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MEMBRANE-BOUND LIPOXYGENASE OF RAT CEREBRAL MICROVESSELS

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SUMMARY: The microvessels isolated from rat cerebral cortex has arachidonate lipoxygenase activity, which was not due to possible contamination of the platelets. The major product was identified to be 12-hydroxyeicosatetraenoic acid. After homogenization and sonication of the microvessel preparations, the lipoxygenase activity was recovered both in the membrane- and the cytosol-fractions, whereas that in the platelets was recovered in the cytosol fraction. Membrane-bound lipoxygenase of the microvessels has apparent Km value of 3.8 μM for arachidonic acid, which was corresponded to 1/5 of that in the platelet enzyme. Microvessel lipoxygenase was inhibited by nordihydroguaiaretic acid but not by indomethacin.

Lipoxygenase metabolism of arachidonic acid to monohydroxyeicosatetraenoic acid has been demonstrated in a variety of mammalian cells and tissues (1-5). HPETE[3], a lipoxygenase product, is a potent inhibitor of prostaglandin biosynthesis in vascular tissues (6) and platelets (7). Greenwald et al. (8) demonstrated the lipoxygenase activity in rabbit aorta and suggested a potential role of the lipoxygenase products as an endogenous regulator of PGI_2 biosynthesis. Brain microvessels are capable to produce PGI_2 (9). PGI_2 and other products of arachidonic acid cascade may alter cerebrovascular tone and thus affect cerebral blood flow (10,11). On the basis of these observations, if brain microvessels have lipoxygenase pathway, it may be physiologically or pathologically important for the regulation of cerebral vascular tone or blood flow. These observations prompted us to determine the lipoxygenase activity in the microvessels islated from rat cerebral cortex.

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[3]The abbreviations used are:HPETE, hydroperoxyeicosatetraenoic acid; BSA, bovine serum albumin; 15-HETE, 15-hydroxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; GSH, glutathione reduced form; LDH, lactate dehydrogenase.

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MATERIALS AND METHODS

Materials: [1-^{14}C]Arachidonic acid (56.9 mCi/mmol) was purchased from the Radiochemical Centre, Amersham. Arachidonic acid, indomethacin, nordihydroguaiaretic acid and soybean lipoxygenase were from Sigma. Pre-coated Silica gel 60 was obtained from E. Merk.

Preparation of microvessels: Male Sprague-Dawley rats of 4 weeks-old were used throughout. The brain vasculature was perfused via left ventricle cannulas with cold saline to remove platelets and other blood components. Cerebral microvessels were isolated by the combination of BSA-density centrifugation and glass beads column chromatography described by Goldstein et al. (12) with modifications (13). After the step of glass beads column chromatography, the final microvessel pellets were suspended in 50 mM Tris-HCl (pH 7.4) and used for lipoxygenase assay. For each preparation, the microvessels corresponding about 4 mg protein was obtained from 10 rats. For sub-fractionation of microvessels, the microvessel suspensions were homogenized and sonicated for 5 min (27kHz, 150 W). These procedures were repeated twice. After centrifugation at 10,000 g for 15 min, the resulting supernatant (cytosol fraction) and the pellets (membrane fraction) were used for the enzyme assay. Sub-fractionation of the platelets was also carried out in the same manner. Purity of the isolated microvessels was examined microscopically and γ-glutamyl transpeptidase activity. Platelets were prepared from the rat by the method of Chang et al. (14).

Lipoxygenase assay: The incubation mixture contained 25 mM Tris-HCl (pH 7.4), 10 μM [1-^{14}C]arachidonic acid, 1 mM GSH and bout 100 to 300 μg protein of the microvessel preparations in a total volume of 600 μl. Reactions were carried out in air at 37°C for 60 min and stopped by the addition of 4 μl of 1 N HCl. The mixture was extracted with 3 ml of cold ethyl acetate, and the organic layer was evaporated under N_2. The residue was dissolved in a small amount of ethyl acetate, and applied to thin-layer chromatography (plate; pre-coated Silica gel 60, solvent; ethyl acetate/isooctane/acetic acid/water=80/50/20/100). The radioactive spots were detected by radioautography, scrapped off, and the radioactivities were determined in a liquid scintillation counter. Platelet lipoxygenase activity was examined in the same manner as described by others (14) except for that 1 mM GSH was present. High-performance liquid chromatographic separation of the lipoxygenase products was carried out on a Zorbax Sil column (0.46 x 25 cm, Shimadsu) with n-hexane/ehtanol/acetic acid (993/6/1) as a carrier solvent. 12-HETE and 15-HETE were purified from the products of platelet- and soybean-lipoxygenases, respectively, with thin-layer and high-performance liquid chromatographies.

γ-Glutamyl transpeptidase activity was assayed as the marker enzyme of microvessels according to the method of Szasz (15). The activities of LDH (16) and alkaline phosphatase (17) were also determined. Protein concentration was determined by the method of Lowry et al. (18).

RESULTS

Microscopic examination of the microvessel preparations with Giemsa staining indicated that the preparations predominantly consisted of long strands of capillaries as well as arterioles and venules (Fig. 1). γ-Glutamyl transpeptidase may be located primarily within capillaries (19). In the present preparations, γ-glutamyl transpeptidase activity was enriched 24-fold when compared to cerebral cortex (Table 1). The microvessel suspensions were

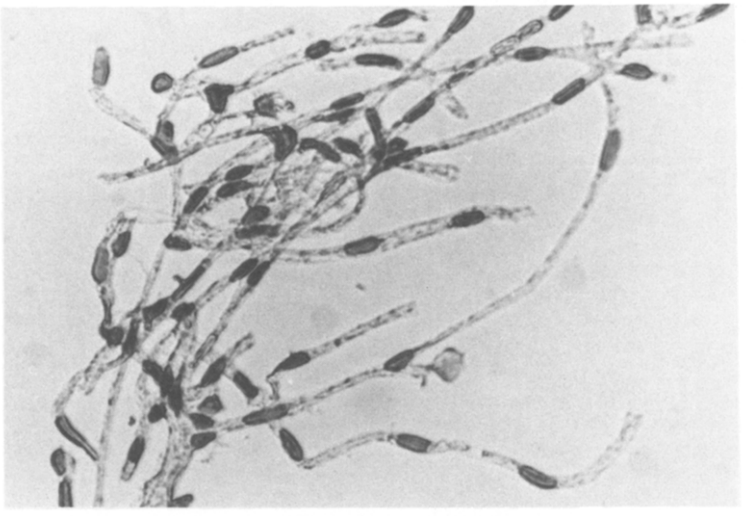

Fig. 1. Micrograph of a typical microvascular fraction with Giemsa staining. Microvessels were isolated by the procedure outlined under Methods. The preparations consisted virtually entirely of capillaries and were essentially free of nonvascular material (×400).

incubated with [1-^{14}C]arachidonic acid at 37°C for 60 min, and the products were subjected to thin-layer chromatography, showing that one major radioactive product was detected of which Rf value is corresponded to that of 12-HETE and 15-HETE (data not shown). The reaction mixture was subjected to high-performance liquid chromatography (Fig. 2). Retention time of the products was coincided with that of authentic 12-HETE. Boiled microvessels suspensions

Table 1. 12-Lipoxygenase activity of the microvessel preparations of rat cerebral cortex. The enzyme activities were determined as described in the Methods. Subfractionation of the microvessels was carried out as described in the Methods. *Each value represents the mean ± S.E. of 6 separate experiments. γ-GTP; γ-glutamyl transpeptidase (nmol/mg protein/hr). N.D.; not determined.

	12-Lipoxygenase* (pmol/mg protein/hr)	γ-GTP*	LDH (%)	Alkaline phosphatase (%)
Cortex				
Homogenates	1.4 ± 0.3	299 ± 41	N.D.	N.D.
Microvessels				
Homogenates	50.9 ± 3.2	7363 ± 1769	100	100
Supernatant	63.3 ± 13.5	N.D.	100	38
Pellets	179.4 ± 73.2	N.D.	0	62

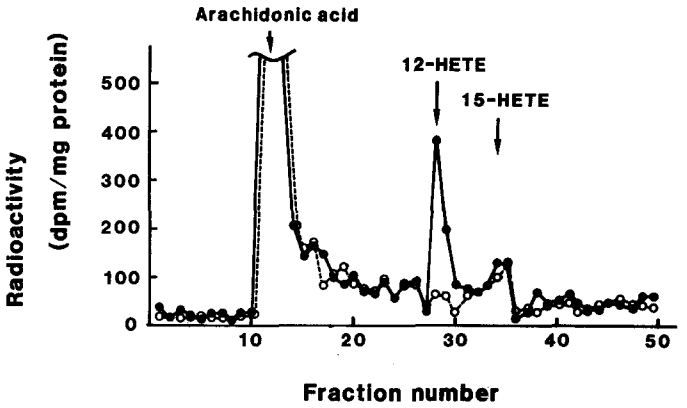

Fig. 2. Straight-phase high performance liquid chromatogram of [^{14}C]arachidonic acid products formed by incubation of the microvessels from rat cerebral cortex. The microvessel homogenates (●) and the boiled homogenates (○) were incubated for 60 min at 37°C in the presence of 3 μM [^{14}C]arachidonic acid. The peaks corresponding to elution of arachidonic acid, 12-HETE and 15-HETE are indicated by the arrows.

had no such an activity. As shown in Table 1, the activity of 12-HETE production (12-lipoxygenase) of microvessels was enriched about 36-fold as compared with that of cortical homogenates. Platelets have soluble 12-lipoxygenase of which activity (9.0 ± 2.5 nmol/mg protein/5 min) is much higher than that of microvessel preparations. To distinguish the microvessel lipoxygenase activity from that of the platelets, some characterization of the enzyme activity in the microvessels were performed. After homogenization and sonication of the microvessels, LDH activity, a marker enzyme in the cytosol, was recovered in the supernatant (Table 1). In addition, microscopic examination showed that the homogenized- and sonicated-microvessels were found to be composed of the destructed membrane fragments (data not shown). After the same treatments, lipoxygenase activity of the microvessel preparations was located both in the membrane- and the cytosol-fractions (Table 1), whereas that of the platelets was predominantly located in the cytosol fraction; microvessels, 54% in the cytosol and 46% in the membranes; platelets, 96% in the cytosol and 4% in the membranes. Km value for arachidonic acid for the lipoxygenase was compared. Km values of the enzymes in microvessels and platelets were 3.8 and 19 μM, respectively. Vmax of the platelet lipoxygenase reaction was about 600

times higher than that of the microvessel enzyme. With respect to the effect of inhibitors of arachidonic acid metabolism, nordihydroguiaretic acid inhibited the activity of microvessel lipoxygenase by 57% and 73% at the concentrations of 10 and 100 μM, respectively. In contrast, indomethacin did not affect the enzyme activity at the concentrations of up to 100 μM.

In the present study, we detected the 12-lipoxygenase activity in the microvessels of rat cerebral cortex. Platelets have soluble 12-lipoxygenase of which activity was extremely high as compared that of microvessel preparations. Therefore, a little contaminated platelet, if so, may account for the lipoxygenase activity in the preparations. To eliminate the possible contamination of the platelets and other blood cells, the brain vasculature was perfused via left ventricle cannulas with cold saline. Glass beads column chromatography also excludes contaminated blood cells from the preparations. Thus, microscopic examination of the microvessel preparations showed that it seemed to be free of blood cells. However, this result is not sufficient to eliminate the possibility of the lipoxygenase activity of the preparations being due to contaminated platelets. If possible contaminated platelets might account for the lipoxygenase activity in the microvessel preparations, most of the enzyme activity should be recovered in the supernatant after homogenization and sonication of the preparations. However, this is not the case; lipoxygenase activity of the microvessel preparations was equally recovered in the cytosol- and the membrane-fractions after the same treatments. Recently, it was found that erythrocytes have 12-lipoxygenase activity (20). We also detected 12-lipoxygenase in rat erythrocytes of which activity is 35 pmol/mg protein/hr. This value is within the same order as that of the microvessel preparations. Therefore, it is reasonable to eliminate the possibility of contaminated erythrocytes accounting for the lipoxygenase activity of the microvessel preparations. In agreement with the previous report (14), Km value for arachidonic acid of rat platelet lipoxygenase was 19 μM. In contrast, that of the microvessels was about 4 μM. This result also indicates the difference between the enzymes in the microvessels and the platelets. Thus, the membrane-

bound 12-lipoxygenase in the microvessels has higher affinity for arachidonic acid than that in the platelets.

Brain microvessels are capable to produce PGI_2 which may regulate cerebrovascular tone (10,11). Since HPETE is a potent inhibitor of PGI_2 biosynthesis, the membrane-bound lipoxygenase in the microvessels may play significant roles of the regulation of the brain vascular system.

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