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Release of Polyunsaturated Fatty Acids From Phospholipids and Alteration of Brain Membrane Integrity by Oxygen-Derived Free Radicals

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We studied the effects of oxygen-derived free radicals on the ultrastructure of brain cortical slices and the release of fatty acids from phospholipids of crude synaptosomes. Xanthine oxidase, hypoxanthine, and ADP-Fe^{3+}, a free-radical-generating system, caused swelling of cellular processes and mitochondria. The oxygen-derived free radicals also caused the rapid release and accumulation of endogenous polyunsaturated fatty acids (PUFA) from membrane phospholipids as determined by high-performance liquid chromatography (HPLC). Furthermore, [^{3}H]-arachidonic acid was also rapidly released from prelabeled phospholipids concomitant with a decrease in radioactivity in various phospholipid fractions. The radioactivities of neutral lipids including diacylglycerols were unchanged by free radicals. These data indicate that the activation of phospholipase A_2 and the release of PUFA may have overt effect on membrane integrity and the subsequent development of cellular injury and brain edema.

Key words: free radicals, high-performance liquid chromatography, arachidonic acid, phospholipids, brain edema

INTRODUCTION

In biological systems, oxygen derived free radicals including superoxide anions ($O_2 \cdot -$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$) are produced by the single-electron reduction of oxygen [Fridovich, 1982]. It has been suggested that these highly reactive oxygen-derived free radicals cause peroxidative damage to membrane phospholipids of CNS and thereby contribute to cellular injury [Demopoulos et al, 1979; Willmore and Rubin, 1982; Yoshida et al, 1982; Chan et al, 1983a; Chan et al 1984; Mead, 1976].

Using brain slices as an in vitro bioassay system, we have previously demonstrated that xanthine oxidase/hypoxanthine/ADP-Fe^{3+}, a widely used in vitro free-radical-generating system [Pederson and Aust, 1973; Kellogg and Fridovich, 1975], induces edema and cellular injury [Chan et al, 1982]. Furthermore, we have shown that in a synaptic membrane preparation exposed to the same free-radical-generating system, phospholipids are degraded. This degradation is accompanied by evidence of

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lipid peroxidation and the release of polyunsaturated fatty acids [Chan et al, 1982]. These data indicate that oxygen-derived free radicals cause severe membrane perturbations in vitro. The rapid accumulation of free unsaturated fatty acids, especially arachidonic acid (20:4) and docosahexaenoic acid (22:6), suggested phospholipase(s) might be involved in the release of fatty acids from purified synaptosomal membranes. The precise mechanism of free-radical–induced fatty acid release is not clear. Using high-performance liquid chromatography (HPLC) to facilitate the rapid separation of free fatty acids, neutral lipids, and various phospholipids, we examined the effects of free radicals on the release of polyunsaturated fatty acids (PUFA) from synaptosomal membranes, and investigated the possible involvement of phospholipase A_2 in this process. We also studied the ultrastructure of brain slices exposed to the free-radical-generating systems.

MATERIALS AND METHODS

Incubation Media and Reagents

The composition of Krebs-Ringer buffer has been reported previously [Chan and Fishman, 1978]. The xanthine oxidase-iron medium (in final concentration, unless otherwise indicated) consisted of xanthine oxidase, 0.4 units/ml, hypoxanthine, 0.1 mM, ADP, 0.1 mM, $FeCl_3$, 0.01 mM. Xanthine oxidase (Buttermilk 33.3 units/ml), hypoxanthine, and ADP were purchased from Sigma, St. Louis, MO. $FeCl_3 \cdot 6H_2O$ was obtained from Baker, Phillipsburg, NJ. Hexane and 2-propanol were purchased from Burdick and Jackson laboratories, Muskegon, MI. H_2O used for HPLC was obtained from house distilled H_2O filtered through a Millipore Milli Qwaterpurificationsystem(MilliporeCorp.Bedford,MA);[5,6,8,9,11,12,14,15,^{3}H(N)] arachidonic acid (61.0 Ci/mmol, 98% purity) was obtained from New England Nuclear, Boston, MA. Fatty acid methyl ester, various phospholipid standards, and silica gel H plates were obtained from Applied Sciences, State College, PA.

Synaptosomes were prepared by homogenizing six single first cortical slices in 4 ml of Krebs-Ringer buffer (Kontes, tissue homogenizer, San Leandro, CA) for 1 min. The homogenates were centrifuged at 1,000g for 20 min and the supernatant was pelleted at 12,000g for 30 min (P_2). Aliquots (1.0 ml) of the pellets (crude synaptosomes 2 mg protein/ml) were incubated with [^{3}H]-arachidonic acid (1.5 μ Ci/ml) in either control or experimental medium containing 2.5 mM and 0.1 mM dithiotreitol [Majewska and Sun, 1982] for 30 min at 37°C. The BSA wash step of prelabeled crude synaptosomes described by Majewska and Sun was omitted in our studies. The [^{3}H]20:4 prelabeled crude synaptosomes were then treated with xanthine oxidase, hypoxanthine, ADP-Fe^{3+} for various times of incubation. The reaction was stopped and lipids were extracted by adding chloroform-methanol (2:1,v/v). The lipid-soluble fractions were washed with an equal volume of Krebs-Ringer and were separated. Occasionally a much higher speed (34,000g) was used to eliminate the emulsion in the lipid fraction. The lipid extracts were dried under N_2 and were resuspended with Hexane/2-propanol (3:4, v/v) containing 4% H_2O.

Separation of Phospholipids, Free Fatty Acids, and Neutral Lipids by High-Performance Liquid Chromotography

The various phospholipids, free fatty acids, and neutral lipids were separated by high-performance liquid chromatography (1082B liquid chromatography, Hewlett Packard, Palo Alto, CA) using a silica column (Micro-Pak Si-5, 30 cm × 4 mm ID,

Varian Assoc, Palo Alto, CA) according to the previous method [Chan et al, 1983a]. The HPLC was equipped with a variable wavelength detector (190–600 nm) and a stop flow scanning spectrophotometer. The gradient began with an initial mobile phase of 4% H_2O in hexane/2-propanol (3:4, v/v), and was increased to 9% H_2O at 10 min and maintained at 9% H_2O for another 10 min, followed by 4% H_2O at 25 min as described previously [Chan et al, 1983a]. Flow rate was maintained at 1 ml/min. Elution was monitored at 206 nm with recorder response at 0.0512 AU/cm. The individual lipid fractions separated from the HPLC column were collected at 0.5-min intervals using a fraction collector (Isco Inc., Lincoln, NE) and the radioactivity of each fraction was measured by a scintillation spectrophotometer (Beckman LS 7,000). The fractions containing various neutral lipids were applied to silica Gel H thin-layer chromatography plates. Triacylglycerols, diacylglycerols, and monoacylglycerols were separated with a solvent system consisting of heptane/diethylether/formic acid (90:60:4 v/v) [Matsuzawa and Hostetler, 1980]. The radioactivity of various neutral lipids was determined by scintillation spectrophotometry.

Fatty Acid Analysis

The free fatty acid peaks eluted from the HPLC were pooled and subjected to methylation according to the method previous described [Chan et al, 1982]. The fatty acid methylesters were analyzed using a gas chromatograph (Hewlett Packard 5830A) equipped with silica capillary column (0.25 mm ID × 30 m., Supelco). The flow rate was 33 ml per minute. The initial column temperature was 40°C for 0.5 min, followed by an increase to 220°C at the rate of 20°C per minute. Temperature was then kept at 220°C for 9 min. Both injector and detector temperatures were set at 250°C. A known concentration of heptadecanoic acid (17:0) was routinely added to the samples as an internal standard.

Ultrastructural Studies

Single first cortical slices, weighing 40–50 mg and in 0.35-mm thickness were prepared from Sprague-Dawley male rats (Simonsen, Gilroy, CA). Following incubation for 30–60 min with the free-radical–generating system, the cortical slices were fixed by immersion in a solution 1% paraformaldehyde, 1% glutaraldehyde, in 100 mM phosphate buffer, pH 7.4, containing 4% sucrose. After fixation overnight at 4°C, small 1 × 1 mm blocks were cut from the center of each slice, rinsed, and postfixed in 1% phosphate-buffered OsO_4 at room temperature for 1 hr. The tissue was then dehydrated in acetone and propylene oxide and embedded in a mixture of Epon and Araldite. The tissue was oriented so that the full thickness (pia to layer 6) of each cortical slice could be sectioned. Ultrathin sections were stained on the grids with lead citrate and examined with a Seimens Elmiskop I electron microscope (EM).

RESULTS

Effects of Oxygen-Derived Free Radicals on the Ultrastructure of Cortical Slices

Our EM observations were confined to the molecular layer because of the severe trauma to the deeper cortical layers inherent in the method for preparing the brain slices. The ultrastructure of the molecular layer of slices exposed to the free-

radical-generating system for 30 or 60 min was dominated by many obviously swollen cellular processes (Fig. 1B). These were generally empty, save for a few remnants of what appeared to be membranes, and abnormal mitochondria. They could not reliably be identified as neuronal or glial. Most mitochondria were grossly swollen, with loss of normal electron density and fragmentation of cristae. Although these changes were widespread, some cellullar processes and an occasional mitochondrion appeared normal. The neuropil of slices incubated in control solutions contained rare dilated processes and occasional processes with dark cytoplasm (which may represent an early form of "dark" degeneration following axotomy [Brodal, 1982]. The striking swelling of cellular processes and changes in mitochondria were not seen in control tissue [Moller et al, 1974] (Fig. 1A).

Release of Arachidonic Acid From Membrane Phospholipids Monitored by HPLC

Figure 2 shows the representative HPLC lipid elution profiles for control and free-radical-treated crude synaptosomes. In the control sample phosphatidylethanolamine (PE—plus plasmalogen), phosphatidylcholine (PC) and neutral lipids are the predominant peaks at UV absorption (206 nm), indicating a higher level of unsaturated fatty acids in these lipid fractions (Fig. 2A). After treatment with free radicals, the absorbance for both PE and PC was decreased concomitant with a significant increase in absorbance of free fatty acids (FFA) (Figure 2B). There were no significant changes in peak areas of other phospholipids or neutral lipids. The release of [^{3}H]-20:4 from labeled lipids induced by free radicals was further monitored by HPLC. Figure 3 shows [^{3}H]-20:4 was released from PC, phosphatidylinositol (PI) and PE and from phosphotidylserine (PS) of free-radical-treated synaptosomes.

Time Course Studies

The time course of free-radical-induced release of [^{3}H]-20:4 from labeled phospholipids was studied further (Fig. 4). The radioactivity of PE was decreased significantly after 5, 10, 30, and 60 min of exposure to the free-radical-generating system. Other phospholipids were affected at 30 and 60 min (Fig. 4). Furthermore, the radioactivity of diacylglycerols was slightly decreased at 10 and 30 min whereas the radioactivity of [^{3}H]-20:4 in both monoacylglycerols and triacylglycerols was not affected (Fig. 5).

Free-radical-induced release of endogenous fatty acids is shown in Figure 6. Figure 6A shows the changes in mole % of five major fatty acids as a function of time of incubation with free radicals. Both palmitic acid (16:0) and stearic acid (18:0) decreased significantly in the first 10 min whereas the unsaturated fatty acids, oleic acid (18:1), 20:4, and 22:6 increased over the same time period. The contents of both 16:0 and 18:0 then remained relatively constant for the remainder of the incubation with free radicals. However, the concentrations of unsaturated fatty acids 18:1, 20:4, and 22:6 increased dramatically throughout the incubation. After 60-min incubation with xanthine oxidase/Fe^{3+} system, 18:1, 22:6, and 20:4 were increased 22-, 44-, and 147-fold, respectively (Fig. 6B).

DISCUSSION

The present studies demonstrate that oxygen-derived free radicals induce changes in brain cell membranes. The exposure of cortical slices to the free-radical-generating

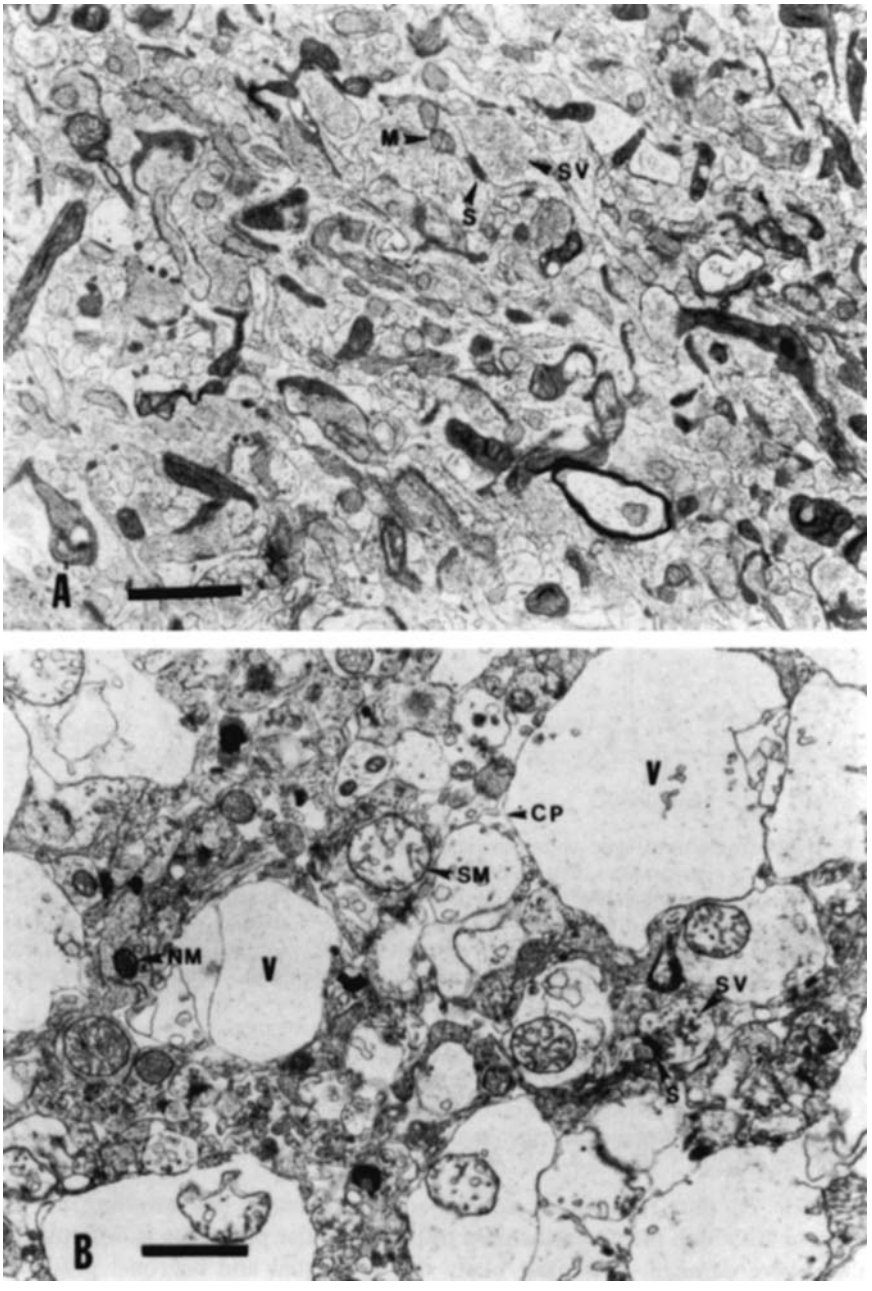

Fig. 1. Effects of oxygen-derived free radicals on ultrastructure of cortical slices. A. Slice incubated in control medium (oxygenated Krebs-Ringer buffer) for 60 min; M, normal mitochondria; S, synapse; SV, synaptic vesicles; V, Vacuole; scale bar, 5 μm. B. Slice incubated in oxygenated xanthine oxidase/hypoxanthine/ADP-Fe^{3+} for 30 min; CP, swollen cellular processes; NM, normal mitochondria; SM, swollen mitochondria; S, synapse; SV, synaptic vesicles. Scale bar, 5 μm.

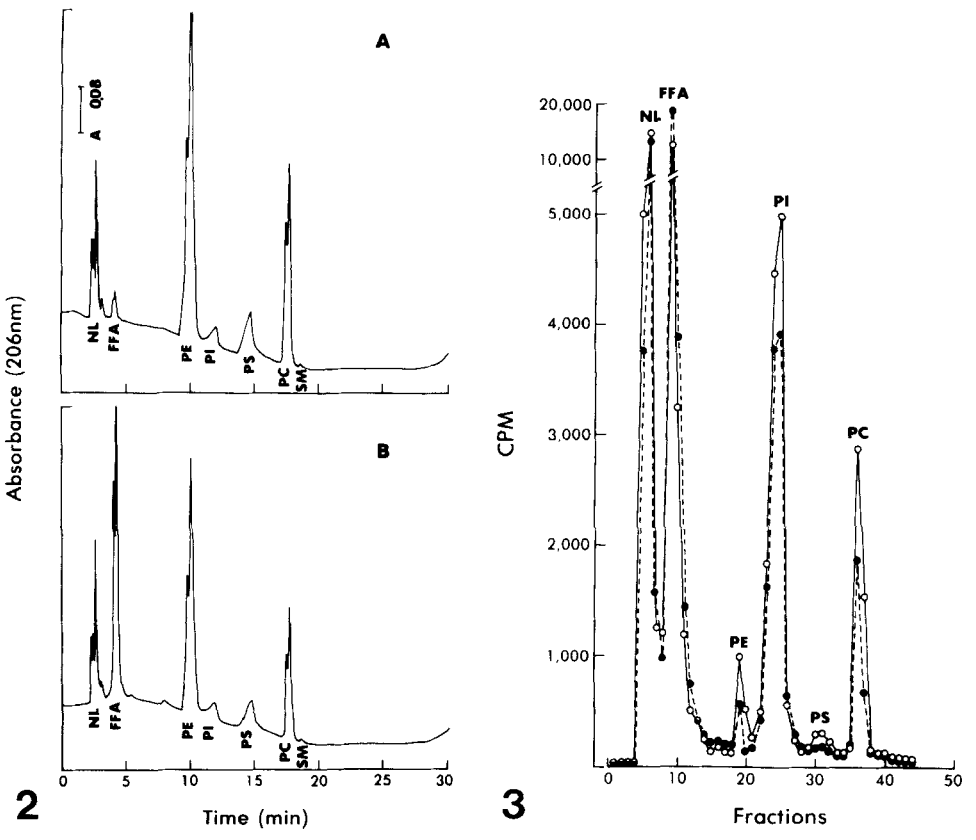

Fig. 2. High-performance liquid chromatography of oxygen-derived free-radical-induced fatty acid release from crude synaptosomes. Crude synaptosomes (P_2) were incubated with either Krebs-Ringer or xanthine oxidase/hypoxanthine/Fe^{3+}-ADP system for 60 min. Phospholipids, free fatty acids, and neutral lipids were separated by HPLC. The absorbance unit full scale is 0.6 cm. A. Control lipid profile. B. Free-radical-treated synaptosomal lipid profile; NL, neutral lipids; FFA, free fatty acids; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin.

Fig. 3. High-performance liquid chromatography of the oxygen-derived free-radical-induced release of [^{3}H]-20:4 from prelabeled membrane lipids. Synaptosomal membrane lipids were labeled with [^{3}H]-20:4 for 30 min prior to a 30 min incubation with either Krebs-Ringer buffer or xanthine oxidase/Fe^{3+} system. ○, control; ●, free radical-treated membranes.

system for 30 min resulted in severe cellular damage and swelling of cellular processes. Although the indentification of these cellular processes is difficult because of the severe damage, it is most likely that both glial and neuronal processes are affected. The swelling, loss of normal electron density, and fragmentation of the cristae of mitochondria indicate that these membraneous structures are also affected by free radicals. Despite this evidence of severe damage to most mitochondria, a few intact mitochondria were present. The reason for this heterogenous response to free radical damage is not clear. These data coincide perfectly with the increased water and sodium contents which characterizes cellular edema in cortical slices observed after 30 min of incubation with an identical free-radical-generating system [Chan et al, 1982].

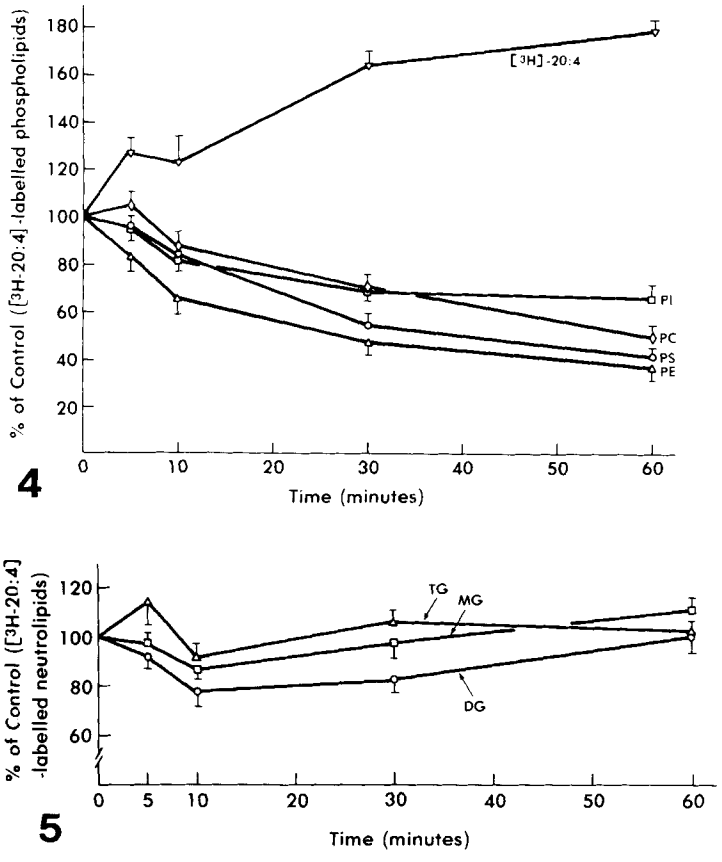

Fig. 4. Time course studies of the effects of oxygen-derived free radicals on the release of [^{3}H]-20:4 from prelabeled membrane phospholipids. The results were obtained from an average of three different experiments. PI, phosphatidylinositol; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine.

Fig. 5. Time course study of the effects of oxygen-derived free radicals on the release of [^{3}H]-20:4 from prelabeled-neutral lipids. TG, triacylglycerols; DG, diacylglycerols; MG, monoacylglycerols.

It is well known that brain in vivo contains no or very low concentrations (0–0.1 μmoles/g wet weight) of free PUFA. The majority of the PUFA is acylated in C-2 position of phospholipids. These PUFA are released from membrane phospholipids very rapidly within seconds to minutes following various pathological insults including ischemia [Bazan, 1970; Marion and Wolfe, 1979; Majewska et al, 1981; Rehncrona et al, 1982; Tang and Sun, 1982; Yoshida et al, 1982], anoxia and hypoxia [Strosznajder and Domanska-Janik, 1980], and cold-injury [Chan et al, 1983c]. Our early preliminary in vitro work also demonstrated that endogenous PUFAs were released from phospholipids by oxygen-derived free radicals [Chan et al, 1982]. These data suggest that the release of PUFAs may foster the secondary cellular damage.

Due to the striking ultrastructural changes in membranes associated with synapses, mitochondria, and other organelles, and since the mode of action of free radicals on membrane perturbation is not clear, we decided to investigate further the biochemical effects of free radicals on crude synaptosomal membranes (P_2). P_2 fractions are known to have mitochondria, microsomes, and myelin membranes. Our

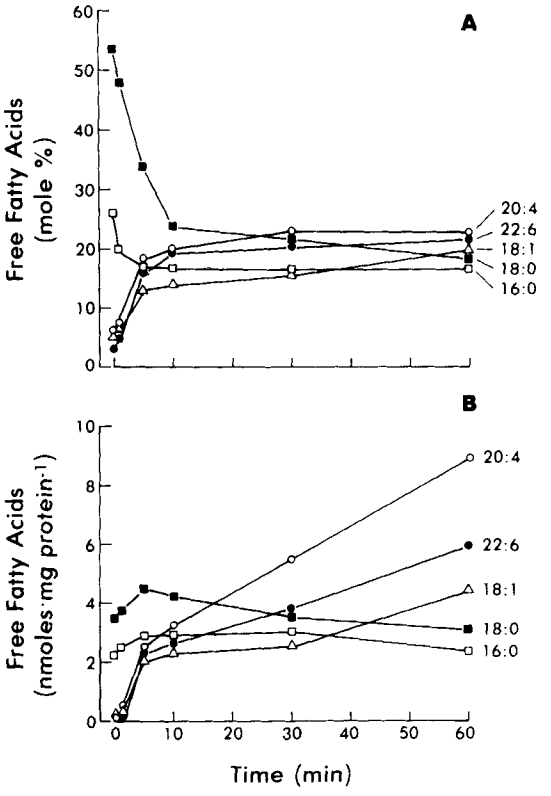

Fig. 6. Time course studies of the release of endogenous fatty acids induced by free radicals. Free fatty acids were separated by HPLC followed by capillary gas chromatographic analysis. A. Free fatty acids composition of brain membranes incubated with free radicals at various times. B. Free fatty acids concentration of brain membranes incubated with free radicals at various times.

present studies demonstrate that free radicals cause a rapid release of PUFA from crude synaptosomal membranes. The release of 20:4 and 22:6 was already increased tenfold at 5 min and increased even further thereafter. Since crude synaptosomes required one–two hr to prepare, they likely had already suffered prolonged ischemic damage with release of their PUFA. Nevertheless, a substantial amount of PUFA was released from these synaptosomal membranes upon further exposure to free radicals. These data suggest that a PUFA pool which is normally not readily accessible to ischemic damage is being affected. Furthermore, the fact that [^{3}H]-20:4 in several phospholipids was affected by free radical treatment, whereas only the 20:4-labeled PC was affected significantly during ischemia [Marion and Wolfe, 1979], further supports the hypothesis that different PUFA pools exist in membrane phospholipids.

Although oxygen-derived free radicals caused a rapid release of PUFAs from membrane phospholipids, our studies do not imply that free radicals had a causative effect on ischemic injury. It has been demonstrated by others that the free radicals are involved at the time of reperfusion of ischemic injury [Yoshida et al, 1980]. Nevertheless, our data suggest that the accumulation of arachidonic acid and its radical metabolites may have a feedback effect on the further deacylation of arachidonic acid from membrane phospholipids.

Ethanolamine phospholipase A_2 in brain is activated during ischemia [Edgar et al, 1982]. Increased phospholipase C activity has also been observed in ischemic brain [Wei et al, 1982]. The possible involvement of both phospholipase A_2 and C at the onset of brain ischemia has been proposed [Bazan and Turco, 1980]. The present study demonstrates the rapid release of arachidonic acid without a concomitant increase in diacylglycerols (DG), which product is formed by phospholipase C. However, phospholipase C could not be excluded as a source of arachidonic acid, since this enzyme is tightly associated with diacylglycerol lipase, which hydrolyzes DG, forming arachidonic acid and monoglycerols [Edgar and Freysz, 1982]. These data suggest that the phospholipase A_2 is the primary target enzyme activated by free radicals. Furthermore, our preliminary studies have indicated that phospholipase A_2 inhibitors could reduce the free-radical–induced 20:4 release in isolated CNS microvessels [Au et al, 1983], thus further supporting the involvement of phosphalipase A_2 in free-radical–induced 20:4 release. Since the accumulation of PUFA, arachidonic acid in particular, fosters the formation of oxygen-free radicals [Chan and Fishman, 1980] and the development of brain injury and edema [Chan and Fishman, 1978, 1984; Chan et al, 1983b], the application of various phospholipase A_2 inhibitors including dexamethasone may provide therapeutic effects: such studies are in progress in our laboratories.

The present study also demonstrates that HPLC methodology can be used to partially quantify the release and accumulation of PUFA, since the 206-nm absorbance used in HPLC is within the maximum absorbance of double bonds (195 nm). However, the oxidized fatty acids which have a maximum absorption at 235 nm would not be detected. These observations led us to scan the free fatty acid peak by a multiwavelength spectrophotometer equipped with HPLC (data not shown). Very little or no oxidized fatty acids were observed by scanning spectrophotometry. An extra peak at 260–265 nm was apparent; this might represent the absorption of carbonyl groups. However, the transient nature and relatively low concentration of the oxidized fatty acids in the small brain sample (1 mg wet weight) may make such determination impossible. It has been shown earlier that the detection of thiobarbituric-reactive malondialdehyde required at least ten times more tissue [Chan and Fishman, 1980; Chan et al, 1982]. The oxidation of PUFA may be a minor event and a transient process induced by free radicals. However, whether the lipid peroxidation process occurred on the PUFA of phospholipids or PUFA which are released from the phospholipids or both is not clear. Further time course studies on both the release of arachidonic acid and the accumulation of malondialdehyde may provide some information regarding the target site(s) of the free radicals. Nevertheless, the present studies support the view that the activation of phospholipase A_2 is a predominant event induced by oxygen-derived free radicals.

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