PART IV. THE ROLE OF CEREBRAL BLOOD FLOW AND ARACHIDONIC ACID METABOLISM IN BRAIN INJURY AND ISCHEMIC DAMAGE

The Role of Arachidonic Acid and Oxygen Radical Metabolites in the Pathogenesis of Vasogenic Brain Edema and Astrocytic Swelling

PAK H. CHAN, SUSAN LONGAR, SYLVIA CHEN, ALBERT C. H. YU, LARS HILLERED, LILLIAN CHU, SHIGERI IMAIZUMI, BRYAN PEREIRA, KI MOORE, VICKI WOOLWORTH, AND ROBERT A. FISHMAN

Neurochemistry Laboratory
Brain Edema Research Center
Department of Neurology
School of Medicine
University of California
San Francisco, California 94143

INTRODUCTION

Alterations of membrane phospholipids and the acyl fatty acid moieties of brain cells have been related to various pathological disorders of the brain, including brain ischemia, hypoxia, blood-brain barrier (BBB) dysfunction, and epileptic seizures. In most cases, the polyunsaturated fatty acids (PUFA), arachidonic acid (AA) (20:4), and docosahexaenoic acid (22:6) are rapidly released from cellular membrane phospholipids as a result of these pathological insults.1-11

Using single rat brain cortical slices as an in vitro bioassay system, our laboratory has demonstrated that free PUFA could induce cellular edema.12 Further studies have indicated that the transient formation of free radicals and lipid peroxides are also involved in PUFA-induced swelling of brain slices and of cultured brain cells.13-15 The mechanism of cellular swelling in these systems is related to altered membrane functions, since both neurotransmitter uptake and Na+, K+-ATPase activity in synaptic membranes are significantly reduced.16 We have further studied the local effects of arachidonic acid on vasogenic edema in rats in vivo. Animals were infused with 0.05 mmol of saturated, monounsaturated, or PUFA into the thalamus, and the edema and cation levels were studied at 24 hours following the injection. Among the fatty acids, AA was the most potent fatty acid in inducing cerebral edema concomitant with the increase in sodium and decrease in potassium contents. Palmitic acid (16:0)

4This work was supported by National Institutes of Health Grants NS-14543 (R.A.F., P.H.C.) and NS-25172 (P.H.C.).

5Address for correspondence: Pak H. Chan, Ph.D., Neurochemistry Laboratory, Brain Edema Research Center, Department of Neurology, Box 0114, University of California, San Francisco, California 94143.
was ineffective in inducing brain edema and cation change. Furthermore, AA and
linoleic acid caused 3-fold and 2-fold increases, respectively in [131-I] albumin space
at 24 hours in the injected hemisphere when compared with Krebs Ringer. Oleic acid
(18:1), palmitic acid (16:0), and stearic acid (9:0) were not effective in altering
blood-brain barrier permeability. Preliminary studies obtained from our laboratory
suggest that superoxide radicals are involved in PUPA-mediated BBB permeability
changes and the development of vasogenic edema, since injection of AA and
liposome-entrapped superoxide dismutase (SOD) intracerebrally inhibited the AA-
induced vasogenic edema and BBB permeability changes.

The aims of our present studies are several. First, we would like to elucidate the
cause-and-effect relationship between oxygen free radicals and the development of
vasogenic edema. Second, the question arises as to whether brain cells like astroglia
will produce superoxide radicals in the presence of AA. Third, the mode of action of
AA and superoxide radicals on astrocytic swelling (cellular edema) and metabolism is
unknown, and is the subject of the present study.

METHODS: COLD-INJURY MODEL

We used a cold-induced brain injury model, which is characterized by the
breakdown of blood-brain-barrier permeability, focal brain necrosis, and development
of vasogenic brain edema. Rats weighing 200 to 250 g were anesthetized with
pentobarbital (50 mg/kg) and placed in a stereotaxic apparatus. A probe with 0.5-cm
diameter, attached to a brass cup (20 cm²) filled with dry ice and acetone (−50 °C),
was applied directly to the right side of the bony skull for 1 minute. This cold-injury
model is a highly reproducible method of inducing brain edema. The injury is
characterized by early changes; within minutes, an increase in BBB permeability is
detected, followed by an increase in brain edema, which is maximal at 24 hours. Our
time-course studies have shown that brain water content following a 1-minute freezing
lesion was increased from a control value of 79.6 ± 0.3 to 81.9 ± 0.5 at 30 minutes,
82.9 ± 0.4 at 2 hours, and 85.1 ± 0.4 at 24 hours. Furthermore, AA was increased by
266%, 476%, and 485% at 1 minute, 30 minutes, and 24 hours, respectively,
following the cold injury.

PRIMARY CELL CULTURE OF ASTROCYTES

Primary cultures of cerebral cortical astrocytes were prepared from newborn
Sprague-Dawley rats as described previously. Cerebral hemispheres were removed
aseptically from the skulls and freed of the meninges. The neopallia were removed and
were cut into small cubes (1 mm³) in a modified Eagle’s minimum essential tissue
culture medium (MEM) containing fetal calf serum (FCS). The tissue was disrupted by
vortex mixing for one minute, and the suspension was passed through two sterile
nylon Nitex sieves with pore sizes of 80 μm (first sieving) and 10 μm (second sieving).
A volume of cell suspension equivalent to one-thirtieth of the brain was placed in a
60-mm Falcon tissue dish. Fresh MEM supplemented with 10% FCS was added to the
dish to a final volume of 3 ml. All cultures were incubated at 37 °C in a 95%:5%
(vol/vol) mixture of atmospheric air and carbon dioxide with 95% humidity. The
culture medium was changed after 3 days of seeding and subsequently two times per
week. After 2 weeks, the cultures reached confluence and were grown in the additional
presence of 0.25 mM dibutyryl cyclic AMP. The cultures were used for experiments
between the ages of 28 and 35 days in vitro.
PREPARATION OF LIPOSOMES AND LIPOSOme-ENTRAPPED 
C u/Zn- SUPEROXIDE DISMUTASE

The procedures for the preparation of positively charged unilamellar liposomes, with a large internal aqueous space and high capture by reverse-phase evaporation, are based on the method of Szoka and Papahadjopoulos and have been adopted by our laboratory (see Fig. 1). The lipids contain L-alpha-dipalmitoyl phosphatidylcholine, cholesterol, and stearylamine with a molar ratio of 14:7:4. The lipids were dissolved in chloroform, followed by the addition of ether and phosphate buffer (0.4 mM), and then sonicated for 5 minutes at 45 °C (bath type, Branson Instruments, St. Louis, MO). The solvent was removed by rotary evaporation at 45 °C, and the unilamellar liposomes were suspended in phosphate-buffered saline. The preparation of liposome-entrapped Cu/Zn-SOD is essentially the same as that for the liposomes. Electrophoretically purified Cu/Zn-SOD, bovine blood 20,000 units/mg (Pharmacia, Uppsala, Sweden),

or human Cu/Zn-SOD produced by genetic engineering method (2,000 units/mg, Chiron Company, Emeryville, CA) at a concentration of $2 \times 10^5$ units was first dissolved in 0.4 mM phosphate buffer and then added to the lipid film (400 μmol phospholipids) before sonication. The yield of liposome-entrapped Cu/Zn-SOD ranged from 25% to 40% and had a value of $2 \times 10^5$ of SOD units/μmol phospholipid. Prior to the enzyme assay, the liposome-SOD (20 μl) was sonicated in the presence of 20 μl of 1% Triton X-100 and diluted with phosphate-buffered saline.

FIGURE 1. Negative-stain electron micrograph of unilamellar liposomes prepared by reverse-phase evaporation technique. The liposomes’ size ranged from 800–4,000 Å.

EFFECTS OF LIPOSOme-ENTRAPPEd Cu/Zn-SUPEROXIDE DISMUTASE ON SUPeroxIDE RADICAL FORMATION IN THE COLD-INJURED BRAIN

The brain level of nitroblue formazan (NBF), a reduction product of superoxide radical and nitroblue tetrazolium (NBT), was increased from a control value of 0.69 ±
FIGURE 2. Effects of Cu/Zn-SOD on superoxide radical formation, vasogenic edema, and blood-brain barrier permeability. (A) Control = contralateral cortex (sham operated); CL = cold lesion; F-SOD = free SOD; L-SOD = liposome-entrapped SOD. * = significance as compared to control; ** = significance as compared to cold lesion; *** = significance as compared to control. (B) A = control brain; B = brain with lesion; C = brain with cold lesion after 5 minutes of pretreatment with free Cu/Zn-SOD; D = brain with cold lesion after 5 minutes of pretreatment with liposome-entrapped Cu/Zn-SOD; E = brain with cold lesion 5 minutes after treatment with free Cu/Zn-SOD; F = brain with cold lesion 5 minutes after treatment with liposome-entrapped Cu/Zn-SOD; * = significance at 0.05 as compared with the group with cold lesions. (C) Effects of liposome-entrapped Ce/Zn-SOD on blood-brain barrier permeability to 125I-labeled serum albumin. Control = contralateral hemisphere; CL = hemisphere with cold lesion; F-SOD (pre) = animals were pretreated with free SOD; L-SOD (pre) = animals were pretreated with liposome-entrapped SOD; L-SOD (post) = animals were posttreated with liposome-entrapped SOD. * = significance as compared to the contralateral control hemisphere; ** = significance as compared to cortex with the cold lesion.

0.04 to 4.44 ± 0.88 nmol per mg of protein at 1 hour following the cold injury (Fig. 2A). The intravenous injection of liposome-entrapped Cu/Zn-SOD (10,000 units) 5 minutes prior to the cold injury significantly reduced the level of superoxide radicals to 1.21 ± 0.14 nmol per mg of protein (Fig. 2A). Both pretreatment (5 minutes prior to the injury) and posttreatment (5 minutes after the injury) of cold-injured animals with
liposome-entrapped Cu/Zn SOD also effectively reduced brain water content from 85.15% ± 0.4 and 82.9% ± 0.4, respectively (Fig. 2B).

Furthermore, cold injury caused a seven-fold increase in BBB permeability, as indicated by \(^{125}\text{I-BSA}\) space 24 hours following injury (Fig. 2C; control \(^{125}\text{I-BSA}\) space = 2.04% ± 0.10). Intravenous injection of liposome-entrapped Cu/Zn-SOD 5 minutes prior to or 5 minutes after the traumatic injury reduced the level of \(^{125}\text{I-BSA}\) space from 14.05% ± 1.3 to 4.69% ± 0.82 and 4.36% ± 0.37, respectively. Pretreatment or posttreatment with free Cu/Zn-SOD did not change the level of superoxide radicals, the degree of brain edema, and the \(^{125}\text{I-BSA}\) space, indicating that only the liposome-entrapped Cu/Zn-SOD was effective in reducing the level of superoxide radical, brain edema, and BBB permeability dysfunction induced by traumatic injury.

EFFECTS OF ARACHIDONIC ACID ON NITROBLUE TETRAZOLIUM REDUCTION, O^-\text{2}, LIPID PEROXIDATION, LACTATE PRODUCTION, AND SWELLING IN PRIMARY CELL CULTURE OF ASTROCYTES

We studied the time and dose-dependent effects of AA on the NBT reduction in intact cultured astrocytes (Fig. 3). The formation of NBF in control astrocytes increased gradually with time. AA at concentrations of 0.1 and 0.5 mM significantly increased the rate of NBF formation to 1.33 ± 0.1 and to 1.93 ± 0.2 nmoles per minute per mg protein, respectively, in astrocytes. The levels of NBF increased linearly within the 30 minutes of incubation with AA and reached a plateau at 60 minutes. Furthermore, other PUFA, including 22:6, linoleic acid (18:2), and linoleic acid (18:3), also increased significantly the level of NBT reduction in intact cultured astrocytes. The increase in NBT reduction was closely associated with the degree of unsaturation, with 22:6 the most effective. Both saturated palmitic acid (16:0) and mono-unsaturated oleic acid (18:1) were not effective. The stimulating effects of PUFA on the level of malondialdehyde (MDA) in astrocytes are shown in Figure 4B. The PUFA 18:3, 20:4, and 22:6 at 0.1-mM concentration increased the formation of MDA by 70%, 100%, and 95%, respectively. Figure 4C shows the effects of various

**FIGURE 3.** Stimulation of nitroblue tetrazolium (NBT) formation by arachidonic acid (AA) in intact cultured astrocytes. Cultured astrocytes were incubated with 1.0 mM nitroblue tetrazolium in the presence of AA (0.1 mM or 0.5 mM) for various times. The extracted NBF was read at 515 nm. Results are means of four different experiments with duplication assays for each experiment. Vertical bars indicate SD.
fatty acids on the lactic acid production in intact astrocytes. PUFA's 20:4, 18:3 and 18:2 at 0.1 mM caused increases in lactic acid production by 240%, 160%, and 60%, respectively, in incubation medium. Both 16:0 and 18:1 were ineffective. Furthermore, AA (0.5 mM) caused a 3-fold increase in intracellular water space, as measured by the uptake of [3H]-3-O-methyl glucose (Chen et al., unpublished data).

EFFECTS OF LIPOsome-ENTRAPped SUPERoxide DISMUTASE ON O2- AND ON LACTATE ACID PRODUCTION

Table 1 shows that when cultured astrocytes were incubated with liposome-entrapped SOD (100 units/ml), the levels of 20:4-induced NBF formation and lactic acid production were reduced significantly. Empty liposome or free SOD (100 units/ml) was not effective in reducing the increased cellular level of NBF or the lactate content in the incubation medium. Our preliminary data also showed that

<table>
<thead>
<tr>
<th>Incubation Medium</th>
<th>Nitroblue Formazan (% Control)</th>
<th>Lactate (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 16</td>
<td>100 ± 11</td>
</tr>
<tr>
<td>20:4</td>
<td>159 ± 15</td>
<td>183 ± 4</td>
</tr>
<tr>
<td>+ Empty Liposomes</td>
<td>180 ± 17</td>
<td>177 ± 10</td>
</tr>
<tr>
<td>+ Free SOD (100 units/dish)</td>
<td>185 ± 13</td>
<td>190 ± 15</td>
</tr>
<tr>
<td>+ Liposome-SOD (100 units/dish)</td>
<td>93 ± 9b</td>
<td>92 ± 9b</td>
</tr>
</tbody>
</table>

*Note: Astrocytes were preincubated with empty liposomes, free SOD, or liposome-entrapped SOD in MEM (serum-free) for 24 hours prior to the addition of 20:4 (0.1 mM) for another hour. Cell pellets were used for nitroblue formazan (NBF) assay, whereas incubation medium was assayed for lactate content. The control values of lactate and lactate were 54.5 ± 10.4 amol/mg protein/h and 0.35 ± 0.04 amol/mg protein/h, respectively. Values are the means ± SD of three different experiments.

*p < 0.01, compared to control group.

*p < 0.01, compared to 20:4 group, using analysis of variance.

liposome-entrapped SOD significantly reduced the astrocytic swelling concomitant with the release of lactate dehydrogenase in the medium (Chen et al., unpublished data). These data clearly indicated the involvement of intracellular superoxide radicals in AA-induced swelling in primary cell culture of astrocytes.

DISCUSSION

The present in vivo studies demonstrate that Cu/Zn-SOD, when entrapped in liposomes and injected intravenously before or immediately after the induction of cold injury, significantly reduced the level of superoxide radicals andameliorated brain edema and BBB permeability changes. However, the intravenous injection of free SOD was not effective. There are many reasons for the failure of free SOD to reduce brain edema. First, Cu/Zn-SOD, a negative-charge protein (isoelectric point ~ 4.2) with a molecular weight of 31,000, is almost completely excluded by endothelial cells and fails
to pass through the normal BBB. Second, even if the free SOD reaches the extracellular space in the injured brain, the brain cells are unable to take up the free enzyme, as was recently shown by our studies using both primary neuronal and astrocytic cultures. Third, the half-life of SOD in plasma is short (about 6 minutes), as indicated by the pharmacokinetic studies of plasma clearance of SOD. By contrast, Cu/Zn-SOD, when entrapped in positively charged sterylamine liposomes, readily was taken up by primary cultures of neurons and astrocytes. The half-life of Cu/Zn-SOD in liposomes is increased from 6 minutes to 4.2 hours. Our data further demonstrated that a single intravenous injection of liposome-entrapped Cu/Zn-SOD caused a twofold increase in the SOD level in the injured brain at 30 minutes and for up to 2 hours thereafter. The level of Cu/Zn-SOD in the contralateral control brain at various time points followed a pattern similar to that of the injured brain, although it was significantly lower than the level in the injured brain, suggesting that liposomes facilitate the transport of SOD into uninjured brain compartments. Furthermore, the transport of Cu/Zn-SOD into endothelial cells was highly facilitated by liposomes, indicating that the breakdown of the BBB may allow a high degree of transport of Cu/Zn-SOD into the extracellular space of the injured brain.

Although the direct involvement of superoxide radicals in the AA cascade in the cold-injured rat brain is not clear at present, the significant increases in AA following cold injury should provide an adequate source of substrate for oxygen radical formation via both cyclooxygenase and lipoxygenase. Furthermore, AA can also co-oxidize with endothelial xanthine oxidase to form superoxide radicals. Thus AA (and other PUFA) are likely to be involved in superoxide radical formation, vasogenic brain edema development, and BBB permeability changes.

Direct evidence of AA-mediated superoxide radical formation comes from our *in vitro* studies using primary cell cultures of astrocytes. The rate of NBF formation, induced by AA in intact cells, is both time- and dose-dependent. Our data further show that the stimulation of NBF formation is not specific for AA, since other PUFA, including linoleic acid (18:2), linolenic acid (18:3), and docosahexaenoic acid (22:6) were also effective (Fig. 4A). These data indicate that PUFA induce oxidative perturbations in cultured astrocytes and support our earlier observations in brain slices. The oxidative stress in astrocytes induced by PUFA was further demonstrated by the measurement of lipid peroxidation. Our data have shown that fatty acids that have three or more double bonds (18:3, 20:4, and 22:6) significantly increased the MDA content in astrocytes (Fig. 4B).

We have suggested previously that the O$_2^-$ formation and subsequent lipid peroxidation via AA cascades cause the membrane perturbation. The initial rate of uptake of glutamate in astrocytes was severely inhibited with 20:4 (concentrations ranged from 0.025-0.1 mM). We have suggested that the perturbation of membrane integrity induced by AA may be responsible for its inhibitory effect on glutamate uptake. Furthermore, our preliminary data also show that AA severely inhibits the (Na$^+$ + K$^+$)-ATPase activity in astrocytes (unpublished data), further indicating the membrane perturbation induced by AA. It is likely that increased level of O$_2^-$ and lipid peroxides are involved in these membrane injuries.

The mechanisms underlying the stimulation of lactate production in astrocytes are not clear at present, and several plausible explanations may be entertained. First, the various enzyme activities of the glycolytic pathway may be enhanced by AA and/or by its oxygen radical intermediates. Second, the normal mitochondrial respiratory activities and metabolic function are affected by AA and/or by oxygen radicals. For example, the enzymes of mitochondrial TCA cycle that are responsible for pyruvate flux and oxidation may be affected by AA. Free fatty acids, particularly PUFA, are potent uncouplers of mitochondrial phosphorylation. Our studies have found that
FIGURE 4. Effects of free fatty acids on the levels of nitroblue formazan (NBF), malondialdehyde (MDA) and lactic acid in astrocytes. (A) NBF formation in intact cultured astrocytes. Astrocytes were incubated with nitroblue tetrazolium in the presence of fatty acid (0.1 mM) for 10 min at 37°C. Results are expressed as percentage of the rate of NBF formation of the control ± SD. The control rate of NBF formation in astrocytes is 0.61 ± 0.02 nmol/min/mg protein. Results are averages of three experiments. * p < 0.01; ** p < 0.01. (B) MDA formation in cultured astrocytes. Astrocytes were incubated with thiobarbituric acid and free fatty acid for 1 h at 37°C. Results are expressed as percentage of rate of MDA formation of the control ± SD. The control rate of MDA formation is 1.78 ± 0.05 nmol/mg protein/h. Results are averaged from four different experiments. * p < 0.01. (C) Lactic acid production in astrocytes. Cultured astrocytes were incubated with individual fatty acid (0.1 mM) for 1 h at 37°C. The incubation medium was used to assay for the lactic acid content. Values are means ± SD of four experiments. * p < 0.05, ** p < 0.001, compared to control.
respiration was inhibited in isolated brain mitochondria with a relatively low concentration of 20.4 (13 nmoles/mg mitochondrial protein), suggesting that the normal mitochondrial function is altered by AA.³¹

Siesjo and his colleagues studied the influence of acidosis on \( \text{O}_2^- \) formation and lipid peroxidation, and have demonstrated that acidic pH in the range of pH 5.0-6.5 significantly induced free radical formation and lipid peroxidation in brain homogenates.²³ These investigators proposed that the effect of acidosis on brain homogenates may involve increased formation of the protonated form of \( \text{O}_2^- \). Our data have shown that AA caused a dose-dependent increase in lactic acid content in astrocytes. Since the increase in lactic acid content will lower the pH and create acidosis, the nature of \( \text{O}_2^- \) induced by AA may be a hydroperoxyl radical (\( \cdot \text{OOH} \)), as suggested by Siesjo et al.²²

The mechanisms underlying the AA-induced NBF formation in astrocytes are not clear at present. Various free radical scavengers and enzyme inhibitors have failed to reduce the level of NBF in astrocytes. Egan et al. found that the EPR signal detected during metabolism of arachidonate or prostaglandin \( \text{G}_2 \) by microsomal fractions and oxygenase was due to the oxidation of adventitious material that was isolated together with microsomal fractions. Their data suggest that the hydroperoxidase rather than the oxygenase of cyclooxygenase can release a reactive oxidant \( [\text{O}\cdot] \) into solution to initiate a radical chain.³³ Our studies have demonstrated that the addition of NADH or NADPH stimulates the NBF production in cultured astrocytes.³³ These data again confirm our previous observation that NADPH-dependent, AA-stimulated oxidases (peroxidases) are involved in \( \text{O}_2^- \) formation in astrocytes. Kukreja et al.³⁴ identified the mechanism by which arachidonate and linoleate metabolism via prostaglandin \( \text{H} \) synthase produced \( \text{O}_2^- \). This mechanism involves the oxygenation of NADH or NADPH to the radicals of \( \text{NAD} \) or \( \text{NADP} \), which then react with oxygen to produce \( \text{O}_2^- \). Our data support this mechanistic scheme, because NADPH or NADH enhances the 20:4-stimulated NBF formation in intact astrocytes. We speculate that the elevated level of NADH and lactic acidosis in the ischemic brain may provide a suitable environment favoring the formation of \( \text{O}_2^- \).

Once it is taken up by the cells, 20:4 is localized primarily in the fluid domains of membranes with preferential incorporation into endoplasmic reticulum or plasma and of mitochondrial membranes.³⁵,³⁶ These studies, together with the fact that the hydrophobic lipid environment is preferable for \( \text{O}_2^- \) and its protonated form (\( \cdot \text{OOH} \)),³⁷ suggest that the \( \text{O}_2^- \) or \( \cdot \text{OOH} \) may be formed or localized in membrane fluid domains. Further studies of \( \text{O}_2^- \) distribution using a cytochemical approach,³⁸ as well as morphological and immunocytochemical studies of the distribution of encapsulated SOD in astrocytes may provide additional information to strengthen this argument. Nevertheless, our studies demonstrate the beneficial effects of liposome-entrapped antioxidative enzymes in ameliorating \( \text{O}_2^- \) formation and lactate production in AA-injured astrocytes.³⁹ Liposome-entrapped antioxidative enzymes have been shown to be effective in ameliorating CNS \( \text{O}_2^- \) toxicity in vivo³⁹ and in preventing oxygen injury in cultured endothelial cells in vitro.⁴⁰ Furthermore, it is noteworthy that vitamin E liposomes have been shown to have beneficial effects in reducing lipid peroxidation and augmenting reactive gliosis in reaggregate cultures of fetal rat brain.⁴¹ Therefore, the present studies provide support for further study of the therapeutic potential of these liposome-entrapped antioxidative enzymes in CNS injury.

ACKNOWLEDGMENT

We thank Dianne Esson for her editorial assistance.
REFERENCES

18. CHAN, P. H., S. LONGAR & S. F. CHEN. Unpublished observations.
formation by arachidonic acid and by polyunsaturated fatty acids in primary astrocytic cultures. J. Neurochem. 59:1185-1191.


