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PREVENTION OF ISCHEMIA/REPERFUSION-INDUCED ALTERATIONS IN SYNAPTOSOMAL MEMBRANE-ASSOCIATED PROTEINS AND LIPIDS BY *N*-TERT-BUTYL-α-PHENYLNITRONE AND DIFLUOROMETHYLORNITHINE

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Abstract—Previous studies in our laboratory demonstrated the alteration in the physical state of synaptosomal membrane lipids and proteins in ischemia/ reperfusion injury using selective spin labels and electron paramagnetic resonance spectroscopy [Hall *et al.* (1995) *Neuroscience* **61,** 84–89]. Since many investigations have provided evidence for free radical generation during ischemia/reperfusion injury, we investigated whether a free radical scavenger would prevent the membrane damage, in gerbils. Further, experiments to determine if a secondary effect of polyamine generation at 14 h reperfusion could be blocked by this free radical scavenger or by an inhibitor of ornithine decarboxylase were also carried out. The alterations in synaptosomal membrane integrity observed during ischemia/reperfusion injury were selectively neutralized by treatment with the free radical spin trap *N*-tert-butyl-α-phenylnitrone or an inhibitor of ornithine decarboxylase, difluoromethylornithine. Administration of *N*-tert-butyl-α-phenylnitrone prior to ischemia totally abrogated both lipid and protein alterations observed at 1 and 14 h reperfusion. Pretreatment with difluoromethylornithine neutralized only the 14 h change in lipid label motion. Treatment with *N*-tert-butyl-α-phenylnitrone at 6 h post ischemia showed only a slight attenuation of the 14 h lipid effect and no change in the protein effect. Difluoromethylornithine treatment at 6 h post ischemia negated the 14 h ischemia/reperfusion injury-induced lipid effect and had no effect on the protein change.

These data support previous suggestions that free radicals and polyamines play a critical role in neuronal damage and cell loss following ischemia/reperfusion injury and that the polyamine effect is dependent upon free radical generation during ischemia/reperfusion injury.

Key words: gerbil, electron paramagnetic resonance, brain, free radicals, polyamines.

Ischemia reperfusion injury (IRI) is a pathophysiological process that affects many organs in the body. One of the most devastating examples of IRI is the condition of stroke, which is estimated to affect over 500,000 people per year in the United States.[56] Although much is known about the early and late events that occur during IRI, the basic mechanisms leading to neuronal death are yet to be determined. For this reason there are few avenues for the prevention of the devastating effects of IRI.

Factors commonly accepted as contributing to the cell damage are the generation of oxyradical species with the onset of reperfusion,[7,13,25] disruption of calcium homeostasis,[73] and an excess of excitatory amino acids,[64] prostaglandins,[2] leukotrenes,[1] and polyamines.[47] The confluence of these factors, however, is not understood. Some combination of these changes is proposed to lead to progression of the infarct and neuronal death.

The polyamines putrescine, spermidine and spermine, from the class of ornithine-derived molecules, play a role in cell growth, cell differentiation,[29] membrane transport functions, protein synthesis, and calcium mobilization.[36,54,58,59] Polyamines have been implicated in the development of cerebral edema by blood–brain barrier breakdown induced by cryogenic injury.[37,69] Polyamines have also been shown to mediate membrane permeability, through intracellular signaling to increase free cytosolic calcium. This is thought to be achieved by enhancing calcium influx

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Abbreviations: DFMO, difluoromethylornithine; EDTA, ethylenediaminetetra-acetate; EGTA, ethyleneglycoltetraacetic acid; EPR, electron paramagnetic resonance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HWHH, half width at half height of the low-field line of the 5-NS EPR spectrum; IRI, ischemia reperfusion injury; MAL-6, 2,2,6,6,-tetramethyl-4-maleimidopiperidine-1-oxyl; NMDA, *N*-methyl-D-aspartate; ODC, ornithine decarboxylase; PBN, *N*-tert-butyl-α-phenylnitrone; PMSF, phenylmethylsulfonyl fluoride; SAMDC, *S*-adenosylmethionine decarboxylase; W/S, the ratio of the amplitudes of the weakly immobilized to the strongly immobilized components of the low-field line of the MAL-6 EPR spectrum.

across the plasma membrane and mobilizing calcium from internal stores by cation exchange reaction.[38]

Polyamine biosynthesis is catalysed by ornithine decarboxylase (ODC), which converts ornithine to putrescine. A second key enzyme is *S*-adenosylmethionine decarboxylase (SAMDC), which converts putrescine to spermidine and spermine. Studies have shown that reversible cerebral ischemia induces changes in polyamine synthesis which include both an increase in ODC activity[20,35,50,52] and a prolonged decrease in the activity of SAMDC.[20,35] The combination of these two changes results in a selective overshoot in putrescine levels.[49 51] In support of polyamine participation in IRI and *N*-methyl-D-aspartate (NMDA)-induced cell damage and loss, pretreatment with the enzyme activated ODC suicide inhibitor α-difluoromethylornithine (DFMO) results in attenuation of damage.[37,45,47,57]

Another compound which offers protection from IRI damage to gerbil brain is the spin trapping agent *N*-tert-butyl-α-phenylnitrone (PBN).[18,25,55,60,63] PBN is thought to protect against damage by reacting with transient free radicals produced at the onset of reperfusion to form stable spin adducts.[55,60] Administration of PBN prior to the onset of IRI has been shown to prevent (i) the loss of glutamine synthetase activity, (ii) the increase in oxidative damage to proteins, and (iii) the lethality commonly observed during intense IRI.[25]

Our laboratory has previously studied changes in the physical state of synaptosomal membrane bound proteins and lipids induced by 10 min ischemia followed by a time course of reperfusion.[28] We found a large decrease in membrane lipid order and motion (increased fluidity) and a large increase in protein–protein interactions at 1 h reperfusion. The initial increase in lipid label motion returned to control values by 6 h reperfusion, and a second increase lipid fluidity occurred at 12 h reperfusion.[28] The purpose of the study presented in this paper was to determine the relative importance of free radicals and polyamines to these membrane changes by investigating the effects of PBN and DFMO.

EXPERIMENTAL PROCEDURES

Materials

Ultra-pure sucrose was obtained from ICN Biochemicals. The protease inhibitors leupeptin, pepstatin A, and aprotinin were obtained from Calbiochem. The protein specific spin label 2,2,6,6,-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) and the lipid bilayer-specific spin label 5-doxylstearic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). DFMO was obtained from (Aldrich Chemical Co., Milwaukee, WI) and PBN was obtained from Centaur Pharmaceuticals (Sunnyvale, CA). All other chemicals were obtained from Sigma in the highest possible purity.

Animals

Male Mongolian gerbils were purchased from Tumblebrook Farms (West Brookfield, MA). Adult gerbils, three months of age, were acclimated to the University of Kentucky Medical Center facility for at least seven days prior to experimentation. The animals were housed under 12 h light/dark conditions with free access to water and standard Rodent Laboratory Chow (Purina). Under pentobarbital anesthesia (40 mg/kg), gerbils were prepared with carotid artery occluders as previously described.[13] Briefly, a loop of dental floss was placed around each common carotid artery and the free ends passed through a double-lumen catheter which exited the dorsum of the neck and was glued in place with cyanoacrylate adhesive. Gerbils were allowed to recover for at least 48 h after surgery. PBN and DFMO solutions were prepared in physiologic saline. Administration of these drugs to gerbils was accomplished through interperitoneal injection 30 min prior to ischemia, 6 h post-ischemia, or at appropriate time points prior to decapitation for controls. PBN was administered at a dose of 300 mg/kg[25] while the DFMO doses ranged from 30 to 300 mg/kg. Ischemia was induced for 10 min by pulling on the free ends of the dental floss, thereby closing the carotid artery against the septum of the double-lumen catheter. Reperfusion was initiated by release and removal of the dental floss. Based on our previous results,[28] reperfusion times were 1 or 14 h, ending with decapitation. The whole brain was removed and quickly dissected on a cold stage. The neocortex was dissected free and suspended in approximately 20 ml of ice-cold isolation buffer (0.32 M sucrose containing 4 μg/ml leupeptin, 4 μg/ml pepstatin A, 5 μg/ml aprotinin, 20 μg/ml type II-S soybean trypsin inhibitor, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA, 2 mM EGTA, 20 mM HEPES at pH 7.4 (buffer A). Cortices from two animals were pooled and homogenized in a Wheaton 30 ml motor-driven Potter-type homogenizer with a Teflon pestle.

Synaptosome preparation

Synaptosomes were purified from homogenized cortices via ultracentrifugation across discontinuous sucrose gradients as previously described,[3,70] and adapted in our laboratory.[71,72] The crude homogenate (in buffer A) was centrifuged at 4°C, 1500 *g* for 10 min, the supernatant carefully removed from the pellet and re-spun at 20,000 *g* for 10 min. The resulting pellet was carefully resuspended in 4 ml of buffer A at pH 8.0. This suspension was then layered on top of sucrose density gradients containing 11.7 ml each of 1.15 M (pH 8.5), 1.0 M (pH 8.0), and 0.85 M (pH 8.0) sucrose, each containing 2 mM EDTA, 2 mM EGTA, and 10 mM HEPES. Samples were then spun at 4°C and 82,500 *g* for 120 min in a SW28 swinging bucket rotor in a Beckman L2-65B refrigerated ultracentrifuge. Synaptosomes were removed from the 1.15 M/1.0 M sucrose interface and resuspended in approximately 20 ml of ice-cold lysing buffer, containing 10 mM HEPES, 2 mM EGTA and 2 mM EDTA, at pH 7.4. The resulting suspension was then spun down at 32,000 *g* for 10 min. The pellet was resuspended in ice-cold lysing buffer and spun down in the same manner two more times to rinse the synaptosomal membranes free of any residual cytoplasm. After the third wash the pellet was resuspended in 2.0 ml of the lysing buffer and assayed for total protein concentration by method of Lowry *et al.*[44] Duplicate samples were taken from synaptosomal suspensions of each independent pool.

Spin labeling

Lysed synaptosomal membranes were labeled with ice-cold protein-specific spin label MAL-6 as previously described.[8,11,30,31,71,72] Synaptosomal membranes containing 5.0 mg of protein were labeled to give a final concentration of MAL-6 of 20 μg/mg protein. The resulting mixture was shaken by hand and incubated at 4°C for 16–18 h. Samples were then washed six times in lysing buffer to remove excess spin label. Samples were brought to a total volume of ~400 μl in lysing buffer and allowed to equilibrate at room temperature for 30 min prior to electron paramagnetic resonance (EPR) analysis. A Bruker 300 EPR instrument, equipped with computerized data acquisition and analysis

capabilities located in a climate-controlled room of constant temperature (20 ± 1°C) and humidity, was used to obtain all EPR spectra. Instrumental parameters were: microwave frequency = 9.78 GHz, modulation amplitude = 0.32 G at 100 kHz, and a time constant of 1.28 ms. The relevant spectral parameter (W/S ratio) was averaged for each duplicate pair of samples.

Spin labeling synaptosomal membranes with the lipid specific spin label 5-doxylstearic acid was done in a similar manner as for erythrocyte membranes.[8] The spin label was dissolved in chloroform to a concentration of 0.2 μM, and 125 μl of this solution was evaporated under nitrogen gas in a glass tube resulting in a thin layer of label on the test tube wall. The synaptosomal membranes were adjusted to a protein concentration of 2.5 mg/ml with lysing buffer and 400 μl added to each spin label tube. Duplicate labeling was performed for each sample. The samples were incubated at room temperature for 30 min prior to spectral acquisition. The instrumental parameters were the same as above. The relevant spectral parameter (HWHH) was averaged for each duplicate pair.

Data analysis

Data were analysed by appropriate Student's *t*-tests, ANOVA, and *post hoc* analysis. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Previous studies have demonstrated IRI induced protein oxidation and changes in bound and free fatty acids in brain. Consistent with this oxidative stress, we have demonstrated significant alterations in protein and lipid spin label motion during IRI using EPR.[28] The protein specific spin label, MAL-6, is known to covalently bind to cysteine sulfhydryl groups of cytoskeletal and transmembrane proteins.[11,72] Two distinct populations of spin label reaction sites are observed in a typical EPR spectrum of MAL-6 labeled cortical synaptosomes.[28] These sites are characterized by their ability to restrict motion of the spin label. The relevant EPR parameter measured is the W/S ratio: the ratio of the spectral amplitude of the $M_I = +1$ low-field weakly immobilized line (W) to that of the $M_I = +1$ low-field strongly immobilized line (S).[28] It is conceivable that the S sites would be located in narrow pockets of the protein which would restrict spin label motion and result in line broadening. The W sites could be located in positions on the protein which would not greatly restrict the motion of the spin label. Consistent with this idea, the electron-nuclear hyperfine coupling constant for the W-peaks indicates that these sites are exposed to an aqueous environment.[8,72] The W/S ratio has been shown to change with perturbations in the normal interactions of cytoskeletal proteins.[4,8,10–12,22,32,75,76] Alterations in the W/S ratio are thought to occur as a result of protein structural changes which affect the steric hindrance to the segmental motion of the spin label at its attachment site to the protein.[28] Experiments designed to decrease cytoskeletal protein–protein interactions in erythrocyte membranes via treatment with polyanions or mild proteolysis led to increased spin label motion and an increased W/S ratio.[32,76] In contrast, addition of polycations or cross-linking agents to erythrocyte membranes resulted in decreased motion of the MAL-6 label and decreased the W/S ratio, likely as a result if increased protein–protein interactions.[22,75] Neocortical synaptosomal membranes possess analogs of most of the major erythrocyte cytoskeletal and transmembrane proteins including spectrin, ankyrin, and Band 3.[6,27,34,66] Results from our laboratory involving protein modifiers and potential Alzheimer's disease therapeutic agents,[11] or protein oxidation,[12,30,68] in both systems, suggest that interpretation of the W/S ratio can be generalized to both erythrocyte and synaptosomal membranes.

Table 1. Effects of pretreatment of gerbils with 300 mg/kg *N*-tert-butyl-α-phenylnitrone or difluoromethylornithine on the W/S ratio of 2,2,6,6,-tetramethyl-4-maleimidopiperidine-1-oxyl labeled synaptosomal membranes prepared following 10 min global ischemia with 1 and 14 h reperfusion*

	Treatment	% Change W/S	S.D.	*N*
1 h Reperfusion				
A	Ischemia/reperfusion only‡	−7.803	±2.173	7
	30 min PBN pre-treatment†‡	2.656	±0.492	5
	30 min DFMO pre-treatment‡	−5.337	±3.109	6
14 h Reperfusion				
B	Ischemia/reperfusion only‡	−5.573	±2.284	6
	30 min PBN pre-treatment†	−0.646	±3.552	5
	6 h PBN post-treatment‡	−6.438	±1.489	5
	30 min DFMO pre-treatment‡	−5.354	±1.901	5
	6 h DFMO post-treatment‡	−6.706	±1.989	5

*All data expressed as % change from non-ischemic control ± standard deviation.

†Represents significant difference between treatment and ischemia/reperfusion alone ($P < 0.01$).

‡Represents change significantly different from non-ischemic control ($P < 0.005$).

$N = 5$–7.

In the current study, MAL-6 was used to investigate the effects of *in vivo* administration of the spin-trap PBN and the ornithine decarboxylase suicide inhibitor DFMO on the membrane protein alterations observed during ischemia reperfusion injury. Table 1 shows the effects of 10 min ischemia followed by 1 and 14 h reperfusion, respectively, with and without either 30 min pretreatment or 6 h post-treatment of 300 mg/kg PBN or 300 mg/kg DFMO. As shown previously,[28] 10 min ischemia followed by reperfusion of 1 or 12–14 h led to significantly decreased W/S ratio ($P < 0.05$) consistent with an increased protein–protein interaction in synaptosomal membranes. DFMO treatment showed no significant attenuation of the W/S ratio at either of these reperfusion time points. In contrast, PBN, when given 30 min prior to ischemia, showed a near complete nullification of the W/S change at both 1 and 12–14 h reperfusion times ($P < 0.01$). When administered 6 h post-ischemia neither PBN nor DFMO could reverse the change in W/S observed at 14 h reperfusion in the absence of any drug, suggesting that earlier events led to the 12–14 h observations. Neither PBN nor DFMO, added at maximal concentrations, caused any significant alteration from control values (i.e. no ischemia/reperfusion) with either spin label at 8 or 14 h post administration, suggesting the agents themselves had no membrane altering effects in the absence of IRI at these time points. When administered 1 h 40 min prior to decapitation at 300 mg/kg, in the absence of IRI, PBN produced a significant increase in the W/S ratio compared with control ($P < 0.001$), but no alteration in the HWHH parameter. This observation suggests some interaction of PBN with the cytoskeletal proteins at early times following administration. DFMO caused no significant alteration in the motion of either spin label at the 1 h 40 min time point under the same conditions.

The IRI-induced alterations in the physical state of neocortical synaptosomal membrane lipids, as monitored by changes in motion of the lipid specific spin label 5-doxylstearic acid, were compared with these changes after treatment of PBN and DFMO to determine if these agents attenuated previously identified IRI alterations. The amphipathic, lipid-bilayer specific spin probe 5-doxylstearic acid is intercalated into both leaflets of the bilayer, with its polar head group near the polar head groups of the phospholipids at the hydrophilic surface of the bilayer and its hydrophobic fatty acyl chain deeply embedded in the hydrophobic bilayer, causing 5-doxylstearic acid to exhibit anisotropic motion.[8,9] This type of motion is different from the isotropic motion exhibited by the non-oriented spin probe MAL-6. The polar head group of 5-doxylstearic acid is thought to be held in place rather firmly by the phospholipid head groups while the hydrophobic tail is free to undergo rapid anisotropic motion inside the bilayer.[8,9] The motion of the EPR active nitroxide group, covalently attached to the alkyl chain of the probe, reflects the intramembrane lipid order and motion (i.e. fluidity), making 5-doxylstearic acid a useful probe for detecting local environmental changes of the hydrophobic portions of phospholipids near the surface of the bilayer.

Measurement of the half-width at half-height (HWHH) of the low-field ($M_I = +1$) line of a typical EPR spectrum of membrane-associated 5-doxylstearic acid provides a good assessment of the motion of the 5-doxylstearic acid probe.[28] Analogous to the observations in chemical exchange, rapid reorientational motion of the 5-doxylstearic acid spin probe results in line broadening, i.e. decreased lipid order, resulting in increased lipid motion, is represented by low-field line broadening.[8,9] The HWHH, unlike other EPR measures of fluidity, is not dependent on the local polarity near the paramagnetic center of the spin label.[8,9] The dynamic range of the HWHH is in the order of approximately 10% of the non-perturbed control value as reported for various membrane systems and perturbations.[8,9]

As described previously from our laboratory,[28] 10 min ischemia followed by 1 and 12–14 h reperfusion led to increased membrane fluidity as observed by significantly increased values of the HWHH parameter of 5-doxylstearic acid. The effects of PBN and DFMO administration on these changes in 5-doxylstearic acid label motion observed during IRI are shown in Table 2. Pretreatment with PBN resulted in complete protection from IRI-induced 5-doxylstearic acid motional changes observed at both 1 and 14 h reperfusion. However, treatment with PBN at 6 h post-ischemia resulted in only a slight attenuation of the increased fluidity observed at 14 h reperfusion, similar to the protein effect noted above. Treatment with 300 mg/kg DFMO at 30 min prior to ischemia resulted in no significant alteration of the 1 h reperfusion effect but complete blockade of the 14 h effect. The increased fluidity seen at 14 h was also abrogated with 6 h post-ischemia treatment of DFMO at this concentration.

In order to determine the dose of DFMO required to attenuate the increased synaptosomal membrane fluidity after 10 min ischemia and 14 h reperfusion, a dose response study was performed (Fig. 1). There was a significant HWHH change versus dose interaction over the concentrations studied ($P < 0.001$). The results indicated that the minimal dose for maximal effect was 100 mg/kg and the dose which resulted in a 50% attenuation was 50 mg/kg.

DISCUSSION

The increased protein–protein interactions and increased lipid fluidity observed in the physical state of synaptosomal membranes at 1 h reperfusion following a 10 min period of ischemia are prevented by pretreatment of PBN but not DFMO. PBN is known

Table 2. Effects of pretreatment of gerbils with 300 mg/kg *N*-tert-butyl-α-phenylnitrone or difluoromethylornithine on the HWHH parameter of 5-doxylstearic acid labeled synaptosomal membranes prepared following 10 min global ischemia with 1 and 14 h reperfusion*

	Treatment	% Change HWHH	S.D.	*N*
1 h Reperfusion				
A	Ischemia/reperfusion only‡	6.981	±1.246	7
	30 min PBN pre-treatment†	0.354	±1.508	5
	30 min DFMO pre-treatment‡	8.493	±1.356	6
14 h Reperfusion				
B	Ischemia/reperfusion only‡	9.613	±1.448	6
	30 min PBN pre-treatment†	−0.042	±1.910	5
	6 h PBN post-treatment‡	7.723	±1.859	5
	30 min DFMO pre-treatment†	−1.092	±1.269	5
	6 h DFMO post-treatment†	1.496	±1.356	5

*All data expressed as % change from non-ischemic control ± standard deviation.
†Represents significant difference between treatment and ischemia/reperfusion alone ($P < 0.00001$).
‡Represents change significantly different from non-ischemic control ($P < 0.001$).
$N = 5$–7.

to be a free radical trapping agent,[14,18,31,55,60,63] while DFMO is a suicide inhibitor of ODC, the enzyme chiefly responsible for putrescine synthesis.[46] These results strongly suggest the involvement of free radicals in these early synaptosomal membrane protein and lipid changes and that polyamine metabolism has little impact at the 1 h reperfusion time point. Based on our observation that pretreatment with PBN completely nullifies the IRI-induced changes normally seen in the proteins and lipids at 1 h reperfusion, we hypothesize that nearly all of the damage to the membrane at this reperfusion time point is due to free radical production. Furthermore, protection against the 14-h post-ischemia damage to both protein and lipid membrane domains suggests that this later change has its origins in the initial IRI-induced free radical damage to membranes, i.e. the polyamine-induced effect at 12–14 h is likely a secondary effect of free radical processes that occur early in IRI. Consistent with this idea, treatment with PBN at 6 h post ischemia showed virtually no protection against IRI-induced membrane protein alterations, and only a slight difference in the physical state of lipids at the 14 h reperfusion time point relative to ischemia/reperfusion alone.

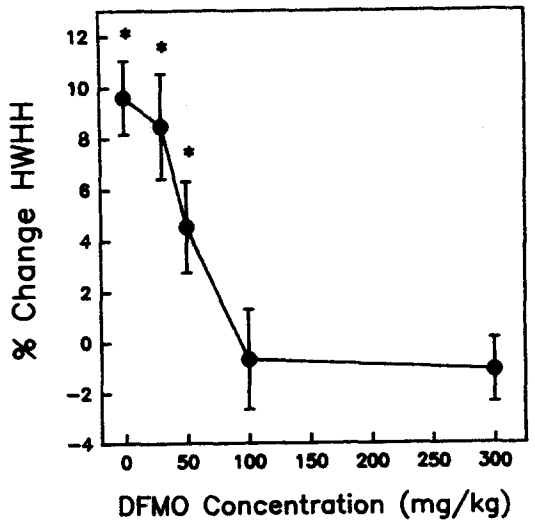

Fig. 1. Effects of pretreatment of gerbils with DFMO concentrations ranging from 0 to 300 mg/kg on the HWHH parameter of 5-doxylstearic acid labeled synaptosomal membranes prepared following 10 min global ischemia with 14 h reperfusion. *Represents significantly different from no-ischemia/reperfusion control ($P < 0.005$). $N = 4$–6.

PBN is known to efficiently cross the blood–brain barrier,[16] and to have protective effects on cells which have been exposed to oxidative damage.[14,18,55,60,63] It has been repeatedly demonstrated that there is a dramatic increase in the production of reactive oxygen free radicals at the onset of reperfusion following ischemia.[7,13,23] These free radicals can be damaging to both the membrane proteins and lipids through protein cysteine sulfhydryl cross-linking and carbonyl formation as well as the production of lipid peroxides and lipid peroxidation products.[7,13,24] This free radical-induced damage has been suggested to be the main contributor to early changes in lipid and protein label motion during IRI.[28] The current results strongly support this suggestion.

Muszynski *et al.* have shown that, in the rat model of IRI, there is an increase in ODC activity which maximizes after 12 h reperfusion.[47] This increase in ODC activity correlates precisely with the second increase in lipid label motion seen at 12 and 14 h reperfusion following 10 min ischemia in the present study and in previous work in the gerbil model.[28] This observation comes from the biphasic change in synaptosomal lipid label motion which we have previously reported.[28] Muszynski's findings have demonstrated that DFMO totally negates the increase both

in ODC mRNA and enzyme activity seen at any time point of reperfusion,[47] consistent with our finding that pretreatment with PBN prevents the 14-h damage to membranes following IRI. Again, this observation correlates with the nullification of the increase in lipid spin label motion observed at 14 h reperfusion by injection with DFMO 6 h post-ischemia. These findings strongly support our hypothesis[28] that the changes observed at this later time point of reperfusion are related to polyamines and phospholipase A_2 activity as consequences of reactive oxygen species production immediately upon reperfusion. Although the precise molecular basis for increased ODC activity 12–14 h after free radical insult during IRI is yet unknown, we offer the following speculative hypothesis.

Tentative hypothesis to account for the 12–14 h ischemia/reperfusion induced changes in the physical and biochemical states of neocortical synaptosomal membranes

ODC may be expressed as a defensive response to the free radical-mediated damage caused by IRI. This expression might result from byproducts of lipid peroxidation or the build up of lipid peroxides in the membrane due to oxidative damage, which is thought to occur at the onset of reperfusion. The increase in ODC activity could indirectly initiate processes which are capable of partial repair of membrane damage. For example, the increase in ODC combined with a decrease in SAMDC activity, observed during reperfusion,[50,53] would have the effect of raising putrescine levels while lowering the levels of spermine and spermidine. A secondary effect of the decrease in SAMDC activity is an increase in mitochondrial Ca^{2+} uptake as a result of the decrease in spermine.[53] Increased putrescine levels could cause changes in the physical state of membrane phospholipids by the mechanisms proposed in Fig. 2. The NMDA receptor has an allosteric binding site for putrescine,[53,67] which increases the NMDA affinity for binding and, therefore, yields an increased influx of Ca^{2+} through these channels.[45] Increased intracellular Ca^{2+} further stimulates ODC activity.[39] Phospholipase A_2 is also affected by both the increased calcium load and the putrescine.[19] It has been shown that there is as much as a three-fold increase in phospholipase A_2 activity in the presence of polyamines.[19] Increasing phospholipase A_2 activity would then feed back to cause a further increase in ODC activity.[26] Since phospholipase A_2 is a calcium-dependent enzyme, the increase in intracellular calcium during reperfusion will enhance its activity to an even greater extent, both directly and through second messenger systems.[41,62]

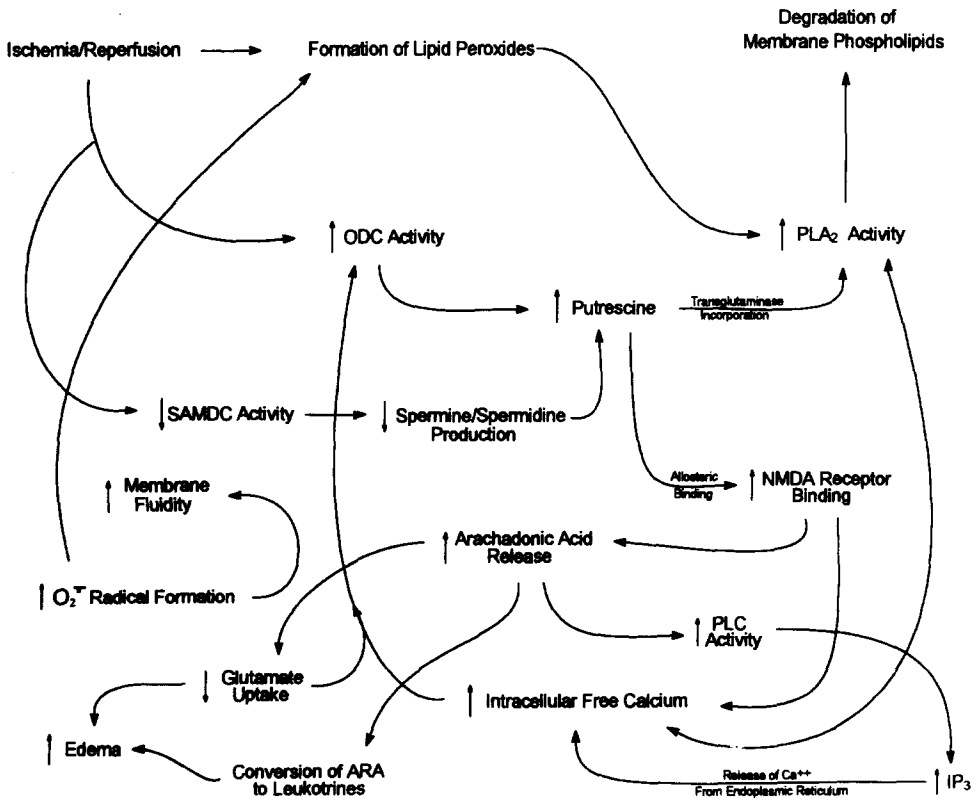

Fig. 2. Schematic representation of the biochemical mechanisms which may be involved in changes of the lipid motion and order observed at 14 h reperfusion following 10 min ischemia. This proposed interrelated series of events centers on an increase in ODC activity, around 14 h reperfusion, which is thought to be a secondary response to free radical damage occurring at early reperfusion times (see text).

Polyamine and calcium dependent increases in phospholipase A_2 activity and the higher affinity of the polyaminated phospholipase A_2 to the lipid bilayer can be combined with what has been termed the interfacial phenomenon or interfacial activation of phospholipase A_2.[19,62] This concept is based on the observation of phospholipase A_2 becoming more active at the lipid interface. Dorn-Zachertz and Zimmer,[21] have shown that phospholipase A_2 increases the motion of 5-doxylstearic acid in erythrocytes, supporting our hypothesis that phospholipase A_2 contributes to the increase in 5-doxylstearic acid motion observed at 12–14 h reperfusion, in the current study. The circle of events involving phospholipase A_2 could easily be conceptualized to have a major effect on the plasma membrane of neurons experiencing IRI. It is important to note that this cycle is greatly dependent on the IRI-induced stimulation of ODC activity and the production of polyamines, a stimulation consistent with the DFMO effects showing abrogation of the 12–14 h alterations in the physical state of the synaptosomal membrane.

NMDA-activated arachidonic acid release during IRI can also be enhanced in the presence of high levels of polyamines.[41,61] Arachidonic acid can be converted to leukotrienes which could then contribute to brain edema.[1] In addition, arachidonic acid can decrease glutamate uptake by the cell, further stimulating the increase in intracellular calcium levels.[5,15,41,65]

Another effect of the build up of arachidonic acid is it can increase the formation of free radical species such as the superoxide radical O_2.[33,74,79] Previous studies have shown that the superoxide radical itself can increase membrane fluidity.[40] Production of superoxide radicals could conceivably be partially responsible for the increases in lipid spin-label motion we observed with IRI in the gerbil. Consistent with this idea, the decrease in SAMDC activity during reperfusion is reported to decrease the spermidine concentration.[53] The decrease in spermidine has been shown to cause an increase in mitochondrial calcium uptake, an effect which can potentiate leakage of superoxide radicals and enhance free radical damage.[48,53]

Associated with the increase in phospholipase activity is an increase in free fatty acids in the brain during IRI. Three of the effects of excess free fatty acid formation are to impair mitochondrial ATP production,[78] increase PLA_2 activity,[5,77] and increase edema,[15,78] all known to occur during IRI.

An important factor which should also be taken into consideration when analysing the changes associated with IRI is a decreased pH. The accumulation of H^+ during IRI induced acidosis has been proposed to preconcentrate in close vicinity to the plasma membrane. It is possible that the hydrogen ions will compete with calcium in competitive binding to cause the release of calcium and higher intracellular free calcium levels. This would yield yet another source of free calcium to feed into the inter-connected framework of mechanisms previously discussed. Lowered pH itself is known to activate lysosomal phospholipases.[17]

An end result of the combination of phospholipase A_2-activating processes described above is the depletion of phospholipids in the plasma membrane by removal of damaged alkyl side chains. This depletion alone can cause a 25–50% increase in passive calcium influx across the plasma membrane.[17]

It is easy to conceptualize this combination of responses by the cell as a frantic attempt to try and save itself by removal and replacement of its damaged components. In the presence of only a mild amount of stress this feat could conceivably be adequately accomplished by the cell with a subsequent return to normal function. However, if there were too much initial damage, the cell may actually bring about its own death in an attempt to save itself. In this model, the amount of replacement and repair would be directly proportional to the amount of membrane damage which would, itself, be directly proportional to the intensity of the IRI insult. It then follows that apoptosis, or programmed cell death,[42,43] conceivably might have more to do with the extent of the cells natural repair processes rather than the cell making a conscious decision to end its own life. In this hypothesis, there would be some threshold of damage to the plasma membrane beyond which a cell could not recover and would ultimately die.

CONCLUSIONS

The above discussion of the results of the current study has been an attempt to help explain the interconnected meshwork of events which cumulatively contribute to the overall cell response to IRI. The results presented here support evidence of free radical-mediated damage resulting from reperfusion following ischemia. It is our view that polyamine production is a key secondary response which is initiated by free radical damage. We believe the above-discussed chain of events, initiated by polyamine production, is a response directed toward repair of free radical damage. The outcome of these repair processes could be potentially lethal to the cell if the initial damage is too great. Others have viewed the cell's response as apoptosis.

The results of the current study provide insight into the membrane-altering mechanisms occurring during IRI. These insights will, hopefully, lead to better therapeutic treatments for stroke and head injury, and research into this is actively being pursued in our laboratory.

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