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DEGRADATION OF MEMBRANE PHOSPHOLIPIDS IN THE CULTURED HUMAN ASTROGLIAL CELL LINE UC-11MG DURING ATP DEPLETION

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Abstract—The exposure of brain cells to adverse conditions, such as ATP depletion, induces the degradation of membrane phospholipids and the accumulation of free fatty acids. We have investigated the mechanism of membrane breakdown in an *in vitro* cell injury model. Confluent cells from the human astroglial cell line UC-11MG were treated with sodium iodoacetate to deplete their intracellular ATP. Large amounts of saturated (palmitic acid) and unsaturated (oleic, linoleic and arachidonic acid) free fatty acids as well as diacylglycerols containing prelabeled fatty acids were released from the cells prior to the loss of plasma membrane integrity. The capacity of the cells to reincorporate free fatty acid into membrane phospholipids decreased in parallel with the loss of intracellular ATP, indicating the failure of the acyltransferase pathway. The addition of the phospholipase A_2 inhibitors manoalide, mepacrine, or U-26384, or the phospholipase C inhibitor U-73122, reduced the severity of cell injury, but did not maintain cell viability. The addition of a battery of protease inhibitors with or without the phospholipase inhibitors had no protective effect. These results suggest that the activation of phospholipases A_2 and C coupled with the loss of the reacylation process lead to the breakdown of membrane components during lethal cell injury.

Under normal conditions, the levels of free fatty acids or 1,2-diacylglycerol in brain are very small [1, 2]. Two balanced enzymatic pathways, the deacylation and reacylation systems [3, 4], are believed to maintain the integrity of membrane phospholipids. During ischemia or traumatic injury, the failure of the reacylation system due to an energy shortage tilts the balance and results in the enhancement of deacylation pathways. The unchecked action of either phospholipase A_2 or phospholipase C will lead to a marked increase of free fatty acids and 1,2-diacylglycerol in the brain [5–9]. The metabolism of released fatty acids by cyclooxygenase or lipoxygenases to potent mediators and reactive oxygen species may aggravate the injury [10].

If the lipases played such a crucial role in the amplification of membrane damage and tissue injury, inhibition of their action should limit the membrane damage and delay the onset of cell death. Sen and coworkers [11], Armstrong and Ganote [12] and Harrison *et al.* [13] have reported that the phospholipase A_2 inhibitors mepacrine and U-26384 partially protect cultured cardiomyocytes or rat hepatocytes from metabolic inhibitor-induced injury. However, since both mepacrine and U-26384 are hydrophobic compounds, at the high concentrations tested (10–100 μM), they may intercalate into the membranes and affect the interaction of phospholipases with their substrates [14], thereby obscuring the mechanism of their antiphospholipase and cytoprotective action. In this study, we examined the cytoprotective action of manoalide [15, 16], a nonsteroidal sesterterpenoid isolated from the marine sponge *Luffariella variabilis* which has been shown to act on the phospholipid binding domain of phospholipase A_2. Monoalide inhibits extracellular phospholipase A_2 from a number of sources, bee venom phospholipase A_2 (IC_{50} 0.05 to 0.12 μM), cobra venom phospholipase A_2 (IC_{50} = 2 μM) and porcine pancreatic phospholipase A_2 (IC_{50} = 30 μM). It effectively blocks eicosanoid production in murine resident peritoneal macrophages [17] stimulated by phorbol myristate acetate, calcium ionophore A-23187 or zymosan (IC_{50} = 0.18 to 0.35 μM). Comparisons were made with the effects of inhibitors of other phospholipases, diacylglycerol lipase and various proteases. Our findings suggest that: (1) phospholipases A_2 and C are major contributors to the membrane damage in energy-depleted astrocytes and (2) inhibition of phospholipase-induced membrane damage by pharmacological means may delay the onset of irreversible injury or cell death.

MATERIALS AND METHODS

Materials. [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid, [1-^{14}C]oleic acid, [9,10-^{3}H]palmitic acid, [1-^{14}C]linoleic acid and chromium-51 (^{51}Cr) were purchased from Du Pont NEN Research Products, Boston, MA. Manoalide was provided by Dr. Vincent Groppi of the Cell Biology Unit, The Upjohn Co. *p*-Bromophenacyl bromide and RHC-80,267 were obtained from the Department of Chemistry, The Upjohn Co. Aristolochic acid, 7,7-dimethyleicosadienoic acid and oleyloxyethylphosphatidylcholine were purchased from Biomol

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Research Laboratory, Inc., Plymouth Meeting, PA. Sodium iodoacetate (IAA)* fatty acid free bovine serum albumin, the protease inhibitors, pepstatin A, Bestatin, amastatin, antipain, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), E-64, phosphoramidon, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK), and HEPES were purchased from the Sigma Chemical Co., St. Louis, MO. Neutral lipid standards were purchased from Nu Chek Prep, Inc., Elysian, MN, and phospholipid standards were bought from Serdary Research Laboratories, London, Ontario. RPMI 1640, fetal bovine serum, Hanks' balanced salt solution (HBSS) and L-glutamine were purchased from Gibco (Grand Island, NY).

Cell culture. The human astrocytoma cell line UC-11MG [18] was grown in various size dishes or plates in RPMI 1640 growth medium containing 4 mM L-glutamine, 10% fetal bovine serum, penicillin, streptomycin and amphotericin B. The cells were incubated in a humidified incubator maintained at 37° and 5% CO_2. The cultures were grown to confluence in 3 or 4 days and subcultured by treating with 0.05% trypsin and 0.53 mM EDTA in saline.

ATP assay. ATP was measured [18] with a luciferase assay (Calbiochem, La Jolla, CA). The cell monolayers were scraped into 1.5 mL of ice-cold 0.4% perchloric acid and centrifuged at 2000 *g* for 10 min. An 0.5-mL aliquot of the supernatant was neutralized with 0.72 N KOH and 0.6 M KCO_3. After centrifugation, 10 μL of the supernatant was added to 1.99 mL of distilled water. ATP measurement was carried out with the ATP constant light signal kit in an LKB-Wallac Luminometer. The concentration of ATP was read from a calibration curve.

Cytotoxicity assay. Confluent cultures of astrocytes grown in 24- or 48-well plates were treated with 0.1 μCi ^{51}Cr/well for 30 min at 37°. The monolayers were washed twice with HBSS without calcium and magnesium ($HBSS^-$) containing 10% fetal bovine serum and once with RPMI culture medium. Labeled cells were treated with test compounds and then with 75 μM IAA to induce the injury. After appropriate time periods, the plates were centrifuged and the medium from each well was quantitated in a Packard Gamma Counter (Packard Instruments, Downers Grove, IL). Total cellular radioactivity was determined from triplicate control wells after dissolving the cells in 0.1% sodium dodecyl sulfate (SDS) in 0.1 M NaOH.

Phospholipid degradation. Confluent cultures of astrocytes grown in 24-well plates were prelabeled with appropriate amounts of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid, [1-^{14}C]oleic acid, [9,10-^{3}H]-palmitic acid, and [1-^{14}C]linoleic acid for 16 hr. The cells were washed twice with 1 mL/well of warm $HBSS^-$ containing 10% fetal bovine serum and placed in fresh RPMI culture medium. They were treated with cytoprotective compounds as required and then with 75 μM IAA as in the cytotoxicity assay. Following incubations of appropriate duration, the plates were centrifuged, the medium was transferred into Ecoscint scintillation fluid, and radioactivity was quantitated in an LKB 1219 Rackbeta scintillation counter. Fresh medium was added to the wells and incubations were continued for 24 hr to assess the long-term survival of the cells. Following this incubation the above quantitation was repeated.

Fatty acid reacylation. Confluent cultures of astrocytes grown in 24-well plates were washed and treated with 75 μM IAA for appropriate durations. The medium in the wells was aspirated and the cell monolayers were washed twice with fresh, warm RPMI medium. Subsequently, appropriate amounts of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid, [1-^{14}C]-oleic acid, [9,10-^{3}H]palmitic acid, and [1-^{14}C]linoleic acid dissolved in 0.5 mL of warm medium were added to the indicated wells. Further incubations were done at 37° for 30 min. The medium was aspirated and the cell monolayers were washed twice with warm medium. Total radioactivity uptake was determined from triplicate wells after dissolving the cells in 0.1% SDS in 0.1 M NaOH.

Analyses of lipid components. Astrocytes were labeled with [^{3}H]arachidonic acid and treated with IAA as in the cytotoxicity assay. Following incubations of appropriate duration, the cells were rinsed with warm $HBSS^-$ and removed from the plates by scraping with a plastic cell scraper into 1 mL of $HBSS^-$. Lipids were extracted from the cell suspensions by the method of Bligh and Dyer [19]. The chloroform layer was washed with saline and evaporated to dryness under a stream of nitrogen. The neutral lipids, fatty acids, and phospholipids were quantitatively separated by successively eluting from an aminopropyl Bond Elut solid phase extraction column with chloroform, diethyl ether containing 1% acetic acid and methanol [20]. The lipid classes were separated by thin-layer chromatography on silica gel H. The solvent system for separating neutral lipids consisted of hexane:diethyl ether:acetic acid, 70:30:1. The solvent system for separating phospholipids consisted of chloroform:methanol:water, 63:30:5. Spots were visualized by placing the plates in an iodine tank and identified by comparison with authentic standards. The spots were then scraped and counted in Ecoscint scintillation fluid.

RESULTS AND DISCUSSION

Exposure of cultured human astrocytoma cells to 75 μM IAA caused a precipitous drop in intracellular ATP, leveling to 40% of the control value within 60 min and then decreasing to a level below detection in 3 hr. Four hours after the addition of IAA, most cells lost their permeability barrier and died. The loss of the permeability barrier as determined by the release of prelabeled intracellular chromium-51 was first observed at 3 hr and rose to 72.3% at 4 hr. The morphology of the 4-hr injured cell appeared grossly altered, with numerous protrusions spreading over the entire surface. These and other altered cell functions have been described in detail in a previous report [18].

Shortly (30 min to 1 hr) before the breakdown of

* Abbreviations: IAA, sodium iodoacetate; and HBSS, Hanks' Balanced Salt Solution.

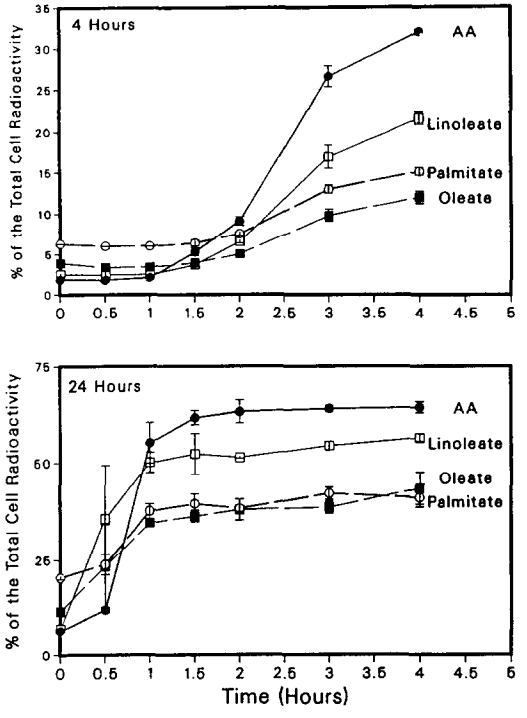

Fig. 1. Time courses of the release of prelabeled [^{3}H]-arachidonic acid (●), [^{14}C]linoleic acid (□), [^{14}C]oleic acid (■) and [^{3}H]palmitic acid (○) from IAA-injured cultured human astrocytoma cells. Confluent astrocytoma cells in 24-well plates were prelabeled with radioactive fatty acids for 16 hr. The unincorporated fatty acids were removed by washing and the amounts of radioactive fatty acids incorporated into the cell monolayers per well were the following: [^{3}H]arachidonic acid (0.075 μCi or 0.31 pmol), [^{14}C]linoleic acid (0.043 μCi or 0.78 nmol), [^{14}C]oleic acid (0.15 μCi or 2.57 nmol) and [^{3}H]palmitic acid (0.024 μCi or 0.48 pmol). The cells were treated with 75 μM IAA for various time durations as indicated. Radiolabeled fatty acids released into the extracellular medium were collected at 4 hr (upper panel) or 24 hr (bottom panel), counted and expressed as the percentage of total cell-associated radioactivity. Results are means ± SEM of triplicate determinations that were repeated three times.

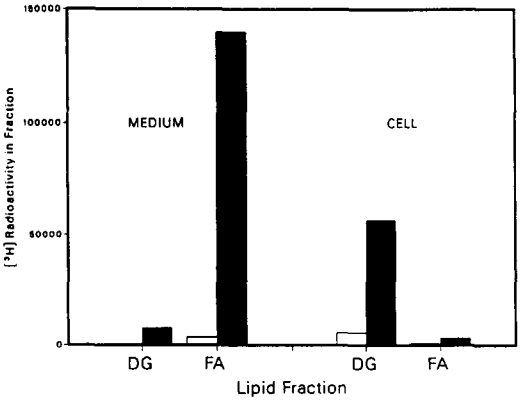

Fig. 2. Accumulation of free fatty acids (FA) and diacylglycerol (DG) in IAA-injured human astrocytoma cells. Confluent astrocytoma cells in 6-well plates were prelabeled with [^{3}H]arachidonic acid for 16 hr. The unincorporated fatty acids were removed by washing and the cells were treated with 75 μM IAA for 4 hr. The medium and monolayers were extracted separately with a chloroform:methanol mixture. The extracts were evaporated and the radioactivity in various lipid fractions was determined. Values represent the mean of counts in fatty acid and diacylglycerol fractions from untreated control cultures (open bar) or IAA-treated cells (solid bars) in triplicate wells from a representative experiment that was performed twice.

the permeability barrier, the membrane phospholipids in the injured cells hydrolyzed and released a substantial amount of free fatty acids into the medium. We monitored this phenomenon by prelabeling the cells with ^{3}H- or ^{14}C-labeled fatty acids and measuring the release of cellular radioactivity into the medium. As shown in Fig. 1, the time courses of the release of prelabeled arachidonic acid, oleic acid, palmitic acid and linoleic acid from IAA-injured astrocytoma cells were very similar, indicating that there was no fatty acid specificity in the breakdown of membrane lipids. Furthermore, when the cells were treated with IAA for various durations of time and the release of fatty acids assessed after 24 hr, all four fatty acids were released to the maximal level after the cells were treated with IAA for 60 min or longer; there was no apparent difference among the four fatty acids tested. The results show that the membrane breakdown was a universal phenomenon and that there was no specificity among molecular species containing the saturated and unsaturated fatty acids. It is well known that most of the unsaturated fatty acids are esterified at the sn-2 position and most of the saturated fatty acids are esterified at the sn-1 position; these results suggest that both are cleaved, probably involving the actions of both phospholipases A_1 and A_2.

To identify the radioactive species we measured, lipids from both the medium and the monolayers of control and 4-hr IAA-injured cells were extracted and analyzed by conventional thin-layer chromatography. The results are summarized in Fig. 2. In both the medium and the monolayers of the control cells, the predominant species was eluted in the phospholipid fraction (data not shown) with very little radioactivity found in the neutral lipids or fatty acids. In the medium of the 4-hr IAA-injured cells, as expected, greater than 80% of the radioactivity co-migrated with authentic fatty acid standard. However, in the monolayers of the 4-hr IAA-injured cells, although 76% of the radioactivity was coeluted with the phospholipids fraction, the radioactivity in the neutral lipid fractions was substantially higher than the control. Thin-layer chromatographic analyses of the neutral lipid fraction showed that the radioactivity was concentrated in the 1,2-diacylglycerol and triglyceride fractions. When compared with control cells, the radioactivity that coeluted with 1,2-diacylglycerol increased 10-fold

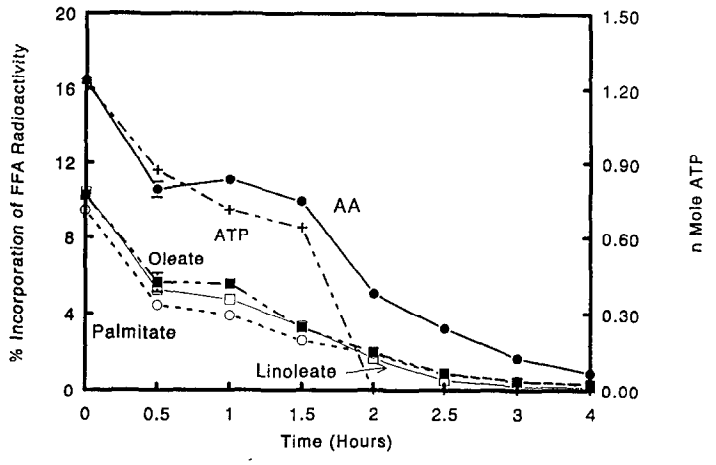

Fig. 3. Time course of the depletion of ATP and the loss of fatty acid reacylation activity in IAA-poisoned cultured human astrocytoma cells. Confluent astrocytoma cells in 24-well plates were treated with 75 μM IAA for various time durations as indicated. The cell monolayers were washed and incubated with [^{3}H]arachidonic acid (0.13 μCi or 0.52 pmol/well; ●), [^{14}C]linoleic acid (0.004 μCi or 71 pmol/well; □), [^{14}C]oleic acid (0.009 μCi or 157 pmol/mL; ■) and [^{3}H]palmitic acid (0.073 μCi or 1.46 pmol/well; ○) for 30 min at 37°. The medium was aspirated and cell monolayers were washed and dissolved in SDS. Radioactivity incorporation into the cell monolayer was counted and calculated as the percentage of total radioactivity added. Cellular ATP levels (nmol ATP/well; +) were analyzed as described in Materials and Methods. Results are means ± SEM of triplicate determinations that were repeated at least three times.

after injury, while that of the triglyceride increased 3-fold (data not shown).

Therefore, the data suggest that phospholipases A_1, A_2 and C may play a role in the disintegration of membrane components of the energy-depleted astrocytes. However, the free fatty acid level in the cells is vigorously controlled by the deacylation–reacylation cycle [3, 4]. Unless the reacylation pathway in the injured cells is obliterated, it is not possible for the free fatty acids to accumulate. We investigated the time course of the reacylation of ^{3}H- or ^{14}C-labeled arachidonic acid, oleic acid, palmitic acid and linoleic acid into the membrane phospholipids of IAA-injured astrocytes. When radioactive fatty acids were added to IAA-injured astrocytes (Fig. 3), the reacylation of all four fatty acids was lost after 2.5 hr. The time course of the loss of reacylation activity mimics that of ATP depletion, indicating the failure of energy-dependent acyl-CoA synthesis. These results suggest that the failure of the fatty acid reacylation system is probably one of the contributing causes in the breakdown of membrane phospholipids and the loss of membrane integrity.

We attempted to inhibit the reacylation of fatty acids in normal, uninjured astrocytes with an acyl-CoA:lysolecithin acyltransferase inhibitor, thimerosal [21]. In our hands, thimerosal inhibited the reacylation of [^{3}H]arachidonic acid into astrocyte lipid at a concentration of 100 μM. However, at this concentration, the compound itself was toxic to astrocytes, inducing 50% release of prelabeled chromium-51 in 4 hr. Since thimerosal is a potent mercurial inhibitor against protein sulfhydryl, we suspect that it may also inhibit ATP production.

If the activation of phospholipases is involved in the breakdown of membrane in the injured cells, the inhibition of phospholipases should protect the cells from injury caused by energy depletion. We tested a battery of phospholipase inhibitors for their cytoprotective activity against IAA-induced release of prelabeled arachidonic acid from cultured astrocytes. As shown in Table 1, mepacrine and U-26384 protected the cells with IC_{50} values of 32.03 and 20.03 μM, respectively. The most potent compound was the nonsteroidal sesterterpenoid inhibitor manoalide which effectively inhibited IAA-induced cell injury with an IC_{50} of 5.05 μM. Furthermore, the phospholipase A_2–phospholipase C inhibitor U-73122 was effective at 40.1 μM. When manoalide was tested in combination with various protease inhibitors including pepstatin, leupeptin, PMSF, soybean trypsin inhibitor, phosphoramidon, E-64, bestatin and antipain, the protease inhibitors did not afford additional cytoprotection. The data suggest that the activation of phospholipases, but not proteases contributes to the disintegration of injured cell membrane. The lack of effect of the diacylglycerol lipase inhibitor RHC-80,267 indicated that the intracellular diacylglycerol was not a precursor of fatty acid released into the medium.

The time courses of IAA-induced membrane phospholipid degradation shown in Fig. 4 revealed that manoalide delayed the disintegration of the membrane phospholipids in the injured cells and, therefore, prolonged the viability of the injured cells. The release of [^{3}H]arachidonic acid in the unprotected cells began at approximately 90 min after the addition of IAA and increased steadily thereafter. After 24 hr, over 80% of the prelabeled

Table 1. Potencies of phospholipase inhibitors on IAA-induced arachidonic acid release from human astrocytes

Compounds	IC_{50} (μM)
Manoalide	5.05
U-26384	20.03
Mepacrine	32.03
U-73122	40.1
Dimethyleicosaidienoic acid	58.7
p-Bromophenacyl bromide	>100.0 (21.2% at 100 μM)
Aristolochic acid	>100.0 (30% at 100 μM)
Oleyloxyethyl-phosphatidylcholine	>100.0 (no effect at 100 μM)
RHC-80,267	>100.0 (no effect at 100 μM)

IC_{50} values were determined by incubating the compounds to be tested with cultured astrocytoma cells for 30 min before the addition of 75 μM IAA to induce ATP depletion. The release of [^{3}H]arachidonic acid into the culture medium was assessed after 4 hr. Radioactivity in triplicate wells of IAA-injured cells treated with test compounds was compared with that in wells of injured cells that were treated only with vehicle, and per cent inhibition was calculated. The IC_{50} values were calculated from four-point concentration–response curves that were repeated at least twice. Each IC_{50} value represents the concentration of a test compound that reduced 50% of the release of prelabeled arachidonic acid from IAA-injured cells that had not been treated with a cytoprotective agent.

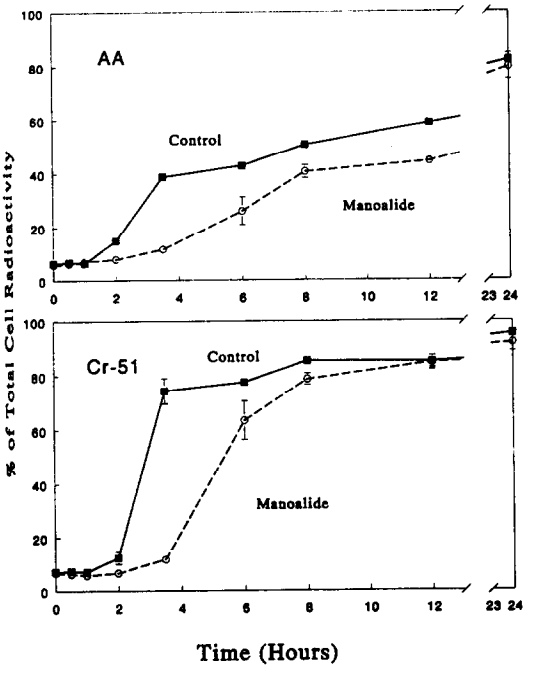

Fig. 4. Time courses of the release of prelabeled [^{3}H]-arachidonic acid (upper panel) and the release of cytoplasmic ^{51}Cr (lower panel) from cultured human astrocytoma cells poisoned with IAA in the absence (■) or presence (○) of 10 μM manoalide. Experimental details are identical to those of Table 1. Extracellular medium at each time point was pipetted out and counted. The same volume of warm, fresh medium was added back to replenish the loss in sampling. Values for the recovered radioactivity are expressed as the percentage of total cell-associated radioactivity (0.05 μCi [^{3}H]arachidonic acid or 0.03 μCi ^{51}Cr per well) released at each time point. Results are means of triplicate determinations that were repeated three times.

arachidonic acid was found in the medium. The manoalide treatment greatly reduced the release of arachidonic acid when assessed at 4 and 6 hr. However, the release of arachidonic acid accelerated after 6 hr and caught up with the untreated cells by 24 hr. These results indicate that the phospholipid breakdown in the IAA-injured cells was delayed significantly, but not prevented totally, by the presence of manoalide, and the membrane integrity was better preserved in the manoalide-treated cells than in the untreated cells. This is a very harsh model of anoxic injury since the effect of IAA is not reversible and in the face of near total energy deprivation eventually the membrane will collapse. However, the inhibition of phospholipases did increase significantly the stability of membrane phospholipids under such adverse conditions.

Extensive research had been done on the disturbance of lipid metabolism during ischemia. It is generally recognized that the alteration of membrane lipid composition is a critical event responsible for the loss of membrane functions and irreversible cell damage. The loss of membrane phospholipids and the accumulation of free fatty acids and 1,2-diacylglycerol have been seen in liver [22], lung [23], heart [24–26], kidney [27] and brain [8] that are subjected to ischemic and reperfusion damage or oxidative stress. Accumulation of other lipid hydrolysis products such as lysoglycero-phosphocholine [28, 29], long chain acylcarnitines [30], hydroxyl fatty acids [31] and lipid peroxides [32,33] in ischemic brain or heart has also been reported. These amphiphilic products can seriously alter membrane structure and functions and exert a detrimental effect on the stressed cells. In heart [34], lysophospholipids and long chain acylcarnitines contribute to the electrophysiological alterations characteristic of early myocardial ischemia. Free fatty acids and their derivatives interfere with mitochondrial function at several sites [35]. This

evidence reiterates the importance of understanding the mechanism that initiates the lipid degradation process. It is perhaps relevant to note that both "membrane destabilization" [36, 37] and free radicals [33] have been postulated as the trigger of injury-induced lipolysis. As we indicated in our previous work [18], the 21-aminosteroid U-74006F and troloxamine U-78517F are very hydrophobic molecules with powerful free radical scavenging activity; this allows them to partition into the lipid bilayers rapidly and stops the onset of lipid degradation. Therefore, it is not surprising that these lipophilic antioxidants protect cells against energy depletion characteristic of ischemia and oxidative stress.

In conclusion, our results indicate that the depletion of ATP caused the failure of fatty acid reacylation, effectively stopping the membrane repair mechanism. The activation of several phospholipases leads to the breakdown of membrane lipids and loss of the permeability barrier and cell viability. The inhibition of phospholipase A_2 or C with manoalide or U-73122, respectively, partially protected the cells from IAA-induced injury.

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