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Ischemia/reperfusion-induced changes in membrane fluidity characteristics of brain capillary endothelial cells and its prevention by liposomal-incorporated superoxide dismutase

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The effect of global cerebral ischemia and reperfusion on cerebral capillary endothelial cell membrane fluidity was examined using electron paramagnetic resonance techniques following 8 minutes of global ischemia and 15 minutes of blood reperfusion. The luminal surface of the cerebral vasculature was perfused with a series of doxyl stearic acid reporters (5-, 12-, 16-doxyl stearic acid) which differ in the site of attachment of the nitroxide free radical on the fatty acid chain. Each doxyl stearic acid reports on membrane fluidity characterstics from different depths within the membrane. Ischemia/reperfusion produced a membrane ordering that was markedly dependent on intramembrane location, and was consistent with changes previously associated with lipid peroxidation. The effect of ischemia/reperfusion on membrane fluidity was maximal in the membrane environment reported by 12-doxyl stearic acid (12-DS). The utilization of a liposomal system was shown to enhance superoxide dismutase delivery to cerebral tissues as well as attenuating the change in membrane order seen following reperfusion-induced lipid peroxidation.

Introduction

Free radicals, reactive substances containing an unpaired electron, occur naturally and are necessary for various physiological processes to occur. However, free radicals can be pathogenic when produced in excessive amounts or occur in inappropriate cellular compartments. In particular, oxygen centered free radicals have been implicated in central nervous system pathology during both regional or global ischemia and subsequent reperfusion [1]. The most susceptible biological structure for free radical attack is the lipid bilayer. Membrane phospholipids are particularly sensitive to oxygen free radical attack for two primary reasons; first, the abundance of polyunsaturated fatty acids provides a good target for free radical attack; and second, the high solubility of molecular oxygen in hydrophobic membranes relative to aqueous environments [2]. Oxygen derived free radicals produced in biological systems have been demonstrated to produce tissue injury such as lipid peroxidation, inflammation and edema [3–5]. The production of lipid peroxidation products has been associated with the loss of membrane integrity following ischemia/reperfusion [6], as well as causing the release of free fatty acids (arachidonic acid) that can promote the induction of edema and other pathologic reactions via arachidonic acid metabolism [7]. Several studies have examined biophysical membrane characteristics following lipid peroxidation with results depending upon methodology [8–10]. Bruch et al. [11], using various electron paramagnetic fatty acid nitroxide probes in model membranes, demonstrated a lipid peroxidation induced membrane alteration that was dependent on the intramembrane location sampled by the nitroxide probes.

Superoxide dismutase (SOD) has been shown to diminish the permeability changes and lipid peroxidation products associated with membrane free radical attack [12,13]. Until recently, SOD was considered limited in its ability to scavenge superoxide generated in the brain for two major reasons: first its half-life ($T_{1/2}$) in circulation is < 6 minutes [14]; and second, the inability of SOD to cross the blood-brain barrier (BBB) or cellular membranes makes it difficult to access the presumed area of free radical production [15]. Recent studies using PEG (poly(ethylene glycol)) conjugated SOD [16] and liposomal incorporated SOD

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[17] have demonstrated a reduction in ischemic brain injury, presumably due to increased uptake of SOD. Similar effects of liposomal SOD have been reported in cultured endothelial cells [15] and lung tissue in vivo [18]. Positively charged liposomes containing SOD have been shown to be readily taken up by primary cultures of neurons and astrocytes, whereas free SOD was not [15]. The effect of positively charged liposomes containing SOD in attenuating ischemic brain injury, such as permeability changes and edema formation has also been demonstrated [17].

The aims of this study are two-fold: First, to examine the depth dependent biophysical membrane changes associated with global cerebral ischemia and reperfusion using an electron paramagnetic resonance reporter technique. Second, to employ a liposomal system for the enhanced delivery of SOD to cerebral tissues in hopes of attenuating the membrane ordering induced by ischemia/reperfusion.

Methods

Preparation of liposomal SOD

The preparation of unilamellar liposomes with a large internal aqueous space was performed using a reverse-phase evaporation technique. Positively charged liposomes were prepared by the addition of L-α-dipalmitoylphosphatidylcholine, cholesterol and stearylamine (Sigma, St. Louis, MO) with a molar ratio of 14:7:4 as described previously by Szoka and Papahadjopoulos [19]. The lipids (250 μmol) were dissolved in chloroform followed by the addition of phosphate-buffered saline (PBS) as an aqueous phase (0.05 M PBS (pH 7.4)) to form a two phase system (3:1, v/v). CuZn-SOD (from bovine erythrocytes 3000 units/mg, Sigma Chemical Corp., St. Louis, MO) was added to the lipid solution just prior to sonication. This mixture was then sonicated under nitrogen with a Branson sonicator (Branson, St. Louis, MO) for 2–5 min at 0–5 °C (60 Watts, pulsed, 50% cycle duration). Sonication continued until the mixture became either a clear or homogenous dispersion that did not separate for at least 30 min post sonication [19]. The solvent was removed by vacuum rotary evaporation at 40 °C and the unilamellar liposomes were resuspended in 0.05 M PBS (pH 7.4). The free enzyme was separated from the liposome entrapped enzyme by centrifugation. The yield of liposome entrapped SOD ranged from 20 to 40% with an average value of $1.2 \cdot 10^2$ units of SOD/μmol of phospholipid as measured by a NBT reduction assay for SOD [20]. A total of 3000 units of SOD entrapped in liposomes was delivered as an i.v. bolus (jugular vein) 5 min prior to induction of global ischemia

Global ischemia / reperfusion model

Male Sprague-Dawley rats (Harlan, Frederick, MD) weighing 200–250 g were anesthetized with pentobarbital (50 mg/kg, i.p.); ventilation was controlled and supplemental O_2 (95% O_2/5% CO_2) was administered through a tracheostomy using a Harvard Rodent Ventilator (TV = 1.5 cm^3/kg, RR = 100). Jugular vein and tail artery catheters were placed to administer SOD, monitor blood pressures, and measure blood gases. A median sternotomy was performed to expose the ascending aorta and inferior vena cava. Rectal temperatures were measured by a YSI thermometer and temperature regulated by heat lamp at 37 ± 1.0 °C. Ischemia was induced by cross clamping the inferior vena cava and ascending aorta. After 8 min of global ischemia, mean arterial blood pressure (MABP) of zero, clamps were released and the animals were reperfused for 15 min. Only those animals not requiring pressor or bicarbonate administration for their resusitation were employed. The MABP returned to 90% of normal within 5 min of reperfusion. This global ischemia model is a modification of the technique developed by others in rats [12,21] and dogs [22–24]. The physiologic confirmation of successful ischemia/reperfusion is provided by the blood gas data summarized in Table I. Immediately following reperfusion, the carotid arteries were cannulated bilaterally, the jugular veins were cut, and the brain perfused clear of blood (0.05 M PBS (pH 7.40), 10 ml) and labeled with 5-, 12- or 16-doxyl stearic acids (100 μM, 10 ml).

TABLE I

Physiologic parameters of rats undergoing ischemic / reperfusion induced damage

Treatment group [a] (*n*)	Blood pressure [b] (mmHg)	Blood gas value ($x \pm$S.E.)		
		pH	pCO_2	PO_2
Control (7)	110.2 ± 9.4	7.34 ± 0.01	35.2 ± 3.8	303.5 ± 37.2
Ischemia (5)	0 ± 0.0	7.12 ± 0.02 *	42.7 ± 6.2	264.7 ± 24.3 *
Reperfusion (5)	115.4 ± 14.3	7.23 ± 0.02 *	46.4 ± 5.3 *	330.1 ± 37.6

[a] Blood gas (arterial) samples drawn at 1 min prior to initiation of ischemia (control); immediately after reestablishment of perfusion (ischemia); 15 min after reestablishment of perfusion (reperfusion). Results expanded as mean ± S.E.

[b] Blood pressure and blood gases obtained from a cannulated tail artery. Blood pressure reported as mean ± S.E.

* $P < 0.05$, compared to control (ANOVA).

Following the final perfusion, a sample plug was removed from the cortex using a 20 μl pipette (Corning, Corning, NY) and the fluidity of the luminal surface of the capillary endothelial cell membrane was measured by electron paramagnetic resonance (EPR) techniques. In those animals undergoing ischemia/hypoxemic reperfusion, the carotids were not allowed to reperfuse with blood, but are immediately cannulated and reperfused with a the deoxygenated buffer with label (0.05 M PBS (pH 7.40)).

Electron paramagnetic resonance measurements

A glass capillary tube (20 μl) containing the cortical sample plug was supported vertically in a quartz tube contained in the cavity of a Bruker EPR 200D-SRC spectrometer. Temperature was regulated by a N_2 controlled variable temperature unit (ER 411VT) and spectra were obtained at 296 K. Data presented is the mean of 4 or more samples for each group. Order parameters ($S_{\parallel}$) were calculated according to the method described by Sauerheber et al. [25],

$$S_{\parallel} = 1/2\left[\frac{3(T_{\parallel} - T_{xx})}{T_{zz} - T_{xx}}\right] - 1$$

T_{zz} and T_{xx} are splitting elements for the doxyl stearic acids obtained from host crystal studies. The values for 5- and 12-DS were obtained from Seelig [27]. Since the splitting elements were not available for 16-DS, we employed the values for 5-DS. Briefly, $S_{\parallel}$ values allow for the expression of membrane fluidity ($S_{\parallel} = 1$ being rigid; $S_{\parallel} = 0$ being fluid) independent of lateral phase separation and probe-probe interactions which can be misconstrued as membrane structural alterations [25].

A reperfusion spectra obtained from the luminal surface of rat brain capillary endothelial cells using 5-DS as the reporter is shown in Fig. 1. The spectral parameters $T_{\parallel}$ and $T_{\perp}$ are demonstrated, as are the chemical structures of the doxyl stearic acids employed as reporters in this study ((*n*, *m*) 5-DS (12, 3), 12-DS (5, 10), 16-DS (1, 14)).

Assay for superoxide dismutase (SOD)

The method used for the measurement of SOD levels in brain homogenates is the nitroblue tetrazolium (NBT) assay based on a modification by Oberley and Spitz [20] on the original assay of Beauchamp and Fridovich [26]. Briefly, a xanthine/xanthine oxidase mixture is used to generate a reproducible amount of O_2^-. Following experimental manipulations, the brain is removed and homogenized in 0.05 M (pH 7.4) using a Dounce homogenizer. Following 20 up and down strokes in the homogenizer various dilutions of the homogenate were made and assayed for maximal inhibition of NBT reduction. The percent inhibition of NBT reduction is a measurement of SOD. One unit of SOD is defined as the amount of protein which gives half the maximal inhibition of NBT reduction. For a 20 tube assay, the following reagents were added as described by Oberley and Spitz [20]: 13.0 ml of 0.05 M phosphate buffer (pH 7.8) with 1.333 mM Detapac; 0.5 ml of 2.24 mM NBT stock solution in phosphate buffer

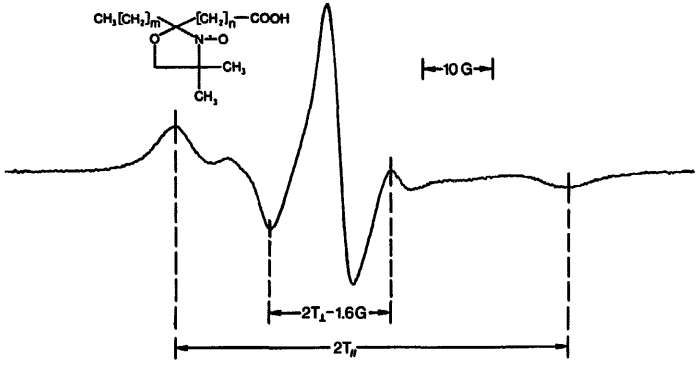

Fig. 1. A representative spectra of 5-doxyl stearic acid (5-DS) incorporated into the luminal surface of brain capillary endothelial cells using the sample-plug technique (23°C). The figure shows the spectral parameters $2T_{\parallel}$ and $2T_{\perp}$ which are used to calculate order parameters. In addition, the chemical structure of the doxyl stearic acids are shown with the following (*m*, *n*) values: 5-DS (12, 3); 12-DS (5, 10); 16-DS (1, 14).

(kept in brown bottle); 1.70 ml of xanthine stock solution (1.8 mM in phosphate buffer, prepared fresh every week); 20 units catalase. This solution is mixed well and aliquots of 0.8 ml are placed in cuvettes and 100 μl tissue homogenate, or 100 μl PBS (Blank) is added. Xanthine oxidase (100 μl) is added (approx. 10^{-2} units/ml) final concentration to give an absorbance rate of 0.015 to 0.025/min at 560 nM in blanks.

Statistics

Group comparisons conducted by Student's *t*-test or ANOVA [28], where significant differences were obtained for $P < 0.05$.

Results

Intramembrane variation in cerebral membrane order following ischemia / reperfusion

Spin labels, containing a paramagnetic nitroxyl moiety at various locations along the stearic acid chain, were employed in an *in vivo* model of ischemia/reperfusion injury to demonstrate site specific effects of lipid peroxidation on membrane fluidity characteristics. The three spin labels used were 5-, 12- and 16-doxyl stearic acids. These spin labels report on membrane fluidity characteristics of the phopholipid head group, mid zone and terminal acyl chain regions of the membrane, respectively. Fig. 2 shows the changes in order parameter ($S_{\parallel}$) following 8 min of global ischemia with 15 min of blood reperfusion. Neither the 5- nor 16-DS reporter groups showed a significant change in membrane order following ischemia/reperfusion. By contrast, the 12-DS reporter, labeling the middle membrane region appeared uniquely sensitive in reporting membrane ordering effects following ischemia/reperfusion. The increase in membrane order reported by 12-DS for the luminal surface of brain capillary endothelial cells following ischemia/reperfusion is equivalent to decreasing the temperature of the membrane by approx. 6 K. This change in membrane order is not normally produced by the amount of pH alterations seen following ischemia.

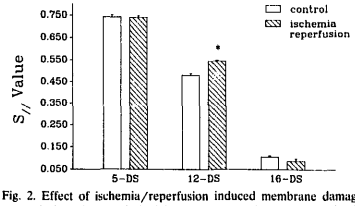

Fig. 2. Effect of ischemia/reperfusion induced membrane damage on cortical capillary endothelial cells as reported by the nitroxyl spin labels: 5-, 12-, 16-doxyl stearic acid (DS). Values represent $x \pm$S.E., with $n = 4$–8 animals. * $P < 0.05$, ANOVA.

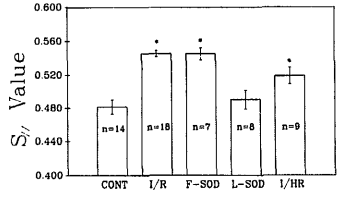

Fig. 3. Effect of free SOD (3000 units) and liposomal incorporated SOD (3000 units) on ischemia/reperfusion induced membrane damage of cortical capillary endothelial cells as reported by 12-doxyl stearic (DS) acid. I/R, 8 min global ischemia with 15 min blood reperfusion ($n = 18$). I/H, 8 min global ischemia followed by 15 min reperfusion with nitrogen saturated buffer. F-SOD, infusion of free SOD (3000 units) intravenously 5 min before ischemia reperfusion. L-SOD, infusion of liposome entrapped SOD (3000 units) 5 min before ischemia reperfusion. Values represent $x \pm$S.E. with $n =$ number of animals. * $P < 0.05$, ANOVA.

Beneficial effects of liposome entrapped SOD on ischemia / reperfusion membrane changes as compared to free SOD

Fig. 3 demonstrates, using a 12-DS spin label, the ability of liposomal entrapped SOD (3000 units) to attenuate the effect of ischemia/reperfusion injury on membrane order. Pretreatment with liposomal-SOD 5 min prior to ischemia completely abolished the ordering effect produced by ischemia/reperfusion. In contrast, free-SOD (3000 units) or empty liposomes (data not shown) given 5 min prior to ischemia were ineffective in attenuating the membrane changes induced by ischemia/reperfusion. It was interesting to note that ischemia/hypoxemic reperfusion resulted in a significant increase in membrane order. This may be due to free-radical formation during the ischemic period, or secondary to trace amounts of oxygen in the deoxygenated perfusion buffer.

Brain SOD levels

The ability of liposomal entrapped SOD to enhance brain SOD levels was measured using the NBT assay. Liposomal SOD and free SOD pretreatments were compared for their ability to elevate SOD levels in the brain following an I.V. bolus (3000 units) and 30 min circulation, a time interval equivalent to the full ischemic/reperfusion protocol. Measurements were made on brain homogenates following experimental manipulations as described previously. The results in Table II demonstrate a > 2-fold increase in whole brain SOD levels in those animals treated with liposomal-SOD relative to control animals. Those treated

TABLE II

Brain concentrations of SOD following intravenous administration of free-SOD and liposomal incorporated SOD

Animal group [a] (n)	Brain SOD levels (units SOD/mg protein)
Control (5)	8.2 ± 0.8
Free SOD (5)	8.2 ± 1.5
Liposomal incorporated SOD (5)	15.0 ± 1.5 *

[a] Whole brain homogenates obtained 30 min after i.v. injection of vehicle (0.9%, NaCl), Free-SOD (3000 units) or Liposomal SOD < 3000 units).

* $P < 0.05$, compared to control (ANOVA).

with free SOD had no significant alteration in whole brain SOD levels. Since the brains are perfused with PBS buffer prior to homogenization, the difference in SOD concentration is not secondary to RBC contamination or elevated intravascular concentrations of liposomal SOD.

Discussion

The data demonstrates a unique depth dependent membrane ordering in the hydrophobic zone of membrane lipids secondary to cerebral ischemia/reperfusion damage. Primary effects of oxygen radicals result in the production of alkyl radicals, which are believed to be initiators in lipid peroxidation reactions. It has been suggested previously that the membrane changes following cerebral ischemia/reperfusion may be associated with free radical production and associated peroxidative lipid cross linking and loss of unsaturation [11,12]. The significance of lipid peroxidative membrane ordering and the attenuation of this ordering by liposomal-SOD in ischemia/reperfusion is relevant to associated brain pathology. Peroxidative processes have been shown to cause the rapid disappearance of membrane unsaturated fatty acids and the release of free fatty acids [29]. The loss of polyunsaturated fatty acids will affect membrane integrity as well as the activity of a variety of membrane associated proteins. In addition to the direct membrane attack by free radicals, free fatty acids released from lipid peroxidation can induce vascular leakage, inflammation and cerebral edema [30,31]. Arachidonic acid release can promote the production of several vasoactive substances with a myriad of vascular effects. Products of arachidonic acid metabolism can result in increased leukotriene production, platelet adherence, leukocyte migration and its associated free radical burst, as well as prostaglandin imbalances and phospholipase activation [32,33]. All of these factors contribute to the inflammation, edema, and vascular permeability changes associated with ischemia and ischemia/reperfusion induced cerebral pathology.

The use of electron paramagnetic resonance reporters to detect peroxidative damage may provide a sensitive assay to compliment existing assays for lipid peroxidation. Previous studies by Bruch et al. [11] in model membrane systems have demonstrated membrane depth dependent alterations using electron paramagnetic resonance reporters in liposomes undergoing peroxidative reactions. Similar results have also been obtained in erythrocytes, microsomes and liposomes using fluorescent probes [8–10]. Studies by Zaleska et al. [34] have recently investigated, using nitroxyl spin labels, the increased microviscosity in striatum synaptosomes following ADP-Fe^{3+}/ascorbate induced peroxidation. In agreement with our data, a signifcant increase in microviscosity was found in the 12-DS region of their membrane model following ADP-Fe^{3+} induced lipid peroxidation. To date, no study has examined the depth dependent membrane order changes seen following global cerebral ischemia/reperfusion damage. Previous studies in our laboratory [12], and by other investigators [29], have demonstrated an increase in lipid peroxidative products following cerebral ischemia/reperfusion insults. Our data presents evidence of possible peroxidative processes occurring deep in the hydrophobic core of the membrane following ischemia/reperfusion, that results in a membrane ordering similar to that seen by others [11] in biological systems undergoing lipid peroxidation. The hypothesis, that the lipid peroxidative reactions in ischemic/reperfused brain occur deep in the membrane, is supported by Braughler et al. [35], in which iron chelators attached to hydrophobic steroids were examined for their ability to inhibit iron-dependent lipid peroxidation in whole rat brain homogenates. It appeared that coupling Fe^{2+} chelators to hydrophobic steroids increased their inhibitory potencies by as much as 10–100-fold. This data demonstrates that deeper intramembrane access of an iron chelator enhances it's inhibitory effect against Fe^{2+} induced lipid peroxidation, suggesting that peroxidative reactions may be occurring deeper in the membrane. Thus, small changes in lipid peroxidation occurring deep in the hydrophobic portions of the membrane that go unnoticed by conventional assays for lipid peroxidation may be detectable by site specific electron paramagnetic resonance reporter techniques.

One question which can be raised about the interpretation of our data, is the location from which our membrane probes are reporting. Because the unique structure of the blood-brain barrier, it appears as though the perfused fatty acids incorporate with the luminal surface of the perfused capillaries. Any intracellular translocation of the spin-labels results in a rapid quenching of their free-radical signal, by intracellular reductive processes, resulting in an inability to measure an EPR spectra. As a result, we feel these

results probably pertain only to membrane characteristics at the luminal surface of the brain capillary endothelial cells. However, our evidence is not yet definitive, and other membrane structures of the brain parenchyma may be involved.

In addition to the electron paramagnetic resonance reporter technique for monitoring membrane changes following ischemia/reperfusion injury, a relatively new liposomal system for SOD delivery [17] was used to attenuate the biophysical membrane changes induced by ischemia/reperfusion and to implicate oxygen free radicals in these reactions. An i.v. bolus of SOD (3000 units, 25 μmol phospholipid) encapsulated in a positively charged liposome was shown to increase the brain SOD levels about 2-fold as compared to controls. A similar i.v. bolus of free SOD did not increase the brain SOD level and was not able to inhibit the membrane alterations in membrane order reported by 12-DS using EPR techniques. The inability of free-SOD to diminish ischemia/reperfusion induced membrane ordering can be attributed to several factors. The negatively charged free-SOD molecule is probably excluded from crossing the BBB. In addition, the half-life of free-SOD in the circulation is only 6 min compared to 4.2 h in liposomes as shown by the clearance studies of Turrens et al. [14]. Further studies need to be performed to determine whether increased half-life, BBB transport or intracellular access provides the pivotal advantage of liposomal-SOD over free-SOD.

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