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Transient Formation of Superoxide Radicals in Polyunsaturated Fatty Acid-Induced Brain Swelling

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Abstract: The involvement of superoxide free radicals and lipid peroxidation in brain swelling induced by free fatty acids has been studied in brain slices and homogenates. The polyunsaturated fatty acids linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4), and docosahexaenoic acid (22:6) caused brain swelling concomitant with increases in superoxide and membrane lipid peroxidation. Palmitic acid (16:0) and oleic acid (18:1) had no such effect. Furthermore, superoxide formation was stimulated by NADPH and scavenged by the addition of exogenous superoxide dismutase in cortical slice homogenates. These *in vitro* data support the hypothesis that both superoxide radicals and lipid peroxidation are involved in the mechanism of polyunsaturated fatty acid-induced brain edema. **Key words:** Superoxide free radicals—Polyunsaturated fatty acids—Brain edema. **Chan P. H. and Fishman R. A.** Transient formation of superoxide radicals in polyunsaturated fatty acid-induced brain swelling. *J. Neurochem.* **35**, 1004–1007 (1980).

We have previously demonstrated that polyunsaturated fatty acids (PUFA) are active in the induction of brain edema, both *in vitro* and *in vivo* (Chan and Fishman, 1978; Prioleau et al., 1979). These edematous rat brain tissues were characterized by increases in water and sodium content. In the *in vitro* model, using cortical brain slices, a decreased extracellular space was observed, indicative of cellular edema. The *in vivo* rat model showed an increased ^{125}I-albumin space, indicative of vasogenic brain edema. The *in vivo* and *in vitro* studies both showed decreased intracellular K^+ levels. Thus, PUFA altered the integrity of brain capillary function *in vivo* and of brain cell membranes *in vitro*. The mechanism of action of PUFA on the formation of brain edema in both models is still obscure, although there is ample evidence suggesting that the formation of lipid peroxide intermediates and free radicals from free arachidonic acid are involved in the inflammatory process associated with polymorphonuclear granulocytes (Higgs et al., 1979; Kuehl et al., 1979). It is well known that superoxide ($O_2^{\cdot -}$) and other radical species may be formed either by a chemical reaction of radical displacement or by the NADPH-dependent oxidase system (Mead, 1976; Gabig et al., 1978; McPhail et al., 1976). These free radicals, particularly $O_2^{\cdot -}$, are known to be directly involved in membrane lipid peroxidation and in the pathological process involving ischemic cell injury in brain, and in carrageenan-induced foot edema (Demopoulos et al., 1979; McCord et al, 1979; Vinegar et al., 1969). Therefore, the possible involvement of $O_2^{\cdot -}$ formation and lipid peroxidation in PUFA-induced brain swelling has been studied further. Our data indicate that the transient formation of $O_2^{\cdot -}$ is involved in the brain swelling induced by PUFA.

MATERIALS AND METHODS

Sprague-Dawley male rats (purchased from Simonsen, Gilroy, California) weighing 100 to 200 g were used. The preparation of single, first cortical slices was described previously (Chan et al., 1978). The initial wet weight of

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Abbreviations used: PUFA, Polyunsaturated fatty acid; MDA, Malondialdehyde; $O_2^{\cdot -}$, Superoxide radical; NBT, Nitroblue tetrazolium; NBF, Nitroblue formazan; Ara, Arachidonic acid; SOD, Superoxide dismutase.

each slice was measured with a Precision Balance (Federal Pacific Electric Co., Northboro, Massachusetts) and each slice was then incubated in 5 ml Krebs-Ringer buffer (K-R) for 20 min at 37°C for reconstitution. Slices were then transferred to the K-R containing the experimental compounds at 37°C for 40 min in an Aquatherm water shaker. After incubation, each slice was lightly blotted on an acid-washed glass Petri dish to remove excess water adhering to the slice and the final wet weight was measured. The tissue swelling (%) was calculated as (Chan et al., 1979):

$$\frac{\text{final wet weight} - \text{initial wet weight}}{\text{initial wet weight}} \times 100$$

After incubation, individual slices were transferred to a ground glass homogenizer containing 0.5 ml K-R and homogenized with 10 strokes by hand. Another 0.5 ml K-R sample was used to rinse the homogenizer. Formation of $O_2^{\cdot-}$ was measured based on the reduction of nitroblue tetrazolium (NBT) to form a purple, insoluble nitroblue formazan (NBF) according to the methods of Rajagopalan and Handler (1964), and Baehner and Nathan (1968). This reaction is inhibited by superoxide dismutase (SOD, EC 1.15.1.1). A sample of 0.4 ml of freshly prepared NBT (1 mg/ml in K-R) was added to tissue homogenates and the mixture was vortexed and then incubated at 37°C for 30 min with vigorous agitation of the surface of the solution by water-saturated 100% oxygen blown through hypodermic needles. The reaction time (30 min) and the NBT concentration (1 mg/ml) were chosen to assure that the rate of NBT reduction was linear. A sample of 30 μg/ml of SOD (bovine blood) was routinely added to the reaction mixture to scavenge the $O_2^{\cdot-}$. The rate of NBT reduction was not affected in control cortical slices by the addition of SOD. The reaction mixture was centrifuged at 10,000 r.p.m. for 10 min and the pellet was extracted with 2 ml of pyridine at 100°C for 1 h and then centrifuged again at 10,000 r.p.m. for 10 min. The supernatant solutions were read at 515 nm. Freshly prepared NBF at concentrations ranging from 5 mg/ml to 40 mg/ml was used for calibration.

For the studies of NADPH-dependent $O_2^{\cdot-}$ formation and the time-course studies, the assay conditions were modified as follows: After the 20 min reconstitution, tissue homogenates (0.5 ml) obtained from cortical slices were directly added into a reaction mixture containing 0.2 μmol NADPH, 0.2 μmol NaN_3, and 0.4 ml of NBT at a concentration of 0.5 mg/ml. Arachidonic acid (Ara) (0.5 mM) and/or SOD (30 μg/ml) was added directly into the assay medium. The final volume of reaction mixture was 2 ml. The NBF extraction and determination procedures were exactly the same as described above.

The determination of lipid peroxidation in tissue homogenates was based on the reaction of malondialdehyde (MDA), the end product of lipid peroxidation, with 2-thiobarbituric acid (TBA) to form a pink-colored substance (Dahle et al., 1962). The MDA assay was composed of 0.5 ml cortical slice homogenate, 1.2 ml of 10% trichloroacetic acid, and 0.5 ml of 0.5% TBA. The reaction mixture was vigorously vortexed, and then heated for 15 min at 100°C. Pink-colored supernatant was obtained by centrifuging the sample at 12,000 r.p.m. for 5 min. The MDA was read at 532 nm. MDA was estimated by using a standard curve of 1,1,3,3,-tetraethoxyl propane, according to Dahle et al. (1962). 1,1,3,3-Tetraethoxyl propane was obtained from Mallinckrodt, Paris, Kentucky; all other chemicals were purchased from Sigma, St. Louis, Missouri.

RESULTS

The effects of free fatty acids on brain swelling and $O_2^{\cdot-}$ formation are shown in Table 1. Sodium palmitate (16:0) and sodium oleate (18:1) did not have an effect on either tissue swelling or NBT reduction. However, sodium linoleate (18:2), sodium linolenate (18:3), and sodium arachidonate (20:4), each being a PUFA, stimulated tissue swelling twofold and NBF formation by 33 to 48%. These PUFA-induced increases in NBF formation were completely inhibited by the addition of SOD (30 μg/ml) in the reaction mixture, further evidence of the presence of $O_2^{\cdot-}$. Table 2 shows that NBF formation in cortical slice homogenates induced by

TABLE 1. *Effect of polyunsaturated fatty acids on brain swelling and superoxide formation*

Medium	Tissue swelling (%)	$O_2^{\cdot-}$ formation (nmol NBF·mg protein^{-1}·30 min^{-1})
Krebs-Ringer	11.3 ± 1.0 (26)	9.7 ± 0.6 (13)
Sodium palmitate (16:0)	13.0 ± 1.9 (5)	10.1 ± 0.5 (5)
Sodium oleate (18:1)	10.5 ± 2.1 (8)	9.0 ± 1.2 (4)
Sodium linoleate (18:2)	25.1 ± 1.1 (4)[a]	13.1 ± 1.3 (4)[a]
Sodium linolenate (18:3)	16.4 ± 2.0 (9)[a]	14.4 ± 0.9 (9)[a]
Sodium arachidonate (20:4)	23.2 ± 1.5 (12)[a]	12.9 ± 0.7 (12)[a]

Mean ± S.E. Fatty acid concentration, 0.5 mM. (n) = number of cortical slices. $O_2^{\cdot-}$ assay was performed in duplicate for each cortical slice. Addition of superoxide dismutase (30 μg/ml) in the reaction medium completely inhibited the increases of NBF in tissue homogenates due to the incubation of PUFA.

[a] $P < 0.001$.

TABLE 2. *Effects of arachidonic acid on NADPH-dependent $O_2^{\cdot-}$ formation in cortical slice homogenates*

Assay medium		$O_2^{\cdot-}$ formation (nmol NBF·mg protein^{-1}·30 min^{-1})
K-R	(14)	27.6 ± 1.1
K-R + SOD	(4)	27.4 ± 0.4
K-R + Ara	(8)	33.8 ± 0.4[a]
K-R + Ara + SOD	(6)	29.8 ± 0.2[b]
K-R + Boiled homogenates + Ara	(6)	6.3 ± 0.02
K-R + Ara, omitted homogenates	(6)	0

Cortical slices were incubated in K-R buffer 20 min for reconstitution. (n), Number of cortical slices. $O_2^{\cdot-}$ assay was performed in duplicate in each cortical slice homogenate. Ara concentration, 0.5 mM; SOD, 10 μg/ml. Mean ± S.E.M.

[a] $P < 0.001$, compared with K-R control.

[b] $P < 0.02$, compared with Ara.

Ara was stimulated by NADPH. This NADPH-dependent NBF formation in cortical slice homogenates was inhibited by SOD, indicating the transient formation of $O_2^{\cdot-}$. There was minimal or no NBF formation when boiled homogenates were included or when homogenates were omitted from the incubation medium. The time-course of this NADPH-dependent $O_2^{\cdot-}$ formation induced by Ara is further shown in Fig. 1. There was no highly significant difference in $O_2^{\cdot-}$ formation between control and Ara-incubated tissue homogenates during the first 10 min of incubation. However, the NADPH-dependent $O_2^{\cdot-}$ formation was increased by 22%, 14%, and 22% at 30, 60, and 120 min, respectively, with the incubation in Ara. The relevance of the observed changes in brain homogenates to those observed in cortical slices and *in vivo* requires further elucidation.

The involvement of $O_2^{\cdot-}$ free radicals in brain swelling induced by PUFA is further demonstrated by the increase of malondialdehyde (MDA), an end product of membrane lipid peroxidation. Table 3 shows that linoleic, arachidonic, and docosahexaenoic acids increased MDA formation by 111%, 154%, and 140%, respectively. Oleic acid did not affect MDA formation.

DISCUSSION

Using rat brain cortical slices as an *in vitro* bioassay system, we had previously demonstrated the induction of brain swelling by PUFA. Both saturated and monounsaturated fatty acids had no such effects on brain water, sodium, and potassium content or inulin space. Free PUFA's that are either transported intracellularly or released from endogenous membrane phospholipids on stimulation (Chan et al., 1980*a*; Samuelsson et al., 1979) are highly susceptible to oxidation to form lipid radical intermediates and other free radical species. Our present studies indicate that the increase in $O_2^{\cdot-}$ formation induced by PUFA occurs concomitantly with the increase in tissue swelling. This does not necessarily imply that lipid peroxidation is a causative factor. The formation of $O_2^{\cdot-}$ in cortical slice homogenates was confirmed further, since it was inhibited by SOD. These data suggest that free PUFAs may serve as precursors for $O_2^{\cdot-}$ and other

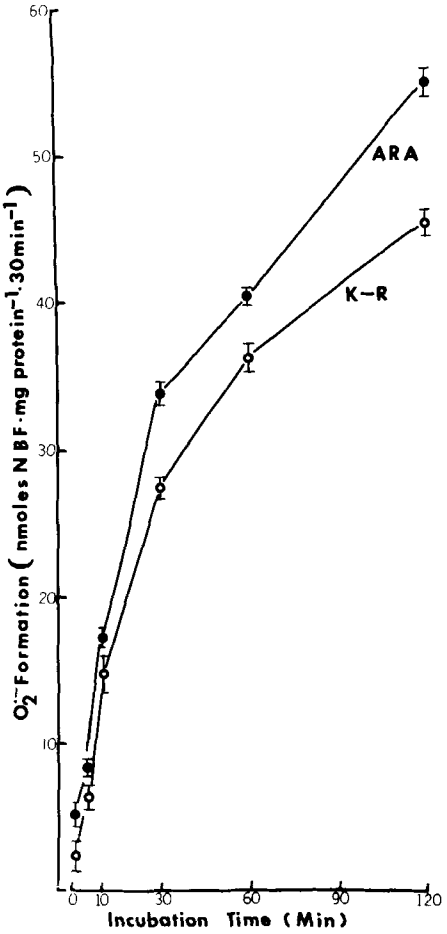

FIG. 1. Time-course studies of arachidonic acid-induced, NADPH-dependent superoxide formation in cortical slice homogenates. Each point represents an average value of duplicate assays obtained from four cortical slice homogenates. K-R, Krebs-Ringer buffer; Ara, arachidonic acid (0.5 mM).

TABLE 3. *Effect of free fatty acids on lipid peroxidation in cortical slices*

Medium	(*n*)	Malondialdehyde Formation (nmol·mg protein^{-1}·15 min^{-1})
K-R	(7)	5.3 ± 0.5
Sodium oleate	(4)	6.6 ± 0.9
Sodium linoleate	(6)	11.2 ± 0.6[a]
Sodium arachidonate	(12)	13.5 ± 0.7[a]
Docosahexaenoeic acid	(12)	12.8 ± 1.7[a]

Mean ± S.E. (*n*), Number of cortical slices. MDA formation was performed in duplicate for each cortical slice. Fatty acid concentrations, 0.5 mM.

[a] $P < 0.001$, using Student's *t*-test for statistical analysis.

free radical formation in cerebral tissue. Tam and McCay (1970) demonstrated that the transient formation of lipid peroxides and the disappearance of endogenous PUFA were involved in NADPH-dependent oxidation of the rat liver microsomal system. The formation of $O_2^{\cdot -}$ induced by PUFA was stimulated by NADPH, indicating the involvement of an NADPH-dependent oxidase in brain tissue. Furthermore, our preliminary studies have shown that the disappearance of PUFA in microsomal phospholipids also occurs in PUFA-treated cortical slices (Chan et al., 1980*b*).

It has been demonstrated recently that a purified preparation of (Na^+ + K^+)-ATPase, the enzyme responsible for the flux of cations and water molecules across cell membranes, was inhibited by $O_2^{\cdot -}$ in rat brain (Hexum and Fried, 1979). Our studies indicate that both microsomal and synaptosomal (Na^+ + K^+)-ATPase were inhibited by free PUFA (Chan et al., 1980*b*), suggesting that the enzyme was affected by the transient formation of $O_2^{\cdot -}$ and lipid peroxidation induced by PUFA, and that such a change might be responsible for the loss of intracellular potassium and the increase in intracellular sodium. The effects of PUFA and $O_2^{\cdot -}$ on the integrity of capillary endothelial cell membranes and their permeability to macromolecules such as albumin require further study.

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