- release. Finally, uncharged cis-unsaturated and trans-unsaturated fatty alcohols, methyl esters, and aldehydes significantly decreased the %Δp values; and yet none of these compounds stimulated O_2^- release. These results indicate that in addition to a limited, calcium-modulated disorganization of the membrane lipid in response to unsaturated fatty acids, activation of the NADPH-oxidase requires the ionized carboxyl group of these fatty acid.

This work was supported by NIH Grants DE-0850, AI2432 and CTR 2065.

5053

ACTIN ASSEMBLY IN ELECTROPERMEABILIZED NEUTROPHILS: ROLE OF INTRACELLULAR CALCIUM. G. Downey, K. Chan, S. Trudel, and S. Grinstein. U. of Toronto, and Hospital for Sick Children, Toronto, Canada.

Assembly of microfilaments involves the conversion of actin from the monomeric to the filamentous (F) form and the role of calcium in this sequence of events remains unclear. Intact and electropermeabilized human neutrophils were used to assess more directly the role of cytosolic calcium ($[Ca^{2+}]_i$) in actin assembly. Staining with NBD-phallacidin or right angle light scattering were used to monitor the formation of F-actin. Though addition of Ca^{2+} ionophores can be shown to induce actin assembly, the following observations suggest that an increased $[Ca^{2+}]_i$ is not directly responsible for receptor-induced actin polymerization. i) Cells in Ca^{2+}-free medium, depleted of internal Ca^{2+} by addition of ionophore, responded to the formyl peptide fMLP with actin assembly despite the absence of changes in $[Ca^{2+}]_i$, assessed by Indo-1. ii) fMLP induced a significant increase in F-actin content in permeabilized cells equilibrated with medium containing 0.1 μM free Ca^{2+}, buffered with up to 10 mM EGTA. iii) Increasing $[Ca^{2+}]_i$ beyond the resting level by direct addition of $CaCl_2$ to permeabilized cells resulted in actin disassembly. Conversely, lowering $[Ca^{2+}]_i$ resulted in spontaneous actin assembly. We investigated whether the ionophore effect on actin assembly might be mediated by a mechanism independent of the increase in $[Ca^{2+}]_i$ and found that the ionophore-induced actin assembly was completely inhibited by the leukotriene B_4 (LTB_4) antagonist L686,675, implying that the ionophore effect was secondary to LTB_4 formation, possibly by stimulation of phospholipase A_2. We conclude that actin assembly is not mediated by an increase in $[Ca^{2+}]_i$, but rather that elevated $[Ca^{2+}]_i$ facilitates actin disassembly, an effect possibly mediated by Ca^{2+}-sensitive actin-severing proteins such as gelsolin.

PULMONARY PATHOBIOLOGY (5054–5055)

5054

INDUCTION OF NEUROENDOCRINE LUNG CANCER IN HAMSTERS BY RISK FACTORS ASSOCIATED WITH SMALL CELL CANCER IN MAN. H.M. Schuller and H.-P. Witschi. Exp. Oncol. Lab., Univ. of TN., Knoxville, TN and Toxic Sub. Prog., Univ. of Calif., Davis, CA.

Small cell cancer is the most malignant type of neuroendocrine lung cancer in man and demonstrates a strong link with cigarette smoking. Efforts to induce this cancer type experimentally have remained unsuccessful until we found that abnormal lung oxygen levels are a necessary co-factor for the induction of this cancer. In our first publications we have shown that simultaneous exposure of hamsters to hyperoxia and N-nitrosamines induces a high incidence of neuroendocrine lung tumors in Syrian golden hamsters. We now report that simultaneous exposure to nitrosamines and hypoxia, as is commonly found in smokers, also causes neuroendocrine lung tumors. Moreover, exposure to abnormal lung oxygen levels and radiation (as is commonly found in uranium miners who smoke) as well as simultaneous exposure to ozone and nitrosamine are also potent inducers of neuroendocrine lung tumors in hamsters. Our data identify for the first time a cell type specific receptor (oxygen receptor) as a crucial factor for cancer induction.

5055

LUNG CARCINOGENESIS BY SILICA: A MODEL FOR STUDYING THE RELATIONSHIP BETWEEN MECHANISMS OF INFLAMMATION AND EPITHELIAL PROLIFERATION. U. Saffiotti and S. F. Stinson. National Cancer Institute, Bethesda and Frederick, MD.

Single intratracheal administrations of Min-U-Sil quartz (QZ) were used to study the pulmonary reactions in SPF animals of 3 species: rats (F344), mice (A/JCr, BALB/cAnNCr and athymic nude NCr/NU) and Syrian golden hamsters (15:16/EHS:Cr). Histogenesis and long-term experiments showed marked species differences in the pulmonary response. Silica-induced macrophage toxicity and granulomatous fibrogenic reactions were marked in rats and mice. Hamsters showed macrophagic storage lesions with minimal further reaction. Alveolar type II epithelial hyperplasia developed early in rats, adjacent to silicotic granulomas, and progressed to adenomatoid lesions and to frank tumors, mostly adenocarcinomas, with a subgroup resembling human pulmonary scar cancer. Mice developed fibrogenic granulomas, often showing necrotic centers, but no progressive alveolar epithelial hyperplastic lesions nor tumors. Hamsters showed no significant epithelial reactions and no lung tumors. Rats, following a single intratracheal installation of 12 mg or 20 mg of QZ or of 20 mg hydrofluoric acid etched QZ, developed high incidences of lung tumors in both sexes, ranging from 78% to 100% of the animals that survived 17 months or longer. Current studies investigate the response to silica toxicity and/or transforming activity in mesenchymal and epithelial cell systems in culture. Mechanisms for the high epithelial proliferative and neoplastic response in rats, but not in mice and hamsters, are investigated with particular interest in the role that inflammatory cellular reactions and their mediators may have in stimulating epithelial cell proliferation.

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EFFECT OF SPHINGOSINE, GANGLIOSIDES, CYCLIC AMP, AND INTERFERONS ON PROGRAMMED CELL DEATH. C. Bai, T.Y. Aw, E. Wang, A.H. Merrill, Jr., D.P. Jones, Emory Univ., Atlanta, GA 30322.

Programmed cell death (PCD) is a cell suicide process marked by extensive DNA fragmentation. Activation of a calcium, magnesium-dependent endonuclease is critical during this process, but the regulatory mechanisms are still poorly understood. Experiments were designed to test whether a group of reagents could affect PCD, as induced in immature thymocyte by the glucocorticoid hormone methylprednisolone (MP). When isolated rat thymocytes were incubated with 10 μM exogenous sphingosine(So) for 5 hr, small but significant increases of MP-induced DNA fragmentation were observed. Sphingomyelinase(0.8u/ml), in contrast to the above results, inhibited MP-induced DNA fragmentation. Brain gangliosides at 1 μM or 10 μM did not show any significant effects on MP-induced DNA fragmentation. Cyclic AMP related reagents, such as dibutyryl cAMP, forskolin, as well as a,r-interferons, did not show any effect on MP-induced DNA fragmentation. No significant degradation of ribosomal RNA was observed from MP-treated thymocyte analyzed by agarose gel electrophoresis. Thus, our data indicate that the endonuclease activation is most potently modulated by exogenous So. Supported by grant 88061709 from Office of Naval Research.

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ISOLATION OF A SECOND FACTOR IN CONDITIONED MEDIUM OF J774 CELLS THAT SUPPRESSES THE FORMATION OF FREE SPHINGOSINE.

L.A. Warden and A.H. Merrill, Jr. Dept. Biochem., Emory Univ. Sch. Med., Atlanta, GA 30322.

Although sphingosine has the potential to influence diverse cell systems, little is known about the formation, movement, catabolism, and function(s) of this molecule in cells. We have found that 2 to 10-fold increases in free sphingosine are obtained when J774 cells are plated in culture dishes for several days, then changed to new medium. The increase lasts for several hours, and is suppressed and reversed by conditioned medium. One of the suppressive factors in conditioned medium appears to be ammonium ion because NH_4Cl at >1 mM inhibited the appearance of sphingosine, and analyses of conditioned medium revealed that the ammonium concentration increases to 3 mM over several days in culture. However, suppression by conditioned medium was consistently greater than the equivalent concentration of NH_4Cl, which suggested that additional factor(s) are present. Fractionation of conditioned medium by cation exchange and gel permeation chromatography yielded a low-molecular weight fraction (ca 100-200 daltons) that migrates as a single material on thin-layer chromatography and contains a ninhydrin- and TNBS-positive amino group. Based on the amount of the amine, this material suppressed sphingosine formation at micromolar concentrations. These findings underscore the complexity of sphingosine formation in cells, and may reflect a mechanism to alter macrophage behavior. Supported by ONR grant N00014-89-J-1027.

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