Lipoxygenase Activity in Rat Kidney Glomeruli, Glomerular Epithelial Cells, and Cortical Tubules*

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(Received for publication, December 21, 1981)

We examined the possibility that renal glomerular and cortical tubular tissue has lipoxygenase activity in addition to the well established cyclooxygenase pathway of arachidonic acid metabolism. Homogenized rat kidney glomeruli, in the presence of meclofenamate (33 μM) and divalent cation ionophore A23187 (3 μM), metabolized octatritiated arachidonic acid to 12-hydroxyeicosatetraenoic acid and lesser amounts of 5- and/or 9-hydroxyeicosatetraenoic acid. These products were identified by thin layer chromatography, high-performance liquid chromatography, and gas chromatography-mass spectroscopy. In order to rule out the synthesis of hydroxylated fatty acids by platelets and leukocytes entrapped in the glomeruli, we studied lipoxygenase products in glomerular epithelial cells after 9 days in cell culture. Homogenized glomerular epithelial cells converted octatritiated arachidonic acid to 12-hydroxyeicosatetraenoic acid solely. The lipoxygenase activity in cortical tubules was substantially less than in glomeruli and only 12-hydroxyeicosatetraenoic acid was synthesized. The production of hydroxyeicosatetraenoic acid by homogenized glomeruli, glomerular epithelial cells, and cortical tubules was inhibited by three lipoxygenase inhibitors, nordihydroguaiaretic acid, 5,8,11,14-eicosatetraenoic acid, and 1-phenyl-3-pyrazolidone. These data demonstrate that there is lipoxygenase activity in rat kidney glomeruli, glomerular epithelial cells and to a lesser extent cortical tubules, and may imply a role of the lipoxygenase products in the regulation of normal glomerular function and inflammatory disease of the kidney.

Lipoxygenase metabolism of arachidonic acid to monohydroxyeicosatetraenoic acids has been demonstrated in a variety of mammalian cells and tissues (Goetzl and Sun, 1979; Siege1, 1979, Herman, 1979; Hamberg, 1976; Hamberg et al., 1980; Borgeat and Samuelsson, 1979a). HETEs' have been found to mediate various physiological processes such as histamine secretion from rat mast cells (Sullivan and Parker, 1979; Stenson et al., 1980), neutrophil degranulation (Stenson and Parker, 1980), chemotaxis (Goetzl and Sun, 1979; Goetzl, 1980; Ford-Hutchinson et al., 1979) and chemokinesis.

Previous studies conducted in this laboratory have demonstrated that isolated rat kidney glomeruli metabolize octatritiated arachidonic acid to an array of prostaglandins and an unidentified substance(s) which has chromatographic mobility consistent with that of a hydroxylated fatty acid (Hassid et al., 1979). The present study was performed to identify this substance. The lipoxygenase products were separated and identified by TLC, HPLC, and GC-MS. Our results show that there is lipoxygenase activity in rat kidney glomeruli and glomerular epithelial cells and to a lesser extent in cortical tubules.

MATERIALS AND METHODS

Isolation of Perfused Glomeruli—Sprague-Dawley rats of either sex (100-200 g) were anesthetized with pentobarbital (30 mg/kg of body weight, intraperitoneal). The kidneys were perfused with isotonic, heparinized saline until blanched (~50-50 ml of saline 2-3 min). The perfused kidneys of two rats were used for each experiment. The cortex from the four kidneys was dissected and minced to a paste-like consistency in PBS containing 150 mM NaCl, 10.6 mM Na₂HPO₄, and 3.8 mM NaH₂PO₄ pH 7.4. The glomeruli were then separated from other cortical tissue by successively passing the minced tissue through 106- and 75-μm metal sieves using the technique previously described (Hassid et al., 1979). The purity of the isolated glomerular fraction was determined microscopically, by counting the number of glomerular and nonglomerular particles suspended in a given volume. The purity of the glomerular fraction varied between 95 and 98%. Virtually no afferent and efferent arterioles could be detected in the glomerular preparation. The isolated glomeruli were then gently homogenized in PBS using a glass homogenizer. In some experiments glomeruli were lysed by several cycles of freeze-thawing instead of being homogenized by hand.

Glomerular Incubations with Tritiated Arachidonate—Homogenized glomeruli were divided into five equal portions. Each portion (0.5 ml, 0.72 mg of protein) was incubated with [3H]arachidonate (20 Ci/mmol, 3-5 × 10⁶ dpm, New England Nuclear), divalent cation ionophore, A23187 (3 μM, Calbiochem-Behring) and sodium meclofenamate (33 μM, Lambert/Parke Davis) in modified Earle's balanced salt solution containing gelatin (0.01 mg/ml) and 25 mM, HEPES, pH 7.4. The total volume of each incubation was 3 ml. Three lipoxygenase inhibitors were used in the study. These included a relatively selective inhibitor of lipoxygenase, NDGA (33 μM, Sigma) (Tappel et al., 1968; Ford-Hutchinson et al., 1979; Hamberg, 1976); and two inhibitors of cyclo-oxygenase and lipoxygenase, ETYA (170 μM, Hoffmann-La Roche, NJ) and phenidone (670 μM, Sigma) (Blackwell and Flower, 1978; Baumann et al., 1980). A fraction of glomerular homogenate was boiled for 5 min prior to incubation and was used as a control for nonenzymatic conversion of C20:4 to lipoxygenase-like products. Incubation was carried out at 37°C for 45 min. Homogenized glomerular protein was determined by the Bio-Rad protein assay (Bradford, 1976).

Lysed Glomeruli: Incubation with Tritiated Arachidonate—Rat kidney glomeruli were isolated as previously described. The isolated
The gas chromatography column was a 6-foot coil packed with 0.8 mg of protein were incubated with [3H]C20:4 (3-4 x 10^7 dpm) and [3H]C20:4 (33 μM) in modified balanced salt solution containing gelatin (0.01 mg/ml) and 25 mM HEPES, pH 7.4. The total volume of each incubation was 3 ml.

Glomerular Epithelial Cell Culture: Incubation with Tritiated Arachidonate—Rat kidney glomeruli were isolated as previously described (Petruis et al., 1981). Glomeruli were then cultured at 37 °C in 35-mm-diameter 6-well plastic clusters (Fisher), containing 2 ml of RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, insulin (0.06 units/ml, Sigma), penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), and buffered with 20 mM HEPES and NaHCO3 (0.2 g/l) (pH 7.2). Cultured media, fetal bovine serum, penicillin/streptomycin solution, and HEPES were purchased from Gibco (Grand Island, NY). Glomerular epithelial cells were identified as previously reported (Petruis et al., 1981). Epithelial cells, after 9 days in culture, were scraped off the dishes and homogenized in PBS, pH 7.4. The homogenized epithelial cells (0.45 mg of protein) were incubated with [3H]C20:4 under the same conditions as those for homogenized glomeruli except that the incubation was carried out for 1 h.

Homogenized Cortical Tubules: Incubation with Tritiated Arachidonate—The cortical tubular fragments separated from the glomeruli after the glomerular isolation procedure (90 to 95% purity determined microscopically) were homogenized in PBS, pH 7.4. The homogenized cortical tubules (1.1 mg of protein) were incubated with [3H]C20:4 as in the homogenized glomerular experiments except that the incubation was carried out for 1 h.

Extraction of Products and TLC—Extraction of the glomerular lipoxynase products was achieved by acidifying the incubation media to pH 3.0-3.5 with 1 N HCl followed by extraction twice with 3 volumes of ethyl acetate. An aliquot (10 μl) of the extracted radioactive materials was spotted on silicic acid thin layer plates (silica gel 60, E. Merck, Darmstadt, Germany) together with 2-3 μg of HETEs standards (Upjohn Co.). HETEs used were the δ-lactone form of 5-HETE, 8-,9-,11- and 12-HETEs. The thin layer plates were developed in a solvent system of hexane:ether:acetic acid (50:50:3, v/v/v). The standards were visualized by exposing the thin layer plates to iodine vapor. The plates were divided into 0.5-cm segments. The silica gel from each segment was transferred to a scintillation vial, and the radioactivity was determined by scintillation counting in a Packard Tri-Carb 4600 TR liquid scintillation system. The instrument used was a Hewlett Packard HP 5992 GC/MS system. The gas chromatography column was a 6-foot coil packed with 1% SE-30 ultraphase on chromosorb W (high performance) (Pierce) operated at 216 °C. Injector temperature was 240 °C.

RESULTS

Incubation of Homogenized Glomeruli—Homogenized glomeruli converted octadecatrienyl C20:4 to products which were not prostaglandins in the presence of a divalent cation ionophore, A23187 (3 μM) and meclofenamate (33 μM). The products were separated and identified by TLC and HPLC. Fig. 1 summarizes the TLC results from one of three similar experiments. The incubation lacking lipoxynase inhibitors generated products which were separated and identified as 12-HETE and lesser amounts of 8- and/or 9-HETE. The radioactive peak corresponding to 12-HETE and 8- and/or 9-HETE had 1.4% and 0.4%, respectively, of the total radioactivity recovered. About 93% of the total recovered radioactivity corresponded with authentic C20:4. Relatively small amounts of radioactivity (0.4% for 12-HETE and 0.2% for 8- and/or 9-HETE) co-migrating with authentic HETEs were also detected in incubations containing boiled glomeruli. The formation of these HETEs by a boiled enzyme preparation presumably indicates nonenzymatic conversion of [3H]C20:4 to HETEs. Incubation of unboiled glomeruli in the presence of NDGA (33 μM) yielded about the same amounts of radioactivity co-migrating with HETE standards as the boiled control. Two other lipoxynase inhibitors, ETYA (170 μM) and phenidone (670 μM), resulted in similar inhibition of lipoxynase activity as NDGA (results not shown). The
enzymatic conversion of octatriated C20:4 to these HETEs was about 3 times greater than that of boiled control for 12-HETE and twice that of 8- and/or 9-HETE. To increase the precision and resolution of the chromatographic separation, we used HPLC to identify further and quantify glomerular lipoxygenase products of C20:4.

Fig. 2 shows a typical HPLC profile of the products. Consistent with the TLC data, two radioactive peaks were observed. One was identified as 12-HETE (retention time = 52 min); another was 8- and/or 9-HETE (retention time = 67 min). The radioactive peak corresponding to free C20:4 was found at a retention time of 6 min. This peak was also identified by co-migrating with authentic \[^{14}C\]C20:4. An unidentified radioactive peak was found at a retention time of 11 min.

Fig. 3 summarizes the HPLC results of incubations, containing or lacking lipoxygenase inhibitors, with control or boiled glomerular preparations. There was a 4-fold increase in the enzymatic production of 12-HETE (8 \times 10^4 dpm) and a 9-fold increase in the formation of 8- and/or 9-HETE (1.8 \times 10^4 dpm) with the boiled controls. The enzymatic formation of these HETEs was completely abolished by the three lipoxygenase inhibitors, NDGA, ETYA, and phenidone. There was no detectable enzymatic formation of 5-HETE and 11-HETE, consistent with the TLC data. The radioactive peak corresponding to 12-HETE had 1.8% and that of 8- and/or 9-HETE had 0.4% of the total radioactivity recovered from the sample lacking lipoxygenase inhibitors (Fig. 2) in an HPLC run. No inhibitor was found in the boiled control; phenidone (670 \mu M); ETYA (170 \mu M); NDGA (33 \mu M).

**TABLE I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity (dpm)</th>
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<tbody>
<tr>
<td></td>
<td>12-HETE</td>
</tr>
<tr>
<td>+ Meclofenamate</td>
<td>55681</td>
</tr>
<tr>
<td>- Meclofenamate</td>
<td>43471</td>
</tr>
<tr>
<td>Boiled control</td>
<td>39614</td>
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Radioactivity (dpm) of all samples was normalized to the sample with meclofenamate (2.47 \times 10^7 dpm). The experiment was repeated twice.

Fig. 4. HPLC profile of the sample without lipoxygenase inhibitors, from homogenized epithelial cells with incubation as described in text. The total radioactivity recovered from an HPLC run was 2.6 \times 10^6 dpm.
analyses. Table I shows the effect of meclofenamate on the enzymatic formation of these HETEs by lyzed glomeruli. In the presence of meclofenamate, the enzymatic production of these HETEs was potentiated by ~30% when compared with the incubation without meclofenamate. Nonetheless, substantial synthesis of HETEs was observed despite an active cyclooxygenase.

Incubation of Homogenized Epithelial Cells—Homogenized glomerular epithelial cells metabolized octatritiated C20:4 to 12-HETE in the presence of the divalent cationophore A23187 (3 μM) and meclofenamate (33 μM). The 12-HETE was identified by TLC and HPLC analyses. Fig. 4 shows a typical HPLC profile of the sample, without inhibitors, from the glomerular epithelial cell experiment. In contrast to the results from the homogenized glomeruli, only 12-HETE was detected. The radioactive peak corresponding to 12-HETE had 1.4% of the total radioactivity recovered from an HPLC run.

Fig. 5 summarizes the results of HPLC analyses of the incubation samples of one of two experiments. There was roughly a 4-fold increase in the formation of 12-HETE (3.7 × 10⁴ dpm) when compared with the boiled control (1.1 × 10⁴ dpm). The enzymatic formation of 12-HETE was significantly reduced by phenidone, ETYA, and NDGA. There was no detectable enzymatic formation of other HETEs.

Incubation of Homogenized Cortical Tubules—Homogenized cortical tubules converted [³H]C20:4 to radioactive products in the presence of A23187 (3 μM) and meclofenamate (33 μM). The products were separated and identified by TLC and HPLC. Fig. 6 shows the results of HPLC analyses of all the incubation samples. There was roughly a 4-fold increase in the formation of 12-HETE (4.5 × 10⁴ dpm). The enzymatic formation of the 12-HETE was abolished by NDGA, ETYA, and phenidone. There was no detectable enzymatic formation of 5-, 11-, 8- and 9-HETEs. The radioactive peak corresponding to 12-HETE had 1.2% of the total radioactivity recovered from an HPLC run. Moreover, cortical tubules produce one-third as much 12-HETE as glomeruli when normalized to equal amounts of protein.

Gas Chromatography-Mass Spectroscopy—To supplement the aforementioned experiments with a more rigorous analytical technique, selected samples from glomerular incubations with nonradioactive arachidonate were subjected to GC-MS analysis following HPLC purification.

The retention time of standard 12-HETE was 6 min. Upon analysis of the 12-HETE sample, purified by HPLC, a peak with retention time of 6 min was observed and identified as 12-HETE based on an intense ion at m/e 295 as well as smaller peak at m/e 173, 229, 316 (M-90), 391 (M-15), and 406 (M+). This is identical with the spectrum of standard 12-HETE. The presumed mixture of 8- and/or 9-HETE purified by HPLC was suggested to be a mixture of both compounds based on intense ions at m/e 255 and 265.

DISCUSSION

The metabolism of arachidonic acid by lipoygenase in mammalian cells generates one or more HETEs, and the HETEs that predominate for each cell type are characteristic of the cellular source (Borgeat et al., 1976; Goetzl and Sun, 1978).
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1979; Hamberg and Samuelsson, 1974; Roberts et al., 1978). We have demonstrated the production of predominantly 12-HETE by rat glomeruli and, to a lesser extent, by cortical tubules. In our studies, homogenized glomeruli were incubated with [3H]C20:4, a high concentration of meclofenamate (33 μM), and the divalent cation ionophore, A23187 (3 μM). Meclofenamate is an inhibitor of cyclo-oxygenase (Hassid et al., 1979) which shifts the metabolism of arachidonic acid from the cyclo-oxygenase to the lipoxygenase pathway. A23187 acts as an electroneutral calcium carrier across cell membranes (Pfeiffer et al., 1974; Pfeiffer and Lardey, 1976) and causes the release of arachidonic acid and lipoxygenase products from human polymorphonuclear leukocytes (Borgeat and Samuelsson, 1979b; Stenson and Parker, 1979) and rat basophilic leukemia cells (Jakschik et al., 1977). It was used here to maximize the formation of lipoxygenase products by glomeruli and cortical tubules even though its potentiating action is unproven in homogenized cells.

The lipoxygenase products of arachidonic acid metabolism, by glomeruli, were identified on TLC as 12-HETE and smaller amounts of 8- and/or 9-HETE (Fig. 1). The radioactivity peak observed at the origin of the TLC plate was not identified. It is unlikely to be a prostaglandin because of the high concentrations of meclofenamate used in the study. It could be phospholipid as suggested previously (Blackwell et al., 1977). The TLC results were confirmed by a chromatographic analytical method having better resolution, namely HPLC (Fig. 3). The combined TLC and HPLC data suggest the existence of lipoxygenase activity in rat kidney glomeruli. This conclusion is based on the following observations.

(i) The production of 12-HETE and 8- and/or 9-HETE was identified by TLC and HPLC. The formation of these HETEs by glomeruli was significantly higher when compared with the boiled controls (Figs. 1 and 3), suggesting an enzymatic process.

(ii) The enzymatic formation of these HETEs was inhabitable by the three lipoxygenase inhibitors, NDGA, ETYA, and phenidone (Figs. 1 and 3).

Additional chemical identification was obtained by GC-MS which unequivocally demonstrated the presence of 12-HETE, and probably 8-HETE and 9-HETE in glomerular incubates. The small amounts of 15-HETE observed probably arose from oxidation of arachidonate during purification of the samples. The aforementioned evidence, however, can be challenged by the possible contamination of perfused glomeruli with entrapped platelets and leukocytes. Platelets have been shown to produce 12-HETE (Siegel et al., 1979; Greenwald et al., 1979); and neutrophils have been shown to produce 8- and 9-HETEs (Goetzl and Sun, 1979; Borgeat and Samuelsson, 1979a). Thus, it is possible that the observed glomerular lipoxygenase activity may have been due to platelets and leukocytes in rat glomeruli. This possibility is unlikely because the glomeruli were perfused with saline prior to homogenization and glomerular epithelial cells were also shown to produce 12-HETE when incubated with [3H]C20:4 in the presence of meclofenamate and A23187 (Figs. 4 and 5). The formation of only 12-HETE by glomerular epithelial cells suggests that the 8- and/or 9-HETE may be formed by glomerular mesangial cells. Though the results do not show any enzymatic 5-HETE formation by rat glomeruli, the existence of 5-lipoxygenase activity is not ruled out. It is possible that the 5-HETE produced by glomeruli is readily converted to leukotrienes during the incubation and not detected in the present studies. We have also investigated the lipoxygenase activity in cortical tubules. Homogenized cortical tubules produced 12-HETE when incubated with [3H]C20:4, A23187, and meclofenamate. The product was identified by HPLC. The formation of this HETE was significantly higher than the boiled control and inhabitable by the lipoxygenase inhibitors, NDGA, ETYA, and phenidone, suggesting an enzymatic process (Fig. 6). Since the tubules contained some glomeruli and vasculature (5–10% of identifiable particles under microscopy) it is likely that some or much of the 12-HETE in the tubular preparations was a result of glomerular lipoxygenase activity.

The physiological significance of the lipoxygenase metabolic pathway of C20:4 in rat kidney glomeruli and cortical tubules is unclear. HETEs have been found to be chemotactic for neutrophils (Goetzl and Sun, 1979; Goetzl, 1980). The chemotaxis of neutrophils by HETEs may increase glomerular vascular permeability since neutrophils can readily penetrate endothelial cell monolayers in response to chemotactic stimuli (Taylor et al., 1981). Furthermore, neutrophils have been shown to release free oxygen radicals (Babirow and Peters, 1981). The accumulation of neutrophils, in response to HETEs, might produce large amounts of oxygen radicals which can stimulate prostaglandin and thromboxane synthesis by rat glomeruli (Baud et al., 1981).

Acknowledgments—Linda Goldberg provided expert secretarial assistance.

Addendum—Winokur and Morrison (1981) have recently identified 12-HETE as a product of rabbit renal medullary cytosol. However, they were unable to find cortical synthesis of 12-HETE.

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