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Induction of Intracellular Superoxide Radical Formation by Arachidonic Acid and by Polyunsaturated Fatty Acids in Primary Astrocytic Cultures

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Abstract: The effects of arachidonic acid and other polyunsaturated fatty acids (PUFAs) on both oxidative and metabolic perturbation were studied in primary cultures of rat cerebral cortical astrocytes. In the presence of 0.1 m*M* arachidonic acid, the rate of the reduction of nitroblue tetrazolium (NBT) to nitroblue formazan (NBF) was stimulated from 0.65 ± 0.10 to 1.43 ± 0.15 and from 0.092 ± 0.006 to 0.162 ± 0.009 nmol/min/mg protein in intact and broken cell preparations, respectively. The rate of superoxide radical formation, as measured by the superoxide dismutase (SOD)-inhibitable NBT reduction was 0.042 nmol/mg protein in broken cells and was negligible in intact cells. The latter is due to the impermeability of SOD into the intact cell preparation. NBF formation in intact astrocytes stimulated by arachidonic acid was both time- and dose-dependent. Other PUFAs, including linoleic acid, linolenic acid, and docosahexaenoic acid, were also effective in stimulating NBF formation in astrocytes, whereas saturated palmitic acid and monounsaturated oleic acid were ineffective. Similar effects of these PUFAs were observed in malondialdehyde formation in cells and lactic acid accumulation in incubation medium. These data indicate that both membrane integrity and cellular metabolism were perturbed by arachidonic acid and by other PUFAs. The sites of superoxide radical formation appeared to be intracellular and may be associated with membrane phospholipid domains, because liposome-entrapped SOD, which was taken up by intact astrocytes, reduced the level of superoxide radicals and lactic acid content, whereas free SOD was not effective. **Key Words:** Arachidonic acid—Astrocytes—Superoxide radicals—Liposomes—Polyunsaturated fatty acids—Lactic acidosis. **Chan P. H. et al.** Induction of intracellular superoxide radical formation by arachidonic acid and by polyunsaturated fatty acids in primary astrocytic cultures. *J. Neurochem.* **50**, 1185–1193 (1988).

Brain cells in situ contain a very low concentration of free polyunsaturated fatty acids (PUFAs), such as arachidonic acid (20:4) and docosahexaenoic acid (22:6; <0.01 μmol/g wet weight) (Lunt and Rowe, 1968; Gardiner et al., 1981), due to a dynamic acylation process of these free PUFAs occurring in cerebral membrane phospholipids (Yau and Sun, 1974). Following various forms of pathological insult, including ischemia, hypoxia, and traumatic injury, 20:4 is released rapidly from cellular membrane phospholipids resulting from the activation of phospholipase C and A_2 (Bazan, 1970; Edgar et al., 1982; Wei et al., 1982; Chan et al., 1983*b*; Yasuda et al., 1985; Yoshida et al., 1986; Abe et al., 1987). Free 20:4 is metabolized readily to prostaglandins and thromboxanes via cyclooxygenase, or to hydroxy or hydroperoxy fatty acids and leukotrienes via lipoxygenase (Gaudet and Levine, 1979; Ellis et al., 1983; Moskowitz et al., 1984). It has been proposed that a highly reactive oxygen radical species is formed during the conversion of prostaglandin G_2 to prostaglandin H_2 and 5-hydroperoxyeicosatetraenoic acid to leukotriene A_4 (Samuelsson et al., 1979; Kuehl and Egan, 1980; Samuelsson, 1983). Kontos and colleagues, using the superoxide dismutase (SOD)-inhibitable reduction of nitroblue tetrazolium (NBT) assay for superoxide

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Abbreviations used: CuZn-SOD, copper-zinc superoxide dismutase; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; MDA, malondialdehyde; MEM, modified Eagle's minimum essential tissue culture medium; Mn-SOD, manganese superoxide dismutase; NBF, nitroblue formazan; NBT, nitroblue tetrazolium; $O_2 \cdot^-$, superoxide radicals; PUFAs, polyunsaturated fatty acids; SOD, superoxide dismutase; 16:0, palmitic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:4, arachidonic acid; 22:6, docosahexaenoic acid.

radicals ($O_2 \cdot^-$), have demonstrated that $O_2 \cdot^-$ are significantly increased in concussive injured brain (Wei et al., 1981; Kontos, 1985). These increases in $O_2 \cdot^-$ levels are associated with abnormality of the pial arterioles and are associated with the prostaglandin synthetic pathway, because topical administration of SOD or indomethacin significantly reduced the level of $O_2 \cdot^-$ formation and ameliorated the 20:4-induced injury of pial arterioles. These studies have suggested further that $O_2 \cdot^-$ are likely being formed intracellularly in capillary endothelial cell walls and subsequently leak out from the endothelial cells via anionic channels (Kontos et al., 1985). However, it is not clear whether the neuronal elements (e.g., astroglia, neurons, etc.) are also involved in $O_2 \cdot^-$ formation in these studies.

We have reported previously that 20:4 or PUFAs induced $O_2 \cdot^-$ formation and lipid peroxidation and increased cellular swelling and lactate production in rat brain slices (Chan and Fishman, 1980; Chan et al., 1983*a*). The increase in lactic acid content may cause an increase in hydrogen ion levels and a decrease in cellular pH (acidic pH), a condition known to generate a protonated form of $O_2 \cdot^-$ ($\cdot OOH$) and lipid peroxidation (Siesjo et al., 1985). However, exogenously supplied SOD or catalase was not effective in reducing the level of $O_2 \cdot^-$ or in ameliorating cellular swelling and lactic acid levels in brain slices (Chan and Fishman, 1978, 1980). On the other hand, exogenously added SOD effectively reduced the level of $O_2 \cdot^-$ in brain slice homogenates incubated with 20:4. These data indicate that $O_2 \cdot^-$ is formed intracellularly and the exogenously added SOD is unable to reach the intracellular targets in intact tissue for their scavenging action.

The present studies are designed to investigate the effects of 20:4 and PUFAs on both oxidative and metabolic alterations in intact cultured astrocytes. The aims of these studies are severalfold: first, attempts are made to confirm the inductive effects of 20:4 and PUFAs on the formation of $O_2 \cdot^-$, lipid peroxidation, and lactic acid production in cultured astrocytes; second, mechanisms underlying $O_2 \cdot^-$ formation in these cultured cells are investigated using radical scavengers and various enzyme inhibitors; and third, we attempt to study the possible beneficial effects of liposome-entrapped SOD in reducing or ameliorating oxidative and metabolic perturbation in 20:4-incubated cultured astrocytes.

MATERIALS AND METHODS

Astrocyte cultures

Primary cultures of cerebral cortical astrocytes were prepared from newborn Sprague–Dawley rats (Simonsen, Gilroy, CA, U.S.A.) as described previously (Yu et al., 1982, 1986). Cerebral hemispheres were removed aseptically from the skulls and freed of the meninges. The neopallia were removed and cut into small cubes (1 mm^3) in a modified Eagle's minimum essential tissue culture medium (MEM) (Hertz et al., 1985) containing fetal calf serum (FCS) (Sterile System, Logan, UT, U.S.A.). The tissue was disrupted by vortex-mixing for 1 min, and the suspension was passed through two sterile nylon Nitex sieves (L. and S. H. Thompson, Montreal, P.Q., Canada) with pore sizes of 80 μm (first sieving) and 10 μm (second sieving). A volume of cell suspension equivalent to one-thirtieth of brain was placed in a 60-mm Falcon tissue dish (Becton Dickinson, Oxnard, CA, U.S.A.). 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 confluency and were grown in the additional presence of 0.25 m*M* dibutyryl cyclic AMP (Sigma, St. Louis, MO, U.S.A.). The cultures were used for experiments between the ages of 28 and 35 days in vitro.

Determinations of NBT reduction, $O_2 \cdot^-$, SOD, lipid peroxidation, and lactic acid

Formation of $O_2 \cdot^-$ was measured based on the SOD-inhibitable reduction of water-soluble, yellowish-colored NBT to water-insoluble, purple-colored nitroblue formazan (NBF) (Chan and Fishman, 1980; Wei et al., 1981). Astrocytic cultures (28–35 days in vitro) were first washed three times with 1 ml of warm Hanks' balanced salt solution (HBSS) (37°C). Broken cells were prepared by scraping and homogenizing the cells for 10 strokes with a homogenizer (0.25 mm clearance). Both intact and broken cells were incubated with 2 ml of HBSS containing 1.0 m*M* NBT (Sigma) plus free fatty acids and CuZn-SOD (100 units/dish) at 37°C. 20:4 or various fatty acids in the form of a sodium salt (Sigma), except 22:6 which is in a free acid form, were first dissolved in ethanol (200 proof; Gold Shield Chemical Co., Hayward, CA, U.S.A.), and the fatty acid solution was then added directly to the tissue culture medium with a final 0.3% ethanol. Identical concentrations of ethanol were always added to the control incubation medium. At the end of the incubation period, medium was removed and intact cells were washed three times with ice-cold HBSS to wash out excess soluble NBT. Cells were scraped off the plates into a test tube, and NBF was extracted with 2 ml of *N,N*-dimethyl formamide in a boiling water bath for 10 min. The pellets were centrifuged at 1,000 *g* and reextracted with 2 ml of the same solvent. In the case of broken cells, the medium was removed by centrifugation and the pellets were washed and then used for NBF assay. The supernatants were read at 515 nm. $O_2 \cdot^-$ formation is defined by the SOD-inhibitable portion of NBT reduction in broken cells. Nitroblue diformazan (Sigma) dissolved in dimethyl formamide with concentrations of 1–5 mg/ml was used for standard calibration.

SOD activity in cell homogenates was assayed by its ability to inhibit $O_2 \cdot^-$-dependent reactions (Crapo et al., 1978; Chan et al., 1987). The assay medium (phosphate-EDTA buffer, pH 7.8) contained 30 μmol cytochrome *c*, 150 μmol xanthine, and cell homogenates, with a final volume of 3 ml. The reaction was initiated by adding 10 μl of xanthine oxidase, and the rate (0.020 per min) in absorbance at 550 nm was recorded. One unit of SOD is defined as the amount of SOD required to inhibit 50% of cytochrome *c* reduction. The astrocytes contain both cytosolic CuZn-SOD and mitochondrial Mn-SOD. For the assay of total SOD activity, 10 μM potassium cyanide was added to the

incubation medium to inhibit cytochrome *c* oxidase activity. For the assay of Mn-SOD activity, 1 m*M* potassium cyanide was added to the incubation mixture to inhibit CuZn-SOD activity. The activity of CuZn-SOD was derived by the subtraction of the Mn-SOD activity from the total SOD activity.

The determination of lipid peroxidation in cultured cell homogenates was based on the reaction of malondialdehyde (MDA), the end product of lipid peroxidation, with 2-thiobarbituric acid to form a pink-colored substance (Dahle et al., 1962). Cultured cells from two 60-mm dishes were combined and homogenized in HBSS, and an aliquot of the cell homogenate was used for MDA assay based on previous methods for brain slice homogenates and tumor cell lines (Chan and Fishman, 1980, 1982).

Lactic acid assay consisted of an aliquot of 0.25 ml of the incubation medium, 1.3 mg β-NAD, and 13.1 units of lactate dehydrogenase (Sigma) in glycine-hydrazine buffer, pH 9.2, with a final volume of 1.5 ml.

Preparation of liposomes and liposome-entrapped CuZn-SOD

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 (1978). The lipids contain L-α-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 m*M*), and then sonicated for 5 min at 45°C (bath type; Branson Instruments, St. Louis, MO, U.S.A.). 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 CuZn-SOD basically is the same as that for the liposomes (Turrens et al., 1984; Chan et al., 1987). Electrophoretically purified CuZn-SOD, bovine blood (20,000 units/mg; Pharmacia, Uppsala, Sweden) or human CuZn-SOD produced by a genetic engineering method (2,000 units/mg; Chiron Co., Emeryville, CA, U.S.A.) at a concentration of 2×10^5 units was first dissolved in 0.4 m*M* phosphate buffer and then added to the lipid film (400 μmol phospholipids) before sonication. The yield of liposome-entrapped CuZn-SOD ranged from 25 to 40% and had a value of 2×10^2 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.

Liposome-entrapped ^{125}I-CuZn-SOD was prepared similarly as described. CuZn-SOD (1 mg/ml) was iodinated with 250 μCi/ml Bolton–Hunter reagent (NaI) in glycine-phosphate buffer at pH 7.4. The free label iodine (^{125}I) and ^{125}I-SOD were separated by Sephadex G-25 (coarse). The specific activity of the ^{125}I-SOD was 3×10^9 dpm/mg SOD. The purified ^{125}I-SOD was redissolved in phosphate buffer, and the liposome-entrapped ^{125}I-SOD was prepared according to the method described above. An aliquot of 5.5 $\times 10^4$ dpm of ^{125}I-SOD in 3 ml MEM was used for uptake studies. Fluorescein isothiocyanate (FITC) SOD was prepared by adding 5 ml of FITC (1.5 mg) to an equal volume of SOD solution (50 mg/ml in 0.6 *M* sodium phosphate buffer), and the solution was allowed to stand overnight. The FITC-SOD was separated from the unreacted dye by Sephadex G-50 (fine). The concentration of FITC bound to SOD was determined by measuring the optical density at 490 nm.

The FITC-SOD preparation was then dialyzed with borate buffer and subsequently with bicarbonate buffer prior to lyophilization. The lyophilized FITC-SOD was entrapped by liposomes according to the method described above.

RESULTS

NBT reduction, $O_2 \cdot^-$, lipid peroxidation, and lactate production

The rate of NBT reduction was 0.092 ± 0.006 and 0.65 ± 0.10 nmol/min/mg protein in broken cells and intact cells, respectively (Table 1). Less than 10% of NBT reduction was inhibitable by SOD in broken cells, whereas a negligible amount of SOD-inhibitable NBT reduction was observed in intact cells. 20:4 (0.1 m*M*) stimulated NBT reduction significantly in both broken and intact cells. About 26% of SOD-inhibitable NBT reduction ($O_2 \cdot^-$ content) with a rate of 0.042 nmol/mg protein/min was observed in broken cells, whereas negligible inhibition of NBT reduction by SOD was detected in intact cells.

Figure 1 shows the time- and dose-dependent effects of 20:4 on the NBT reduction in intact cultured astrocytes. The formation of NBF in control astrocytes increased gradually with time. 20:4 at concentrations of 0.1 and 0.5 m*M* significantly increased the rate of NBF formation to 1.33 ± 0.1 and to 1.93 ± 0.2 nmol/min/mg protein, respectively, in astrocytes. The levels of NBF increased linearly within the 30 min of incubation with 20:4 and reached a plateau at 60 min. The effects of unsaturation of free fatty acids on NBT reduction in cultured astrocytes were studied further. Figure 2 shows that 22:6, linolenic acid (18:3), and linoleic acid (18:2) increased significantly the level of NBT reduction in intact cultured astro-

TABLE 1. *Stimulation of NBT reduction by 20:4 in broken cell homogenates and intact cells of primary astrocytes*

	NBF (nmol/min/mg protein)	
Incubation condition	Broken cells	Intact cells
Control	0.092 ± 0.006	0.65 ± 0.10
+ SOD	0.082 ± 0.002	0.71 ± 0.08
SOD-inhibitable	0.01	<0
20:4	0.162 ± 0.009[a]	1.43 ± 0.15[a]
+ SOD	0.120 ± 0.002[b]	1.52 ± 0.03
SOD-inhibitable	0.042	<0

Cultured astrocyte homogenates or intact cells were incubated with 20:4 (0.1 m*M*) in the absence or presence of SOD (100 units/dish). The SOD-inhibitable NBF formation represents the rate of $O_2 \cdot^-$ formation in these preparations. Experiments are averaged from triplicate assays obtained from two different batches of astrocytes. Values are means ± SD.

[a] $p < 0.01$, compared to control.

[b] $p < 0.05$, compared to 20:4-treated cells.

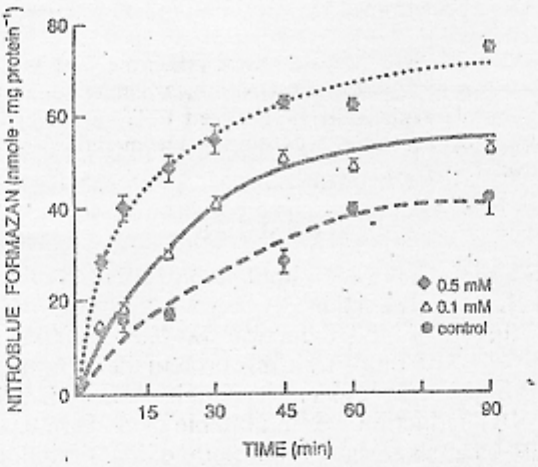

FIG. 1. Stimulation of NBF formation by 20:4 in intact cultured astrocytes. Cultured astrocytes were incubated with 1.0 m*M* NBT in the presence of 20:4 (0.1 m*M* or 0.5 m*M*) for various times. The extracted NBF was read at 515 nm. Results are means of four different experiments with duplication assays for each experiment. Vertical bars indicate SD.

cytes. 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 monounsaturated oleic acid (18:1) were not effective.

The stimulating effects of PUFAs on the level of MDA in astrocytes are shown in Fig. 3. 18:3, 20:4, and 22:6 at 0.1 m*M* concentration increased the formation of MDA by 70%, 100%, and 95%, respectively.

Figure 4 shows the effects of various fatty acids on the lactic acid production in intact astrocytes. 20:4, 18:3, and 18:2 at 0.1 m*M* caused increases in lactate

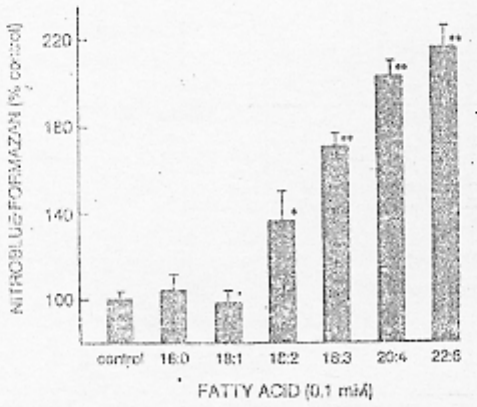

FIG. 2. Effects of free fatty acids on NBF formation in intact cultured astrocytes. Astrocytes were incubated with NBT in the presence of fatty acid (0.1 m*M*) for 10 min at 37°C. Results are expressed as percentage of the rate of NBF formation of the control ± SD. The control rate of NBF formation in astrocytes is 0.61 ± 0.02 nmol/min/mg protein. Results are averages of three experiments. *$p < 0.01$, **$p < 0.001$, using Student's *t* test for statistical analysis.

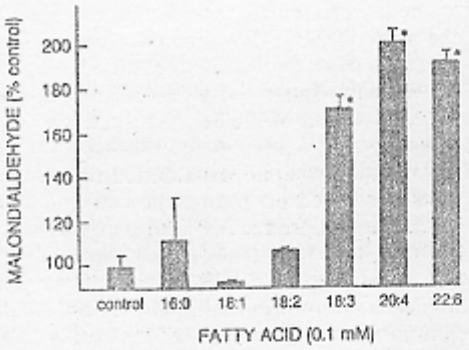

FIG. 3. Effects of free fatty acids (0.1 m*M*) on MDA formation in cultured astrocytes. Astrocytes were incubated with thiobarbituric acid and free fatty acid for 1 h at 37°C. Results are expressed as percentage of rate of MDA formation of the control ± SD. The control rate of MDA formation is 1.78 ± 0.06 nmol/mg protein/h. Results are averaged from four different experiments. *$p < 0.01$, compared to control, using Student's *t* test for statistical analysis.

production of 240%, 160%, and 60%, respectively, in incubation medium. Both 16:0 and 18:1 were ineffective. The increases in lactic acid production by 20:4 were dose-dependent (Fig. 5).

The mechanisms underlying 20:4-induced NBT reduction (or NBF formation) in cultured astrocytes were investigated (Table 2). Various free radical scavengers, including tryptophan, histidine, thiourea, vitamin E, catalase, glutathionine peroxidase, and SOD, and other enzyme inhibitors, including allopurinol, indomethacin, monoamine oxidase A and B inhibitors, pargyline, imipramine, and *trans*-2-phenylcyclopropylamine, were not effective in ameliorating NBT reduction. However, NADH or NADPH stimulated the 20:4-induced NBF formation by 40% and 33%, respectively. 20:4 alone (without astrocytes) did not induce NBF formation.

The increased formation of NBF in astrocytes prompted our studies on the possible inhibitory effect of 20:4 on SOD activity. Table 3 shows that normal

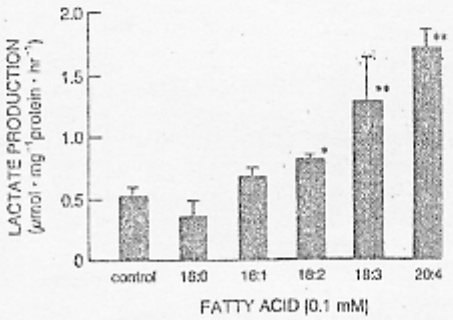

FIG. 4. Effects of free fatty acids on lactic acid production in astrocytes. Cultured astrocytes were incubated with individual fatty acid (0.1 m*M*) for 1 h at 37°C. The incubation medium was used to assay for the lactic acid content. Values are means ± SD of four experiments. *$p < 0.05$, **$p < 0.001$, compared to control, using Student's *t* test for statistical analysis.

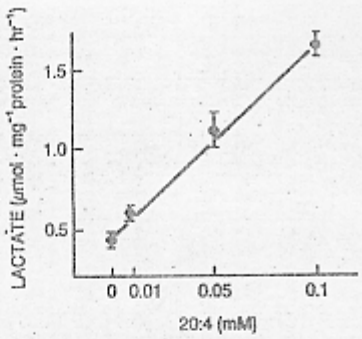

FIG. 5. Dose-dependent curve of lactate production in astrocytes induced by 20:4. Astrocytes were incubated with various concentrations of 20:4 for 1 h at 37°C. Cultured medium was used to assay for lactate content. Values are means ± SD of three experiments.

cultured astrocytes have a higher activity of cytosolic CuZn-SOD (3.90 ± 1.12) than mitochondrial Mn-SOD (1.30 ± 0.42). Incubation with 20:4 (0.1 m*M*) for 1 h slightly, although not significantly, decreased the CuZn-SOD activity by 18%, whereas Mn-SOD activity was not affected by 20:4.

Uptake of liposome-entrapped SOD into cultured astrocytes

The uptake of free ^{125}I-SOD and liposome-entrapped ^{125}I-SOD in astrocytes is shown in Fig. 6. The uptake of liposome-entrapped ^{125}I-SOD was time-dependent. More than 70% of radioactivity was associated with the extensively washed astrocytes at 90 min following the incubation. Free ^{125}I-SOD activity was not associated with astrocytes. Similar uptake studies were performed in astrocytes with free FITC-conjugated SOD and liposome-entrapped FITC-conjugated SOD in cultured astrocytes (Fig. 7). Figure 7B shows the uptake of fluorescent SOD at 6 h following the incubation of cells with liposome-entrapped FITC-conjugated SOD. However, there was a lack of significant uptake of fluorescent SOD in astrocytes when the free FITC-conjugated SOD was used (Fig. 7D).

TABLE 2. *Effects of antioxidants and enzyme inhibitors on 20:4-induced $O_2\cdot^-$ formation in astrocytes*

Incubation medium	Concentration (m*M*)	NBF content (% control)
20:4	0.1	100 ± 6.0
+ Indomethacin	1	90 ± 7.0
+ Allopurinol	1	104 ± 7.6
+ Tryptophan	5	101 ± 5.5
+ Histidine	5	114 ± 4.5
+ Thiourea	5	99 ± 5.0
+ Vitamin E	0.1	102 ± 5.0
+ SOD	(100 units/dish)	94 ± 6.5
+ Catalase	(100 units/dish)	100 ± 6.0
+ Glutathione peroxidase	(100 units/dish)	101 ± 5.0
+ Pargyline	0.1	96 ± 8.0
+ Imipramine	0.1	91 ± 2.0
+ *trans*-2-Phenylcyclopropylamine	0.1	90 ± 5.0
+ NADPH	0.1	133 ± 5.3[a]
+ NADH	0.1	140 ± 2.8[a]
20:4 alone (without cells)	0.1	0

Cultured astrocytes were incubated with various compounds in the presence of 20:4 for 30 min. Experiments are averaged from triplicate assays obtained from two different batches of astrocytes. The rate of NBF formation in 20:4-incubated astrocytes was 0.65 ± 0.02 nmol/min/mg protein. Values are the means ± SD.
[a] $p < 0.01$, Student's test.

TABLE 3. *Effects of 20:4 on SOD activities of astrocytes*

Medium	CuZn-SOD	Mn-SOD	Total
Control	3.9 ± 1.2	1.3 ± 0.4	5.1 ± 0.8
20:4	3.2 ± 0.4	1.3 ± 0.1	4.5 ± 0.8

Astrocytes were incubated with 20:4 (0.1 m*M*) or MEM medium for 1 h at 37°C. Results are averaged from three different experiments. Values are means ± SD in units per milligram of protein.

Effects of liposome-entrapped SOD on $O_2\cdot^-$ and on lactic acid production

Table 4 shows that when cultured astrocytes were incubated with liposome-entrapped SOD (100 units/ml and 2,000 units/ml, respectively), 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 lactate content in the incubation medium.

DISCUSSION

The present studies have demonstrated clearly that 20:4 caused both oxidative and metabolic perturba-

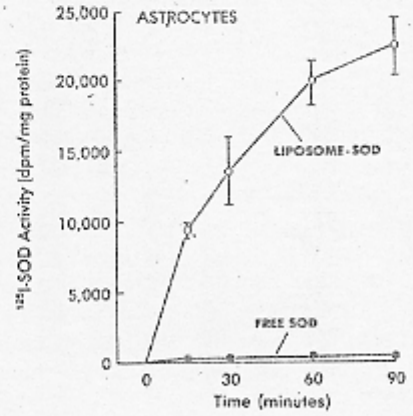

FIG. 6. Uptake of free ^{125}I-CuZn-SOD and liposome-entrapped ^{125}I-CuZn-SOD into astrocytes. Cultured astrocytes were incubated with 55,000 dpm/dish for both free ^{125}I-CuZn-SOD and liposome-entrapped ^{125}I-CuZn-SOD for various times at 37°C. After the incubation, astrocytes were washed three times with saline phosphate buffer, and the cells were solubilized with NaOH and counted for radioactivity. Results are averaged from triplicate assays from two different patches of cells. Values are means ± SD.

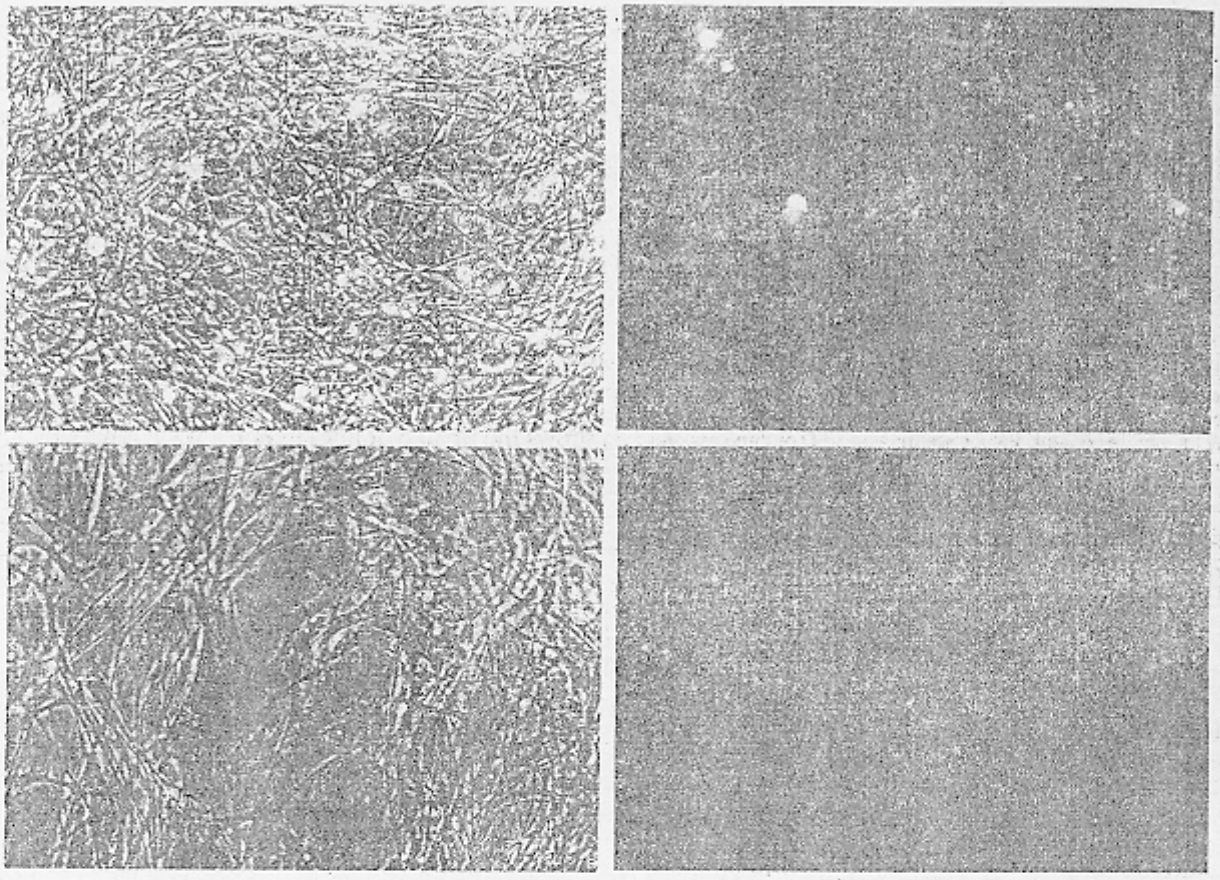

FIG. 7. Uptake of free FITC-CuZn-SOD and liposome-entrapped FITC-CuZn-SOD into astrocytes. Cultured astrocytes were incubated with 100 units of either free FITC-CuZn-SOD or liposome-entrapped FITC-CuZn-SOD for 6 h at 37°C. A: Phase-contrast photograph of astrocytes. Cells were incubated with liposome-entrapped FITC-CuZn-SOD. B: Fluorescence photograph for the same field of cells as shown in A. Note the uptake of FITC-SOD into astrocytes and some fluorescence distribution in astrocytic processings. C: Phase-contrast photograph obtained with FITC-CuZn-SOD–incubated astrocytes. D: Fluorescence photograph obtained from same field of cells shown in C. Note the limited uptake of FITC-CuZn-SOD into astrocytes. (Magnification ×144).

tion in primary cell cultures of astrocytes. The rate of NBF formation, induced by 20:4 in intact cells, is both time- and dose-dependent (Fig. 1). Although the NBF formation assay is commonly used for measuring $O_2 \cdot^-$, only the SOD-inhibitable portion of NBT reduction (or NBF formation) is $O_2 \cdot^-$-specific (Kontos et al., 1985). Thus, in broken cell preparations, exogenously added SOD inhibited 20:4-induced NBT reduction by 26%, indicating this portion of NBT reduction is $O_2 \cdot^-$-specific (Table 1). However, the rate of NBF formation in broken cells is significantly lower than that in intact cells. These data suggest that the structural integrity of cell membranes and subcellular mitochondrial membranes may account for the lower level of NBF formation in broken cells. On the other hand, there was no SOD-inhibitable portion of NBT reduction in intact astrocytes. However, these data do not indicate the absence of $O_2 \cdot^-$-specific NBT reduction. Rather, they suggest that the exogenously added SOD was not able to penetrate the intact cell membrane and react with intracellular $O_2 \cdot^-$.

Our data further show that the stimulation of NBF formation is not specific for 20:4, because other PUFAs, including 18:2, 18:3, and 22:6, were also effective (Fig. 2). On the other hand, saturated 16:0 and monounsaturated 18:1 were not effective. These data indicate that PUFAs induce oxidative perturbations in cultured astrocytes and support our early observations in brain slices (Chan and Fishman, 1980). The oxidative stress in astrocytes induced by PUFAs was demonstrated further by the measurement of lipid peroxidation. Our data have shown that fatty acids which have three or more double bonds (18:3, 20:4, and 22:6) significantly increased the MDA content in astrocytes (Fig. 3), whereas 18:2 was not effective. The observation of the ineffectiveness of 18:2 on MDA formation is not surprising, because 18:2 also has a much lesser effect on both NBF levels ($O_2 \cdot^-$ formation) and lactate production than other PUFAs

TABLE 4. *Effects of liposome-entrapped CuZn-SOD on 20:4-induced $O_2 \cdot^-$ formation and lactate production in astrocytes*

Incubation medium	NBF (% control)	Lactate (% control)
Control	100 ± 16	100 ± 11
20:4	199 ± 15[a]	183 ± 4[a]
+ Empty liposomes	180 ± 17	177 ± 10
+ Free SOD (100 units/dish)	185 ± 13	190 ± 15
+ Liposome-SOD (100 units/dish)	93 ± 9[b]	92 ± 9[b]
+ Liposome-SOD (2,000 units/dish)	90 ± 10[b]	20 ± 3[b]

Astrocytes were preincubated with empty liposomes, free SOD, or liposome-entrapped SOD in MEM (serum-free) for 24 h prior to the addition of 20:4 (0.1 m*M*) for another hour. Cell pellets were used for NBF assay, whereas incubation medium was assayed for lactate content. The control values of NBF and lactate were 54.3 ± 10.4 nmol/mg protein/h and 0.35 ± 0.04 μmol/mg protein/h, respectively. Values are the means ± SD of three different experiments.

[a] $p < 0.01$, compared to control group; [b] $p < 0.01$, compared to 20:4 group, using analysis of variance.

(Figs. 2 and 3). Further studies on diene conjugation for early peroxidation may provide information to discern this difference. Nevertheless, these data confirm our previous findings that 20:4 and PUFAs induce lipid peroxidation in intact C-6 glioma cells and in brain slice preparations (Chan and Fishman, 1980, 1982). Furthermore, the $O_2 \cdot^-$ formation and subsequent lipid peroxidation may cause the membrane perturbation. We have demonstrated earlier that the initial rate of uptake of glutamate in astrocytes was inhibited severely with 20:4 (concentrations ranged from 0.025 to 0.1 m*M*) (Yu et al., 1986). We have suggested that the perturbation of membrane integrity induced by 20:4 may be responsible for its inhibitory effect on glutamate uptake. Furthermore, our preliminary data also show that 20:4 severely inhibits the Na^+,K^+-ATPase activity in astrocytes (unpublished observations), further indicating the membrane perturbation induced by 20:4. It is likely that increased levels of $O_2 \cdot^-$ and lipid peroxides are involved in these membrane injuries.

It has been shown previously that 20:4 and other PUFAs, but not saturated and monounsaturated fatty acids, cause a significant increase in lactic acid production in brain slices and in tumor cell lines, including rat C-6 glioma and mouse N18-TG-2 neuroblastoma cells (Chan and Fishman, 1980, 1982; Chan et al., 1983*a*). Similar findings are observed in the present studies, because PUFAs, including 20:4, cause a 50–300% increase in lactic acid production in primary cultures of astrocytes (Fig. 4). The mechanisms underlying such a 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 20:4 and/or by its oxygen radical intermediates. Second, the normal mitochondrial respiratory activities and metabolic function are affected by 20:4 and/or by oxygen radicals. For example, the enzymes of the mitochondrial tricarboxylic acid (TCA) cycle which are responsible for pyruvate flux and oxidation may be affected by 20:4. It is well known that free fatty acids, particularly PUFAs, are potent uncouplers of mitochondrial phosphorylation. Our preliminary studies have found that respiration was inhibited in isolated brain mitochondria with a relatively low concentration of 20:4 (13 nmol/mg mitochondrial protein) (Hillered and Chan, 1987), suggesting that the normal mitochondrial function is altered by 20:4. On the other hand, oxygen radicals generated enzymatically, also inhibit respiratory activities in isolated brain mitochondria (Hillered and Ernster, 1983). Thus, the inhibitory effects of 20:4 on normal mitochondrial function may account for the increase of lactic acid production in astrocytes.

Siesjo and his colleagues (1985) studied the influence of acidosis on $O_2 \cdot^-$ formation and lipid peroxidation, and have demonstrated that acidic pH in the range of 6.0–6.5 significantly induced free radical formation and lipid peroxidation in brain homogenates. These investigators proposed that the effect of acidosis on brain homogenates may involve increased formation of the protonated form of $O_2 \cdot^-$. Our data have shown that 20:4 caused a dose-dependent increase in lactic acid content in astrocytes. Because the increase in lactic acid content will lower the pH and create acidosis, the nature of $O_2 \cdot^-$ induced by 20:4 may be a hydroperoxyl radical ($\cdot OOH$), as suggested by Siesjo et al. (1985).

The mechanisms underlying the 20:4-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 (Table 2). Egan et al. (1981) found that the electron paramagnetic resonance signal detected during metabolism of arachidonate or prostaglandin G_2 by microsomal fractions and oxygenase was due to the oxidation of adventitious material which was isolated together with microsomal fractions. Their data suggest that the hydroperoxidase, rather than the oxygenase of cyclooxygenase, can release a reactive oxidant $[O_x] \cdot$ into solution to initiate a radical chain. On the other hand, addition of NADH or NADPH stimulates the NBF production in cultured astrocytes. These data again confirm our previous observation that NADPH-dependent, 20:4-stimulated oxidases (peroxidases) are involved in $O_2 \cdot^-$ formation in astrocytes (Chan and Fishman, 1980). Recently, Kukreja et al. (1986) identified the mechanism by which arachidonate and linoleate metabolism via prostaglandin H synthase produced $O_2 \cdot^-$. This

mechanism involves the oxygenation of NADH or NADPH to the radicals of NAD or NADP which then react with oxygen to produce $O_2 \cdot^-$. 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 ischemic brain may provide a suitable environment favoring the formation of $O_2 \cdot^-$.

The inability of free exogenous antioxidative enzymes, including SOD, to inhibit NBT reduction may suggest that $O_2 \cdot^-$ are formed intracellularly. Our strategy for this problem has been to employ liposomes to deliver the SOD intracellularly and to scavenge these $O_2 \cdot^-$. We have shown recently that intravenous injection of liposome-entrapped SOD effectively ameliorates the $O_2 \cdot^-$ formation, blood-brain barrier permeability changes, and vasogenic brain edema in cold-injured rat brain (Chan et al., 1987). We have suggested that endothelial cells may take up the liposomes and the encapsulated SOD. These antioxidative enzymes may then interrupt the 20:4 cascade by scavenging the $O_2 \cdot^-$ intermediates. The present studies further support the usefulness of the liposome-entrapped SOD in reducing intracellularly formed $O_2 \cdot^-$ in astrocytes. Furthermore, the uptake of FITC fluorescence and the distribution of fluorescence in astrocytic processes (Fig. 7B) indicate the endocytosis of liposome-entrapped SOD and subsequent internalization of these compounds. However, the fate of the liposomes and the internalized SOD is not clear at present and is a subject of further investigation.

20:4, once taken up by the cells, is localized primarily in the fluid domains of the membranes with preferential incorporation into endoplasmic reticulum or plasma and mitochondrial membranes (Klausner et al., 1980; Neufeld et al., 1985). These studies, together with the fact that the hydrophobic lipid environment is preferable for $O_2 \cdot^-$ and its protonated form ($\cdot OOH$) (Pryor, 1976; Fridovich, 1986), suggest that $O_2 \cdot^-$ or $\cdot OOH$ may be formed or localized in membrane fluid domains. Further studies of $O_2 \cdot^-$ distribution using a cytochemical approach (Briggs et al., 1986), as well as morphological and immunocytochemical studies of the distribution of encapsulated SOD in astrocytes, may provide additional information to strengthen this argument. Nevertheless, present studies demonstrate the beneficial effects of liposome-entrapped antioxidative enzymes in ameliorating $O_2 \cdot^-$ formation and lactate production in astrocytes. Liposome-entrapped antioxidative enzymes have been shown effective in ameliorating CNS $O_2 \cdot^-$ toxicity in vivo (Yusa et al., 1984) and preventing oxygen injury in cultured endothelial cells in vitro (Freeman et al., 1983). 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 (Halks-Miller et al., 1986). Therefore, the present studies provide support for further study of the therapeutic potential of these liposome-entrapped antioxidative enzymes in CNS injury.

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