Localization of Arachidonate 12-Lipoxygenase in Parenchymal Cells of Porcine Anterior Pituitary*

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Natsuo Ueda‡, Akihiko Hiroshima‡‡, Kiyoshi Natsui, Fukiko Shinjo§§, Tanihiro Yoshimoto¶¶, Shocho Yamamoto††, Kunio Ii**, Kiryaki Gerozissis$$, and Fernand Dray†††

From the Departments of ‡Biochemistry and ‡‡Pathology School of Medicine, Tokushima University, Kuramoto-cho, Tokushima 770, Japan and the $$Unite de Radioimmunologie Analytique, Institut Pasteur, Paris, 75724 France.

12-Lipoxygenases oxygenate arachidonic acid producing its 12S-hydroperoxy derivative and are well known as platelet and leukocyte enzymes. When a peroxidase-linked immunoassay of the enzyme according to the avidin-biotin method was applied to cytosol fractions from various parts of porcine brain, a considerable amount of the enzyme was found in the anterior pituitary. The enzyme level (about 200 ng/mg cytosol protein) corresponded to about 6% of the enzyme content in porcine peripheral leukocytes. Posterior and intermediate lobes showed about one-tenth of the enzyme level of anterior pituitary. Other parts of porcine brain contained the 12-lipoxygenase in amounts below 7 ng/mg cytosol protein. The cytosol fraction (0.7 mg of protein) of anterior pituitary produced 12S-hydroxy-5,8,10,14-eicosatetraenoic acid from 25 μM arachidonic acid in about 34% conversion at 24 °C for 5 min, giving a specific enzyme activity of about 3 nmol/min/mg protein. Furthermore, various octadecapolyenoic acids were oxygenated almost as fast as the arachidonate 12-oxygenation. When anterior pituitary was investigated immunohistochemically with anti-12-lipoxygenase antibody, most of the immunostained cells were certain parenchymal cells with granules, which were not blood cells. These biochemical and immunohistochemical results provide a good reason for considering that 12-lipoxygenase does play an important role in pituitary function.

Arachidonate 12-lipoxygenase has been found in various mammalian tissues, especially in platelets (1, 2) and leukocytes (3, 4). As pointed out by Brash (5), “the pathways of arachidonic acid metabolism that are understood lead to the formation of potent biological mediators such as the prostaglandins and leukotrienes. However, there is no obvious analogy to these mediators in the 12-lipoxygenase pathway.” Nakan et al. (6) reported an extremely potent activity of 12-HETE (but not 5-HETE and 15-HETE), which stimulated the migration of rat aortic smooth muscle cells at a concentration in the order of 10 fm. A more recent work by Piomelli et al. (7) demonstrated that the neurotransmission in the marine mollusk, Aplysia californica, was mediated by the 12-lipoxygenase product, 12-HETE, or its metabolites such as 12-keto-5,8,10,14-eicosatetraenoic acid (8) and 8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid (9). Furthermore, 12S-HETE (but not 12R-HETE) was proposed to participate in the expression or activation of a tumor cell glycoprotein receptor (10). Generalization of these intriguing observations to many other mammalian tissues has not yet been established. Thus, the general physiological function of the 12-lipoxygenase pathway is still an open question.

Previously, we prepared monoclonal antibodies against the 12-lipoxygenase of porcine leukocytes and developed a peroxidase-linked immunoassay for determination of the enzyme amount in various porcine tissues (11). However, the occurrence of the 12-lipoxygenase in brain remained unclarified due to the insufficient sensitivity of the assay. As detected by transformation of cyclooxygen arachidonic acid, the 12 lipoxygenase activity was found previously in pituitary (12-16), pineal gland (16), and other parts of the rat brain (17). These previous observations do not necessarily indicate the occurrence of 12-lipoxygenase in the parenchymal cells of these organs rather than contaminating or infiltrating blood cells. Recently, we improved the enzyme immunoassay of 12-lipoxygenase and increased its detectability by introducing the solid-phase and avidin-biotin methods and applied the modified sensitive assay to detect the 12-lipoxygenase in various parts of porcine brain. In addition, we performed immunohistochemical studies on the distribution and localization of 12-lipoxygenase in brain tissues.

**EXPERIMENTAL PROCEDURES**

Materials—Various unsaturated fatty acids were purchased as described previously (18). High molecular weight standard mixture for polyacrylamide gel electrophoresis was obtained from Sigma, Immunoprobe 1 from Nunc (Roskilde, Denmark), horse-rasidase peroxidase-avidin D and N-hydroxysuccinimidobiotin from Pierce,Vectastain ABC-kit, goat biotinylated anti-rabbit IgG antibody, and normal goat serum from Vector Laboratories (Burlingame, CA), o-phenylenediamine from Nakarai (Kyoto), 3,3’-diaminobenzidine 4 HCl from Dojin (Kumamoto), and P-10 column from Pharmacia (Uppsala).

1The abbreviations used are: 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 5-HETE, 5-hydroxy-6,8,10,14-eicosatetraenoic acid; 9-HETE, 9-hydroxy-5,7,11,14-eicosatetraenoic acid; 12-HPETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13E-eicosatetraenoic acid; HETE, hydroxy-eicosatetraenoic acid; HPLC, high performance liquid chromatography.
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Quotol 812 was supplied from Nissin EM (Tokyo), Entellan Neu and amium tetroxide from Merck (Darmstadt), and O.C.T. compound from Miles (Elkhart, IN). Monoclonal antibodies (lox-1 and lox-2) against porcine leukocyte 12-lipoxygenase were raised in mice as described previously (11). Both species reacted with 12-lipoxygenase without the loss of enzyme activity. As described in Ref. 19, a polyclonal anti-12-lipoxygenase antibody was raised in rabbits using the purified enzyme. The antibody was then affinity-purified in an activated cyanogen bromide-streptavidin column. In Western blotting of the porcine leukocyte cytosol, this antibody recognized only one band corresponding to the purified leukocyte 12-lipoxygenase (19). 5-Lipoxygenase, which was also purified from porcine leukocytes (20), did not react with the polyclonal anti-12-lipoxygenase antibody. The cytosol fraction and the purified 12-lipoxygenase (specific enzyme activity, 4 amol/mg min) were treated at 30 °C with enzyme from porcine leukocytes as described in Ref. 19. 5-HETE (21), 12-HETE, SHETE, 13-hydro(pero)xy-9,11-octadecadienoic acid (18), and 15-hydroxy-11,13-eicosadienoic acid (4) were prepared as described previously.

Enzyme Immunoassay of 12-Lipoxygenase—Each part of brain (totally about 1 g wet weight) collected and combined from several pigs was homogenized in 5 volumes of phosphate-buffered saline, pH 7.4, by the use of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 27,000 × g for 10 min and then at 105,000 × g for 60 min. The supernatant solution was stored at −80 °C as the cytosol fraction. Protein concentration was determined by the method of Lowry et al. (22) with bovine serum albumin as a standard.

The polyclonal anti-12-lipoxygenase antibody (lox-1 and lox-2), which recognized different sites of 12-lipoxygenase (11), were utilized. lox-2 (1.0 mg) was incubated with 0.1 mg of N-hydroxy-succinimido-biotin at room temperature for 1 h. The remaining N-hydroxy-succinimido-biotin was removed by washing with 50 mM phosphate-citrate buffer, pH 5.0, containing 1% glutaraldehyde. The glass slides were immersed in 0.1% osmium tetroxide dissolved in 0.1 M phosphate buffer, pH 7.4, at 4 °C for 1 h, dehydrated with ethanol, and embedded in Quetol 812. Ultrathin sections were prepared by an LKB model 8800 ultramicrotome and investigated with a Hitachi H-300 electron microscope.

Immunostaining for electron microscopy was almost the same as that described above for light microscopy except for the minor modifications. After incubation with a peroxidase-labeled biotin-avidin complex, the specimen were treated for 5 min with 0.1 M phosphate buffer, pH 7.4, containing 1% glutaraldehyde. The glass slides were immersed in 0.1 M phosphate buffer, pH 7.4, containing 1% gelatin for 2 min, air-dried, and further fixed with the 1% glutaraldehyde for 5 min. After the glass slides were preincubated at room temperature for 30 min with 3,3′-diaminobenzidine only, the peroxidase reaction was performed for 5 min at room temperature in the presence of hydrogen peroxide. Then the glass slides were treated with 2% osmium tetroxide dissolved in 0.1 M phosphate buffer, pH 7.4, at 4 °C for 1 h, dehydrated with ethanol, and embedded in Quotol 812. Ultrathin sections were prepared on a LKB model 8800 ultramicrotome and investigated with a Hitachi H-300 electron microscope.

RESULTS

Predominant Occurrence of 12-Lipoxygenase in Porcine Pituitary—The standard peroxidase-linked immunooassay was performed to screen the 12-lipoxygenase contents in various parts of porcine brain. As listed in Table I, the highest enzyme content was found in anterior pituitary. The enzyme level was about 6% of that of peripheral leukocytes which were known as the richest source of 12-lipoxygenase in pig (11). About 10% of the enzyme contents of anterior pituitary was found in posterior and intermediate lobes of pituitary which were excised together. Other parts of porcine brain tested showed a very low content of 12-lipoxygenase (about 0.1% of that in peripheral leukocytes). Although rat pineal gland was reported to have a relatively high 12-lipoxygenase activity (16), it should be noted that only a low enzyme content was found in porcine pineal gland. The cytosol fraction of most regions of porcine brain listed in Table I was also incubated with 25 μM [14C]arachidonic acid. Only anterior and posterior pituitary showed a detectable 12-lipoxygenase activity.

Catalytic Properties of 12-Lipoxygenase of Porcine Anterior Pituitary—The cytosol fraction of anterior pituitary was incubated with arachidonic acid. The hydroperoxy products were reduced with borohydride and analyzed by reverse-phase HPLC. As monitored at 235 nm for a conjugated diene, a major peak appeared at 18.3 min (Fig. 1).
The cytosol fraction of each part of porcine brain was prepared, and the 12-lipoxygenase content was determined by the standard enzyme immunoassay. The cytosol fraction of each tissue was included in various amounts in the assay mixture, and the enzyme content was determined from the linear range of the results. The listed values were corrected for the background determined in the absence of IgI. Values are means ± S. D. for n experiments, each with a sample from several animals; low values presented without S.D. were determined with several samples, and the mean values are listed.

| Tissues          | 12-Lipoxygenase content (ng/mg cytosol protein) |
|------------------|-----------------------------------------------|
| Neocortex       | <1                                            |
| Corpus callosum | 6                                             |
| Olfactory bulb  | 5                                             |
| Hippocampus     | <1                                            |
| Caudate nucleus | 2                                             |
| Globus pallidus | <1                                            |
| Thalamus        | 2                                             |
| Hypothalamus    | 6 ± 3 (n = 4)                                 |
| Pituitary       |                                               |
| Anterior lobe   | 221 ± 116 (n = 6)                             |
| Posterior and middle lobes | 22 ± 8 (n = 5)                           |
| Pineal body     | 2 ± 1 (n = 3)                                 |
| Cerebellum      | 3                                             |
| Cerebellar peduncle | 4                                       |
| Superior colliculus | 4                                    |
| Inferior colliculus | 4                                 |
| Pons            | 3                                             |
| Medulla oblongata | 7                                      |
| Peripheral leukocytes | 3891 ± 1645 (n = 4)                          |

1B), and cochromatographed with authentic 12-HETE (Fig. 1A). A minor peak (retention time, 16.4 min), which was coeluted with authentic 13-HETE, was also found. A peak corresponding to 5-HETE was hardly detected even in the presence of calcium ion (24) and ATP (25), which are known as 5-lipoxygenase activators. When arachidonic acid was incubated with the cytosol fraction of cerebellum, no significant amount of 12-HETE was detected in sharp contrast to the anterior pituitary (Fig. 1C). The specific 12-lipoxygenase activity of the cytosol fraction of anterior pituitary was calculated from the experimental result presented in Fig. 1B and was found to be 5.0 nmol/5 min/mg protein at 24 °C with arachidonic acid as a substrate. This value corresponded to 6% determined by the enzyme immunoassay. The 12-HETE synthesized by the cytosol fraction of anterior pituitary, was purified by reverse-phase HPLC and then derivatized to its methyl ester. Absolute configuration of the 12-HETE methyl ester was determined by chiral phase HPLC as described in Ref. 26. The ratio of the optical isomers (S/R) was approximately 96:4.

As examined by reverse-phase HPLC monitoring at 235 nm for a conjugated diene of oxygenated products, the cytosol fraction of anterior pituitary was active with the following three octadecapolyenoic acids: linoleic acid, 5.5 nmol/5 min/mg protein at 24 °C (110% of the activity with arachidonic acid); α-linolenic acid, 4.6 (92%); γ-linolenic acid, 5.7 (114%). The products from these three fatty acids were chromatographically indistinguishable from the oxygenated products obtained by the purified enzyme of porcine leukocytes (18).

Localization of 12-Lipoxygenase in the Parenchymal Cells of Porcine Anterior Pituitary—The specificity of the polyclonal anti-12-lipoxygenase antibody to be utilized in the following immunohistochemical studies was examined by immunoblotting. The cytosol fraction of porcine anterior pituitary showed a major colored band (Fig. 2). The band appeared at the position corresponding to a molecular weight of about 68,000, which was slightly lower reproducibly than that of the purified 12-lipoxygenase of porcine leukocytes (72,000) (18). The band was not detected in the absence of the antibody. The anterior pituitary cytosol was incubated with a monoclonal anti-12-lipoxygenase antibody lox-2, and 12-lipoxygenase was immunoprecipitated with the aid of protein A-bearing S. aureus. Immunoblottting of the 12-lipoxygenase-free cytosol fraction thus prepared failed to detect the above-mentioned positive band by the use of the polyclonal antibody. Disappearance of the major band in this experiment supported its identity with 12-lipoxygenase protein. It was possible that the 12-lipoxygenase contained in porcine pituitary was derived from infiltrating or contaminating leukocytes. Therefore, the localization of 12-lipoxygenase in porcine pituitary was investigated immunohistochemically using the polyclonal anti-12-lipoxygenase antibody. As examined by light microscopy, about 7% of anterior pituitary parenchymal cells found in Fig. 3A were positively stained. These cells were distinguishable from granulocytes in blood vessels, which were also positively stained. No stained cells were found in anterior pituitary in a control experiment with an IgG fraction of the anti-12-lipoxygenase antiserum was incubated with the purified leukocyte 12-lipoxygenase at 4 °C overnight, and the IgG fraction thus treated failed to stain the parenchymal cells of anterior pituitary. As shown in Fig. 4, immunoelectron mi-
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FIG. 2. Immunoblotting of 12-lipoxygenase contained in porcine anterior pituitary. The cytosol fraction of anterior pituitary (58 μg of protein) (lane A) and the purified 12-lipoxygenase of porcine leukocytes (40 ng protein) (lane B) were subjected to 7.5% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (1-mm thick) (37). Electrophoretic transfer of protein bands from the gel to a nitrocellulose membrane (38) and immunostaining with polyclonal anti-12-lipoxygenase antibody were performed as described previously (26).

FIG. 3. Light microscopy of porcine anterior pituitary stained with polyclonal anti-12-lipoxygenase antibody (A) or non-immunized rabbit IgG (B). Bar = 50 μm. Arrow, a granulocyte in blood vessel.

FIG. 4. Immunelectron microscopy of a porcine anterior pituitary cell stained with polyclonal anti-12-lipoxygenase antibody. Bar = 1 μm. N, nucleus.

DISCUSSION

Our improved peroxidase-linked immunoassay of 12-lipoxygenase allowed the determination of 12-lipoxygenase content in porcine brain, which was impossible by the previous less sensitive method (11). The highest enzyme content was found in anterior pituitary. In agreement with this observation, incubation of the cytosol fraction of porcine anterior pituitary demonstrated the 12-lipoxygenase activity with exogenous arachidonic acid as a substrate. Several previous papers described the conversion of exogenous arachidonic acid to 12-HETE in rat pituitary. Pilote et al. (12) incubated 50 μM [14C]arachidonic acid for 10 min with rat anterior pituitary as such. 12-HETE was produced as the major product from added radioactive arachidonic acid in a 0.1% conversion rate. When our group reported a 12-lipoxygenase activity in rat pineal gland, we also detected a very low rate of conversion from 10 μM arachidonic acid to 12-HETE (only less than 0.2%) in rat pituitary (16). Vanderhoek et al. (13) prepared a gonadotroph-enriched cell fraction from rat pituitary. Incubation of this cell fraction with radioactive arachidonic acid produced a variety of metabolites of the cyclooxygenase pathway and several lipoxygenase products including 12-HETE as a minor component. The cyclooxygenase products were predominant by at least 3-4-fold over the lipoxygenase products. In all these earlier works the 12-oxygenation of arachidonic acid was observed to a minor extent, and it was unclear whether such a minor conversion was attributed to the parenchymal cells of pituitary or to the contaminating platelets or leukocytes. Indeed, our recent immunohistochemical work demonstrated the distribution of 12-lipoxygenase in porcine alimentary tract and lymphatic organs in addition to leukocytes in peripheral blood. Only various types of resident leukocytes were positively stained in the former organs (19). By the use of cloned tumor cells of rat pituitary, Rabier et al. (15) mentioned the production of 12-HETE (in a yield of 14% of all the products) together with 15-, 5-, and 9-HETEs (66, 11, 9%, respectively) from exogenous arachidonic acid. The production of a mixture of HETEs suggests a lack of stereospecificity usually associated with non-enzymic oxygenation. Alternatively, a small percentage of 12-HETE could arise as a minor product of a 15-lipoxygenase reaction as is known to occur with the reticulocyte enzyme (27).

Thus, these previous papers described a minor extent of 12-HETE production from a quantitative view and a heterogeneous composition of products from a qualitative view. In contrast, our present work demonstrated clearly the occurrence of 12-lipoxygenase in porcine pituitary on the basis of not only the arachidonate transformation but also the peroxidase-linked immunoassay. Furthermore, we demonstrated...
the localization of the 12-lipoxygenase in some parenchymal cells of anterior pituitary rather than contaminating or resident blood cells. We attempted to identify the 12-lipoxygenase-containing pituitary cells by the use of antibodies specific for luteinizing hormone, follicle-stimulating hormone, thyroid-stimulating hormone, and adrenocorticotropic hormone of porcine origin. We have not yet tested antibodies against growth hormone and prolactin. The use of these antibodies has not clarified as yet whether the 12-lipoxygenase-containing cells are composed of several subgroups each secreting different hormone or they are associated with a group of pituitary cells secreting a specific type of hormone.

Selective production of S-isomer of 12-HPETE is known as a common feature of mammalian 12-lipoxygenase of platelet (1) and leukocyte (26), whereas cytochrome P-450 was reported to produce predominantly 12R-HPETE over 12S-epimer in a ratio of 81:19 (28). Since the 12-lipoxygenase of porcine anterior pituitary produced selectively 12S-HPETE, the enzyme is distinguishable from the cytochrome P-450 as a 12R-hydroxylase, which might be contained in the microsome of porcine anterior pituitary. Casepiedeva and others (14) reported the production of 12-HETE without a mention of stereochemistry as one of various oxygenated products when the microsome of rat anterior pituitary was incubated with arachidonic acid.

It was proposed that there were two types of 12-lipoxygenase, a leukocyte type and a platelet type (4, 26, 29). The high reactivities of anterior pituitary 12-lipoxygenase with linoleic and linolenic acids were similar, if not identical, to the activities of porcine leukocyte 12-lipoxygenase with these octadecapolyenoic acids (18). In contrast, platelet 12-lipoxygenase was almost inactive with these octadecapolyenoic acids (2, 26, 29). Thus, the 12-lipoxygenase of porcine anterior pituitary is a leukocyte-type enzyme in terms of the catalytic properties. Incidentally, it should be noted that 12-lipoxygenase was not detected in porcine platelets by the enzyme immunoassay and the activity assay (11). It cannot be ruled out that the platelet-type 12-lipoxygenase is also present in the cytosol of porcine anterior pituitary in such a small amount that does not significantly affect the results of substrate specificity study. Incubation of arachidonic acid with the cytosol fraction of various parts of porcine brain except the cytosol of porcine brain did not produce 12-HPETE. Thus, in these areas for pituitary did not produce 12-HPETE. Thus, in these areas.

Several papers have reported stimulatory effects of arachidonic acid on the hormone release from anterior pituitary cells of rat: luteinizing hormone by 5,6-epoxyeicosatrienoic acid (31), leukotriene C4 (32) and 5-HPETE (33), and prolactin by 5-HETE (44). The presence of 12-lipoxygenase in porcine anterior pituitary suggests a possible role of 12-HPETE or its metabolites in the hormone release from porcine pituitary cells.

A previous finding that 12-HETE stimulated the release of luteinizing hormone-releasing hormone from rat median eminence (35) prompted us to carefully examine the 12-lipoxygenase content in porcine median eminence. However, only a very small amount of the enzyme was detected in the porcine hypothalamic area containing median eminence. Earlier we screened lipoxygenase activities in rat brain tissues using 14C-labeled arachidonic acid. The 12-lipoxygenase activity was by far the highest in pineal gland, and less than 5% of the activity was found in pituitary gland and hypothalamus (16). In relation to this observation, 12-HETE, but not 12-HETE, stimulated melatonin synthesis in rat pineal gland (36). However, in the present work the enzyme immunoassay showed only a low enzyme content in porcine pineal gland. The cytosol of porcine pineal gland did not show the 12-lipoxygenase activity as examined by incubation with arachidonic acid. Thus, porcine pineal gland does not appear to contain significant amount of 12-lipoxygenase of both the leukocyte-type and the platelet-type.

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