Journal of Cellular Biochemistry 27:449–453 (1985)

Stimulation of Phospholipase A_2 Activity by Oxygen-Derived Free Radicals in Isolated Brain Capillaries

A.M. Au, P.H. Chan, and R.A. Fishman

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

An exogenous free radical generating system added to isolated brain capillaries induces degradation of phospholipids. This inductive effect reflects increased phospholipase activities as measured by fatty acid composition of various phospholipid fractions. The correlation of phospholipid degradation with stimulation of phospholipases was further investigated by using cationic amphiphilic agents, which are known to be phospholipase A_2 inhibitors. The breakdown of phospholipids was inhibited by the pretreatment of isolated capillaries with these drugs.

Key words: phospholipid degradation, phospholipase A_2, free radicals, brain capillaries

Several recent in vitro [1,2] and in vivo [3,4] studies using free radical generating systems suggested that free radicals may alter the permeability of the blood-brain barrier and may change intracellular water, sodium, and potassium content, as well as induce phospholipid degradation and free fatty acid release. Furthermore, free radicals have been shown to alter the blood-brain barrier following cerebral trauma [5–8]. The in vivo work has shown that brain capillary endothelial cells are vulnerable to free radical damage.

The present study was undertaken to determine the effects of a free radical generating system on isolated brain capillaries. Preliminary observations have shown perturbation of phospholipids in isolated capillaries upon treatment with a free radical generating system. The present experiments are designed to elucidate those effects.

MATERIALS AND METHODS

All reagents were analytical grade and were obtained from a variety of commercial sources. Phospholipid standards were from Sigma Co. (St. Louis, MO), and free fatty acid standards were from Applied Sciences (State College, PA). Thirty-day-old Sprague-Dawley rats were from Simonsen (Gilroy, CA).

Received April 5, 1984; revised and accepted December 12, 1984.

© 1985 Alan R. Liss, Inc.

The protocol for isolation of a fraction enriched for small blood vessels has been adopted from the method pioneered by Goldstein et al [9]; however, the method has been modified with the use of a Pharmacia Percoll (Piscataway, NJ) density gradient to eliminate cell debris and myelin contamination.

Suspension of the capillary fraction was performed at a concentration of 0.5 mg/ml in Krebs-Ringer solution (pH 7.4) containing 2.5 mM adenosine triphosphate (ATP), 0.1 mM dithiothreitol (DTT), and 0.1 mM coenzyme A (CoA) in the presence or absence of 10 mM amphiphilic cationic agents or other drugs as indicated. Aliquots (0.5 ml) of capillary suspension were incubated at 37°C for 30 min. At the end of the incubation period, 0.5 ml Krebs-Ringer solution containing 1 mM hypoxanthine, 0.1 mM $FeCl_3$ and 1 mM adenosine diphosphate (ADP) were added to various aliquots, while control aliquots contained 0.5 ml Krebs-Ringer solution alone. A second incubation at 37°C for another 30 min was carried out.

Upon termination of the second incubation, extraction of phospholipids was performed twice with 2 ml $CHCl_3$:CH_3OH (2:1). Extracts were pooled and washed before concentration and separation on a Hewett Packard model 1082B high performance liquid chromatography (HPLC) system with a silica gel column (4 mm × 30 cm, Micropack Si-5, Varian, Palo Alto, CA). The mobile phase consisted of 4% water to 9% water in hexane:2-propanol (3:4) at a rate of 0.5%/min [10]. The flow rate was 1 ml/min at ambient temperature, and the elution was monitored at 206 nm with record response set at 0.0512 absorbance units (AU)/cm.

Free fatty acid fractions collected from HPLC were further analyzed by gas chromatography. A 0.25-mm internal diameter (i.d.) × 30-m fused silica capillary column was used. The flow rate was 33 ml/min, and the detector and injector temperature was 250°C. Temperature programming was used: an initial temperature of 40°C was maintained for 0.5 min, then increased to 220°C at the rate of 20°C per min; the final temperature was maintained for 9 min. The column was calibrated with free fatty acid standards.

RESULTS

Figure 1 shows the HPLC profile of lipids present in chloroform/methanol extracts of isolated brain capillaries treated with buffer (A), xanthine oxidase (B), xanthine oxidase in the presence of chloroquine (C), or mepacrine (D). As compared to the control sample (A), a decrease of several phospholipids and substantial increase of free fatty acids in xanthine oxidase treated capillaries were observed (Fig. 1B). Chloroquine, however, effectively inhibited the action of xanthine oxidase, preserving the phospholipid profile pattern (Fig. 1C) characteristic of the control sample (Fig. 1A). Mepacrine, a comparatively weaker inhibitor in this system, also was capable of inhibiting free fatty acid production (Fig. 1D).

Contrary to the enzymatically generated oxygen-derived free radicals, the action of paraquat, a nonenzymatic free radical generator [11], appeared to have no effect as an inducer of fatty acid production at various concentrations. In addition, denatured xanthine oxidase had no inductive effect.

Gas chromatographic analysis of free fatty acid fractions collected from HPLC from the free radical treated capillary fraction further indicated that polyunsaturated fatty acids, especially arachidonic acid and docosahexaenoic acid were the major

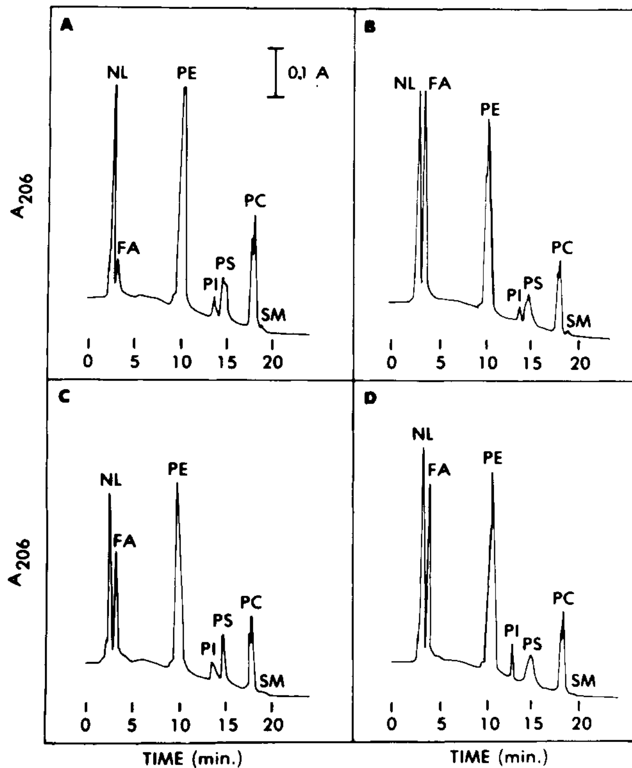

Fig. 1. High performance liquid chromatographs illustrating the effects of xanthine oxidase/hypoxanthine/ADP-Fe^{3+} treated isolated brain capillaries in the presence of buffer (B), chloroquine (C), and mepacrine (D), with (A) as untreated control. Capillaries isolated from 20 rat brains were resuspended at a concentration of 0.5 mg protein/ml in Krebs-Ringer solution and were incubated at 37°C for 30 min as discussed in Materials and Methods. NL, neutral lipids; FA, free fatty acids; PE, phosphatidylethanol amine, PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; SM, sphingomyelin.

components in that fraction (Table I). A decreased polyunsaturated fatty acid release was observed in fractions collected from capillaries pretreated with chloroquine or mepacrine and treated with xanthine oxidase. On the other hand, lipophilic heterocyclic compounds pyrimethanine and 5,7 diiodo-8-hydroxy-quinoline, when used in place of chloroquine or mepacrine, were unable to elicit any effect on xanthine oxidase induced phospholipid degradation (data not shown).

DISCUSSION

In vivo changes in vascular permeability can be initiated by enzymatically generated oxygen-derived free radicals [1,3]. In the present communication, we have studied these effects on brain capillaries. The integrity of the capillaries was established by electron microscopy; in addition, they were found to be metabolically active by glucose uptake and glucose transport studies, respectively [unpublished results].

TABLE I. Results of Free Fatty Acid Fractions Analysis*

	Free fatty acid (ng)/sample					
Samples	16:0	18:0	18:1	18:2	20:4	22:6
A	7.2	6.3	1.7	0.9	2.3	3.1
B	9.2	8.7	8.9	2.0	31.8	72.4
C	31.3	9.5	8.2	3.1	1.5	27.5
D	2.1	3.5	1.5	0.9	2.8	60.3

*These results of gas chromatographs show the effects of xanthine oxidase/hypoxanthine/ADP-Fe^{3+} treatment on the free fatty acid composition of isolated brain capillaries. A, buffer treated control; B, xanthine oxidase/hypoxanthine/ADP-Fe^{3+} treated capillaries in the presence of buffer; C, chloroquine; D, mepacrine. For experimental details, see Materials and Methods. 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid, 18:2, linoleic acid; 20:4, arachidonic acid; 22:6, docosahexaenoic acid. The results are the average of those obtained in two different experiments.

Data obtained using HPLC showed that xanthine oxidase is a potent inducer of phospholipid degradation. The fact that paraquat and denatured xanthine oxidase were ineffective suggests that this phenomenon was xanthine oxidase specific. Xanthine oxidase has been used commonly as a free radical generating system [12–14].

Subsequent to xanthine oxidase treatment, concomitant appearance of lysophospholipids as well as free fatty acids should be detected. However, the monitoring wavelength in this study was 206 nm, which selectively favored the detection of unsaturated fatty acids. The absorbance of lysophospholipids, possibly having lost their unsaturated fatty acids by the action of phospholipase A_2, would be extremely low at this absorbance even at high concentrations. Hence, our HPLC method would not be able to detect lysophospholipids with saturated acyl chains. Other studies undertaken in this laboratory [unpublished results] have indicated that the peak area of high concentrations of lysophospholipids in phospholipase A_2 treated brain homogenates is very small.

Chloroquine and mepacrine, amphiphilic cationic inhibitors of phospholipase A_2 [15–17], both blocked free fatty acid release in capillaries, suggesting that these responses may be dependent on phospholipase A_2 activities. This conclusion is supported by the finding that lipophilic heterocyclic compounds, which have not been shown to be phospholipase A_2 inhibitors, do not inhibit the release of free fatty acids or phospholipid degradation. Perhaps the amphiphilicity of the compound is important in the manifestation of this effect. There was no apparent effect of chloroquine or mepacrine on buffer treated capillaries alone. However, preliminary study does not allow discrimination between the interaction of these drugs with phospholipids and their interaction with phospholipases. Thus, we cannot unequivocally rule out the former possibility.

Preliminary studies where EGTA was included showed that it abolished the action of free radicals on free fatty acid release. Other laboratories have shown that calcium mobilization in a cell-free system is crucial for the expression of phospholipase A_2 activity [18]. At present, it is not possible to determine the exact mechanism by which calcium acts.

An interesting perspective is provided by comparing these data with similar studies on whole brain homogenates; the induction and inhibition effects are not as

dramatic [1]. Perhaps this is an indication that these effects on the target site (microvascular membrane) are masked by other compounds in the total homogenate.

It is clear that a free radical generating system has profound effects on membrane phospholipid degradation. This report also demonstrates that this effect can be inhibited by phospholipase A_2 inhibitors (chloroquine or mepacrine). The release of free fatty acid, especially arachidonic acid, is predominant. Arachidonic acid, once released from the cellular membrane, is rapidly converted either by cyclooxygenase to form prostaglandins, thromboxanes, and other radical intermediates or by lipoxygenase to form hydroperoxy fatty acids and leukotrienes [19,20]. These metabolites have been involved in capillary permeability changes and in inflammation [21-23]. Therefore, our findings are significant in view of the fact that vasogenic brain edema stems from changes in permeability of the blood-brain barrier [24].

ACKNOWLEDGMENTS

Our thanks to Drs. J.W. Schmidley, and S.F. Chen, and Ms. S.M. Longar for their assistance. This study was supported by NIH grant NS 14543.

REFERENCES

1. Chan PH, Yurko M, Fishman RA: J Neurochem 38:525-531, 1982.
2. Chan PH, Fishman RA: J Neurochem 35:1004-1007, 1980.
3. Chan PH, Schmidley JW, Fishman RA, Longar SM: Neurology (NY) 34:315-320, 1984.
4. Chan PH, Fishman RA, Caronna J, Schmidley JW, Prioleau G, Lee J: Ann Neurol 13:625-632, 1983.
5. Flamm ES, Demopoulos HB, Seligman ML, Posner RG, Ransohoff J: Stroke 9:445-447, 1978.
6. Demopoulos HB, Flamm E, Seligman M, Pretronigro D: "Pathology of Oxygen." New York: Academic Press, 1982, pp 127-155.
7. Demopoulos HB, Yoder M, Gutman EG, Seligman ML, Flamm ES, Ransohoff J: Scanning Electron Microsc 2:667-682, 1978.
8. Yoshida S, Abe K, Busto R, Watson BD, Kogure K, Ginsberg MD: Brain Res 245:307-316, 1982.
9. Goldstein GW, Wolinsky JS, Csejtey J, Diamond I: J Neurochem 25:715-717, 1975.
10. Chan PH, Fishman RA, Chen S, Chew S: J Neurochem 41:1550-1557, 1983.
11. Moody CS, Hassan HM: Proc Natl Acad Sci USA 79:2855-2859, 1982.
12. Kellogg E, Fridovich I: J Biol Chem 250:8812-8817, 1975.
13. Pederson TC, Aust SD: Biochem Biophy Res Commun 52:1071-1078, 1973.
14. Del Maestro RF, Thaw HH, Bjork J, Planker M, Arfors KE: Acta Physiol Scand 492:43-57, 1980.
15. Matsuzawa Y, Hostetler KY: J Biol Chem 225:5190-5194, 1980.
16. Lapetina EG: Trends Pharmacol Sci 3:115-118, 1982.
17. Dise CA, Burch JW, Goodman DBP: J Biol Chem 257:4701-4704, 1982.
18. Derksen A, Cohen P: J Biol Chem 250:9342-9347, 1975.
19. Sammuelsson B, Hammarstrom S, Borgeat P: Adv Inflam Res 1:405-411, 1979.
20. Wolfe LS: J Neurochem 38:1-14, 1982.
21. Moncada S, Vane JR: N Engl J Med 300:1142-1147, 1979.
22. Kuehl FA, Egan RW: Science 210:978-984, 1980.
23. Dahlen SE, Bjork J, Hedqvist P, Arfors KE, Hammarstrom S, Lindgren J-A, Sammuelsson B: Proc Natl Acad Sci USA 78:3887-3891, 1981.
24. Chan PH, Fishman RA: Fed Proc 43:210-213, 1984.