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Alterations of Membrane Integrity and Cellular Constituents by Arachidonic Acid in Neuroblastoma and Glioma Cells

PAK HOO CHAN and ROBERT A. FISHMAN

Brain Edema Clinical Research Center, Department of Neurology, University of California, San Francisco, CA 94143 (U.S.A.)

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Effects of arachidonic acid on cellular metabolism, cation content, lipid peroxidation, sodium pump activities and release of labeled arachidonic acid were studied in C-6 glioma cells and N18TG2 neuroblastoma cells. Arachidonic acid caused a significant increase in intracellular sodium levels concomitant with a decrease in intracellular potassium in both cell lines. Both $(Na^+ + K^+)$-ATPase and *p*-nitrophenyl phosphatase of glioma cells were inhibited by arachidonic acid whereas only the *p*-nitrophenyl phosphatase of neuroblastoma cell was inactivated. Low concentrations of arachidonic acid stimulated lactic acid release whereas high concentrations had an opposite effect. In addition, the lipid peroxide content of glioma cells was increased abruptly by 50 μM arachidonic acid whereas only a slight increase of malondialdehyde was observed in neuroblastoma cells. When the cultured cells of both cell lines were incubated with exogenous labeled arachidonic acid, 78–95 % of the label was incorporated into membrane phospholipids. Only a very small fraction of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ was synthesized. Exogenous arachidonic acid and free radicals generated with xanthine–xanthine oxidase caused a significant release of endogenous labeled arachidonic acid from cellular membrane phospholipids. These data further support our hypothesis that the arachidonic acid and its oxygen radical metabolites induce pathological alterations in membrane permeability and cellular volume.

INTRODUCTION

Following various forms of pathological insult, arachidonic acid (20:4, the major structural polyunsaturated fatty acid (PUFA), is released from cellular membrane phospholipids[2,21,27]. Once released from the cellular membrane, arachidonic acid is converted either by cyclo-oxygenase to prostaglandins and lipid peroxides or by lipoxygenase to form hydroxy fatty acids and slow reacting substances (leukotrienes)[22,24]. Although the exact physiological function of these arachidonic acid-derived metabolites is not well understood, it is generally accepted that prostaglandins and free radicals play a role in the inflammatory process in various biological systems[15,19]. Using rat brain cortical slices as a bioassay system, we have reported earlier that arachidonic acid caused a three-fold increase in tissue swelling concomitant with the increase of intracellular sodium and decrease of intracellular potassium (refs. 3,5). Membrane $(Na^+ + K^+)$-ATPase, the enzyme that regulates the distribution of cellular cations, is also inhibited by arachidonic acid[6]. This model of cellular edema is also associated with changes in cellular metabolism since lactic acid levels were elevated and high energy nucleotides were reduced[10].

We have reported that arachidonic acid induced the transient formation of superoxide radicals and lipid peroxides in cortical slices[4]. Further studies have shown that free radicals caused a four-fold increase in free PUFA content concomitant with increase of malondialdehyde (MDA)[8]. The free PUFAs, especially arachidonic acid, are released from phospholipids as a result of the stimulation of phospholipase(s) by free radicals. Although the experimental data support the view that arachidonic acid participates in membrane perturbations associated with brain edema, its specific effect on different neural elements is not clear. Using the established brain cell cultures of mouse N18TG2 neuroblastoma cells and rat C-6 glioma cells as in vitro

neuronal and glial models, the present studies report the pathological effects of arachidonic acid on the membrane integrity, intracellular cations and lactic acid release of these neoplastic brain cells.

MATERIALS AND METHODS

The original stocks of C-6 rat glioma cells and N18TG2 mouse neuroblastoma cells were obtained from the National Institutes of Health and maintained by the Cell Culture Facility at the University of California, San Francisco. The cells were either grown in Falcon petrie dishes (60 mm diameter) or Falcon T75 flasks and grown with Dulbecco Modified Eagle Medium (DME) supplemented with 10% fetal calf serum. For neuroblastoma cells, 6-thioguanine was added in DME medium. The cells were kept at 37 °C in a humidified atmosphere of 5% CO_2–95% O_2. Cells were grown to confluency and about 2 to 3 $\times$ 10^6 cells and 20 $\times$ 10^6 cells were grown in a petrie dish and in a flask, respectively. The cells were rinsed twice with 2 ml of DME medium each time. Glass beads (3 mm diameter) were used to dissociate the cells from the container. The cells obtained from this method are more viable than from the previous method[7]. These cells have a higher level of intracellular potassium and a lower level of intracellular sodium as compared to the cells obtained by the previous method (Fig. 1). The cells were suspended in Krebs–Ringer or DME medium for assays. The viability of these dissociated cells was measured by trypan blue exclusion. About 95% of these cells were viable.

About 1.5 ml of incubation medium with Krebs–Ringer or Krebs–Ringer with arachidonic acid was added to an equal volume of cell suspension (10^6 cells/ml) and then incubated at 37 °C for 30 min. The cells were pelleted by centrifugation at 5,000 *g* for 15 min, the supernatant was removed and 1.5 ml analyzed enzymatically for lactic acid content according to the previous method[5]. The pellet was rinsed with 300 mM sucrose and digested with 1.5 ml of 2N HNO_3 for 5 days. The nitric acid extracts were diluted 20 times with distilled water, and sodium and potassium were measured by atomic absorption spectrophotometry (Perkin–Elmer 560). Intracellular cations were expressed as Eq cation per mg of protein. Protein was measured according to the method of Lowry et al.[20].

Thiobarbituric acid reactive malondialdehyde (MDA) was measured as an index of lipid peroxidation in brain tissue according to the method of Chan and Fishman[4]. 0.5 ml of glioma cells (5.5 mg protein/ml) or 0.5 ml of neuroblastoma cells (9.6 mg protein/ml) were incubated with either Krebs–Ringer or Krebs–Ringer with 50 μm arachidonic acid at 37 °C for 30 min. After the incubation, an aliquot (0.5 ml) of the cells was assayed for lipid peroxidation. A separate tube containing either Krebs–Ringer or 50 μm arachidonic acid was also assayed and used as a blank. MDA was estimated by using a standard curve of 1,1,3,3,-tetraethoxylpropane, according to Dahle et al.[9].

(Na^+ + K^+)-ATPase activity of cultured cells was assayed according to the method of Skou and Esman[26]. The assay medium contains 80 mM imidazole, 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, pH 7.5 and the assay carried out at 37 °C for 30 min. K^+ was omitted and 0.1 mM ouabain was added to the incubation medium to obtain ouabain-inhibitable ATPase activity. Mg^{2+}-ATPase was derived from the ATPase activity not inhibited by ouabain. The K^+-stimulated *p*-nitrophenyl phosphatase activities in cell preparations were measured in 100 mM KCl, 20 mM $MgCl_2$, 30 mM histidine, 0.2% BSA at a pH of 7.6[23].

Prostaglandins, phospholipids and free fatty acids of cultured cells were separated by one-dimensional thin layer chromatography on silica gel G according to the method of Isakson et al.[16] along with known standards. Individual phospholipids were separated two-dimensionally on silica gel H based on the method of Kimelberg and Papahadjopoulos[18].

The composition of Krebs–Ringer buffer was reported previously[6]. [1-^{14}C]arachidonic acid (specific activity, 52.7 mCi/mmol), [1-^{14}C]prostaglandin E_2 (specific activity 40 mCi/mmol), [1-^{14}C]prostaglandin $F_{2\alpha}$ (specific activity 40 mCi/mmol), and [1-^{14}C]prostaglandin E_1 (specific activity 40 mCi/mmol) were obtained from New England Nuclear, Boston, MA. Sodium arachidonate, PGE_2, $PGF_{2\alpha}$, xanthine, xanthine oxidase (milk, 10.8 units/ml), lactate dehydrogenase, *p*-nitrophenol, ouabain, vanadate-free ATP, ADP, thiobarbituric acid were purchased from Sigma, St. Louis, MO. Phospholipid standards, silica gel H, and silica gel G were obtained from Applied Sciences, State College, PA.

1,1,3,3,-tetraethoxylpropane was obtained from Mallinckrodt, Paris, KY.

RESULTS

Effects of arachidonic acid on cation content and lactic acid production

Fig. 1 shows the effect of various concentrations of arachidonic acid on intracellular sodium and intracellular potassium of C-6 glioma and N18TG2 neuroblastoma cells. Arachidonic acid, at a concentration of 0.01 mM, did not induce significant changes in intracellular cation content of either groups of cells. However, when the brain cells were incubated with arachidonic acid at higher concentrations, a significant increase of intracellular sodium concomitant with a decrease of intracellular potassium was observed in both cell lines. Arachidonic acid at concentrations of 0.05 mM and 0.5 mM,

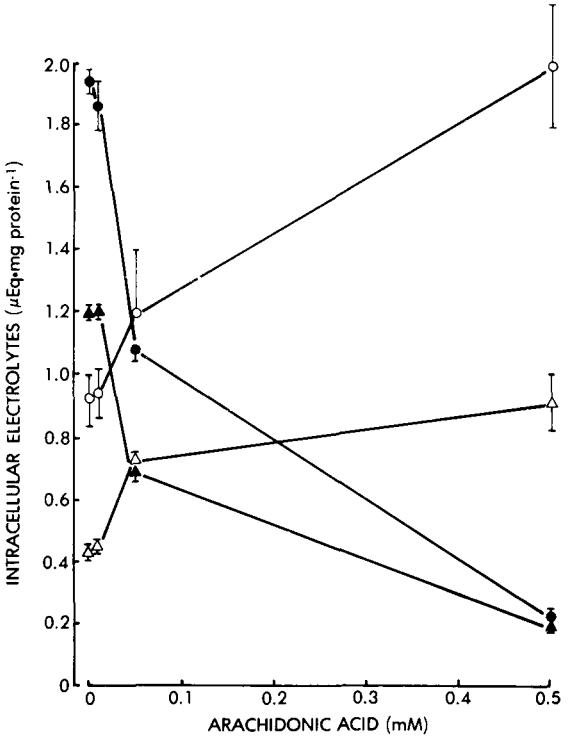

Fig. 1. Effects of arachidonic acid on intracellular cations of glioma and neuroblastoma cells. 1.5 ml of glioma cells of neuroblastoma cells at 10^6 cells/ml were incubated with various concentrations of arachidonic acid at 37 °C for 30 min. After rinsing with sucrose, the cells were pelleted by centrifugation and were extracted by 2N nitric acid for 5 days. The vertical bars represent the S.E.M. obtained from 4 experiments. Glioma cells: ○, Na^+; ●, K^+. Neuroblastoma cells: △, Na^+; ▲, K^+.

increased the Na^+/K^+ ratio from 0.47 (control) to 1.11 and 9.09 and from 0.35 (control) to 1.06 and 4.55 for incubated glioma cells and neuroblastoma cells, respectively.

Arachidonic acid at 0.01 mM slightly increased the levels of lactic acid in the incubation medium with both neoplastic brain cell cultures. The release of lactic acid was further stimulated by 0.05 mM concentration of arachidonic acid. However, higher concentration of arachidonic acid (0.5 mM) inhibited the cellular release of lactic acid in both cell lines (Fig. 2).

Inhibition of (Na^+ + K^+)-ATPase and p-nitrophenyl phosphatase of C-6 glioma cells by arachidonic acid

Table I shows that the cation-dependent enzymes of C-6 glioma cells were inhibited by arachidonic acid. (Na^+ + K^+)-ATPase and K^+-dependent *p*-nitrophenyl phosphatase were inhibited by 59 % and 56 %, respectively. No significant changes in (Na^+ + K^+)-ATPase activity of incubated neuroblastoma cells were observed. However *p*-nitrophenyl phosphatase was inhibited 59 % by arachidonic acid. The Mg^{2+}-ATPase activities of both incubated cell lines were not affected by arachidonic acid.

Effects of arachidonic acid on membrane lipid peroxidation

The induction of membrane lipid peroxidation in both neuroblastoma cells and glioma cells is shown in Fig. 3. The content of thiobarbituric acid-reactive malondialdehyde (MDA) has been used in the present studies as an index of membrane lipid peroxidation. C-6 glioma cells have a higher basal value of MDA levels than neuroblastoma cells. After incubation with Krebs–Ringer buffer for various times, the MDA levels of both glioma and neuroblastoma cells were unchanged. The MDA levels of glioma cells were stimulated by 75 to 118 % with the incubation of arachidonic acid (0.05 mM) whereas the MDA contents of neuroblastoma cells were slightly increased by 30 to 53 %.

Distribution of [^{14}C]arachidonic acid in chloroform-methanol extracted lipid fractions

The metabolic fate of exogenous [^{14}C]arachidonic acid in incubated brain cells was studied. The distribution of [^{14}C]arachidonic acid in membrane phos-

TABLE I

Effects of arachidonic acid on ($Na^+ + K^+$)-ATPase and p-nitrophenyl phosphatase of glioma and neuroblastoma cells

Both glioma and neuroblastoma cells were grown to confluency stage. An aliquot of 0.1 ml cell suspension (glioma cells: 2.4×10^6 cells/ml; neuroblastoma cells: 6.7×10^5 cells/ml) was used for the enzyme assays. The values represent an average of 3 experiments. Values are means ± S.E.M.

Incubation	*($Na^+ + K^+$)-ATPase (μmol pi.mg protein^{-1}.h^{-1})*	*Mg^{2+}-ATPase (μmol pi.mg protein^{-1}.h^{-1})*	*p-Nitrophenyl phosphatase (μmol p-NP.mg protein^{-1}.h^{-1})*
Glioma cells + Krebs–Ringer (control)	1.35 ± 0.10	1.86 ± 0.1	2.25 ± 0.14
Glioma cells + arachidonic acid	0.79 ± 0.03**	1.81 ± 0.34	1.25 ± 0.03**
Neuroblastoma cells + Krebs–Ringer (control)	1.60 ± 0.45	6.36 ± 0.20	2.15 ± 0.29
Neuroblastoma cells + arachidonic acid	1.50 ± 0.35	6.30 ± 0.25	1.27 ± 0.17*

* $P < 0.05$; ** $P < 0.01$; Student's t-test.

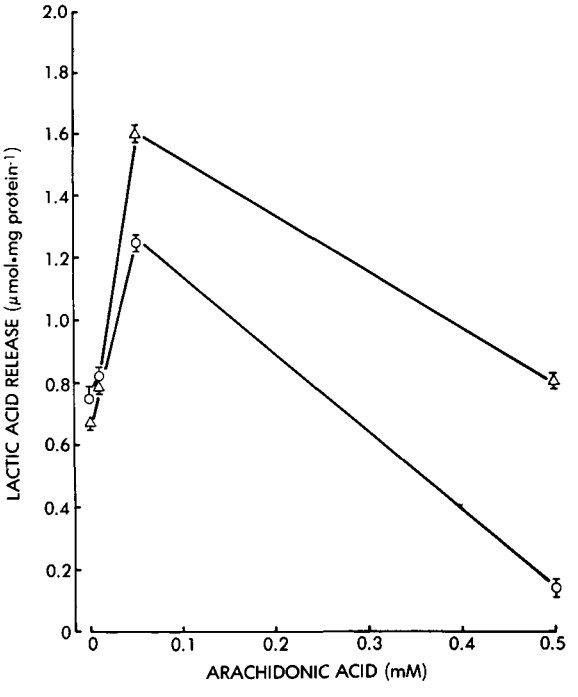

Fig. 2. Effects of arachidonic acid on the release of cellular lactic acid of glioma and neuroblastoma cells. Cellular lactate in the incubation medium was measured enzymatically. The vertical bars represent the S.E.M. obtained from 4 experiments. ○, glioma cells; △, neuroblastoma cells.

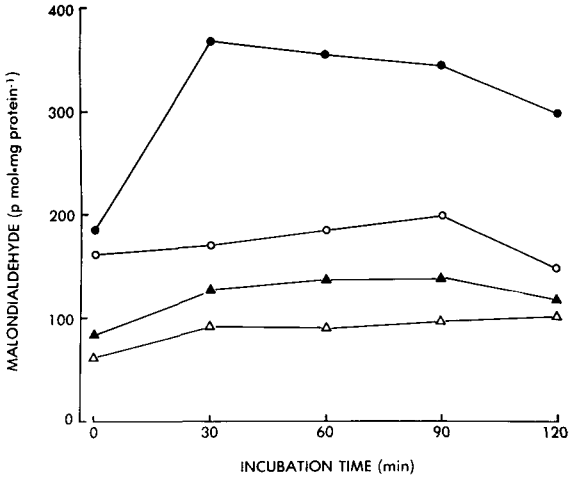

Fig. 3. Time course studies of arachidonic acid-stimulated malondialdehyde formation in glioma and neuroblastoma cells. Cells were incubated with 50 μM of arachidonic acid at 37 °C for various times. Glioma cells: ○, control; ●, experimental. Neuroblastoma cells: △, control; ▲, experimental.

pholipids, prostaglandins and other chloroform-methanol extractable fractions after 16 h incubation is shown in Table II. The majority of the labeled arachidonic acid was incorporated into the membrane phospholipids which account for 78.5% and 94.6% for glioma and neuroblastoma cells, respectively. Among the individual phospholipids, the majority of the arachidonic acid was incorporated into phosphatidylcholine followed by phosphotidylethanolamine. However, a large portion of arachidonic acid (21%) was incorporated into phosphatidylinositol (PI) in neuroblastoma cells whereas only 1.3% label was incorporated in the PI of glioma cells. Only a small fraction of incorporated arachidonic acid was localized in phosphatidylserine and sphingomyelin in both cell lines. Furthermore, a negligible amount of arachidonic acid was converted to prostaglandins E_2 and $F_{2}\alpha$.

TABLE II

Distribution of [^{14}C]arachidonic acid in chloroform-methanol extracted lipid fractions

Glioma cells and neuroblastoma cells were grown to confluency (about 2 × 10^6/petrie dish) and were incubated with 0.25 μCi of [1-^{14}C]arachidonic acid at 37 °C for 16 h. About 80% of radioactivity was incorporated into cellular fractions. After the incubation, cells were washed with DME medium and were pelleted by centrifugation at 9,000 *g* for 2 min. The cells were extracted by chloroform-methanol (2:1, v/v) and the extracts were subjected for phospholipid and prostaglandin separation by thin layer chromatography.

Fractions	*Glioma cells*	*Neuroblastoma cells*
	% of total radioactivity	
Phospholipids	78.5	94.5
Phosphatidyl ethanolamine	19.9	11.4
Phosphatidyl choline	55.2	60.0
Phosphatidyl serine	1.8	2.7
Phosphatidyl inositol	1.3	20.8
Sphingomyelin	0.3	0.7
Cholesterol — neutral lipids	19.3	3.7
Prostaglandin E_2	0.3	0.3
Prostaglandin $F_2\alpha$	0.7	0.5
Free arachidonic acid	1.1	1.0

TABLE III

Effects of arachidonic acid, prostaglandins and free radicals on the release of preloaded [^{14}C]arachidonic acid from glioma cells

The cells were preloaded with 0.25 μCi of [^{14}C]arachidonic acid at 37 °C for 16 h. After the incubation, the cells were washed twice with DME medium and were incubated with various agents for 60 min at 37 °C. The radioactivity in incubation medium was extracted by chloroform and methanol and was separated by thin layer chromatography and the spot corresponding to arachidonic acid was counted by a scintillation counter. The concentration used: arachidonic acid, 50 μM; PGE_2, $PGF_2\alpha$, 1 μg/ml; xanthine oxidase 0.08 units/ml, xanthine, 0.1 mM, $FeCl_3$, 0.01 mM; ADP, 0.1 mM. The number of observations was indicated in parentheses. Values are means ± S.E.M.

Release medium	*[^{14}C]Arachidonic acid release (cpm.10^5 cells^{-1}.h^{-1})*
Krebs–Ringer	1388 ± 49 (9)
Arachidonic acid	3368 ± 20 (4)*
Xanthine oxidase — xanthine — — Fe^{3+}-ADP	2124 ± 39 (4)*
PGE_2	1545 ± 51 (4)
$PGF_2\alpha$	1373 ± 12 (4)

*$P < 0.01$; Students *t*-test.

Effects of arachidonic acid, prostaglandins and free radicals on the release of preloaded [^{14}C]arachidonic acid from glioma cells

We have demonstrated earlier that both exogenous arachidonic acid and xanthine oxidase-Fe^{3+}, a free radical generating system, stimulated the release of endogenous arachidonic acid from membrane phospholipids[5,8]. These observations prompted our further studies on the effect of arachidonic acid, prostaglandins and free radicals on the release of preloaded [^{14}C]arachidonic acid from cellular membrane phospholipids. Exogenous arachidonic acid, at a concentration of 50 μM, caused a 143% increase in [^{14}C]arachidonic acid release (Table III). Free radicals, generating from xanthine oxidase and ferric ions, stimulated arachidonic acid release by 53%. Both PGE_2 and $PGF_2\alpha$ were ineffective.

DISCUSSION

The present studies demonstrate that arachidonic acid induced significant biochemical changes in membrane-associated functions and in cellular metabolism of neoplastic brain cells. Arachidonic acid at a concentration of 50 μM, induced the increase of intracellular sodium and the decrease of intracellular potassium in both cell lines. The Na^+/K^+ ratio of both cell lines was also increased. Only the $(Na^+ + K^+)$-ATPase of glioma cells was inhibited by arachidonic acid, whereas the enzyme of neuroblastoma cells was unaffected. K^+-stimulated *p*-nitrophenyl phosphatase, an enzyme associated with $(Na^+ + K^+)$-ATPase, was inhibited by arachidonic acid in both glioma and neuroblastoma cells. The inhibition of *p*-nitrophenyl phosphatase by arachidonic acid was much greater than the effect on $(Na^+ + K^+)$-ATPase in both glioma and neuroblastoma cells. This might indicate that one of the subunits of $(Na^+ + K^+)$-ATPase possessing nitrophenyl phosphatase activity is first modified by arachidonic acid, followed by the total modification of both subunits which could result in the inactivation of $(Na^+ + K^+)$-ATPase. This mechanism has been suggested by Hansen et al.[14], using thimerosal as a lipid removal agent. Although the mode of action of arachidonic acid might be like that of thimerosal, the actual effect of arachidonic acid on the lipid micro-environment of the $(Na^+ + K^+)$-ATPase re-

quires further investigation. On the other hand, the present studies provide evidence that arachidonic acid induces perturbation in membrane lipids, as observed by the increased levels of MDA. In this regard, the MDA content of glioma cells induced by arachidonic acid was much higher than that of neuroblastoma cells. The differences in response to arachidonic acid between glioma and neuroblastoma cells are not clear. Whether these are due to quantitative differences between neuroblastoma cells and glioma cells in superoxide dismutase levels and/or endogenous antioxidant contents requires further study[12].

Our data further show that both exogenous arachidonic acid and xanthine oxidase-Fe^{3+}, a free radical producing system[17], increase lipid peroxide content and the release of endogenous arachidonic acid from phospholipids. Furthermore, Marion et al. have demonstrated that the exogenous arachidonic acid and the endogenous counterpart do not mix appreciably in the intact brain slice[22]. Furthermore, only a very small amount of free arachidonic acid (5–20 nm/g tissue) is presented in brain[13,21]. Thus, the exchange between exogenous and endogenous arachidonic acid is unlikely to be the mechanism totally accountable for release of endogenous labeled arachidonic acid. The increased release of endogenous prelabeled arachidonic acid would be explained by the activation of phospholipase(s) by free radicals. The intermediate metabolites of arachidonic acid would be increased by the activity of both cyclo-oxygenase and lipoxygenase[8]. We have hypothesized earlier that both arachidonic acid and free radicals participate in the pathogenesis of brain edema[11]. This hypothetical mechanism has been extended by others[25]. It has been demonstrated by Siesjö's group that the damage of brain cells by severe hypoxia and by hypoglycemia involved both free radicals and free arachidonic acid[1,13]. Although the direct involvement of free radicals in membrane damage induced by arachidonic acid has not been proven, the present studies indicate that the oxygen radical metabolites formed by arachidonic acid are responsible for the increased amount of MDA which reflects lipid peroxidation. Although the release of MDA was greater in glioma than in neuroblastoma cells, such a differential response may not occur in vivo where it is not known whether glial or neuronal elements have differences in their susceptibility to free radical damage.

It seems unlikely that prostaglandins, the arachidonic acid-derived metabolites by cyclo-oxygenase, are involved in membrane perturbation for two reasons. First, the rate of conversion of arachidonic acid to PGE_2 and $F_2\alpha$ by cyclo-oxygenase in both neoplastic brain cells is slow. The accumulation of both PGE_2 and $F_2\alpha$ in both cell lines was approximately 1 % after the cells were incubated with arachidonic acid for 16 h (Table II). The free radical-treated synaptic membranes accumulated four-fold more arachidonic acid than the untreated membrane preparations[8], indicating the turnover rate of the newly released arachidonic acid to prostaglandins by cyclo-oxygenase was slow. Second, both PGE_2 and $PGF_2\alpha$ do not stimulate the release of endogenous arachidonic acid from membrane phospholipids (Table III). We have also demonstrated earlier that PGE_2 and $PGF_2\alpha$ were not involved in arachidonic acid-induced swelling in brain slices[3]. We speculate that the release and accumulation of arachidonic acid and of oxygen radical intermediates may cause the perturbation of the membranes of neurones, glial and endothelial cells in some forms of cellular injury and brain edema in vivo.

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