Role of cytochrome P-450 arachidonate metabolites in endothelin signaling in rat proximal tubule

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Escalante, Bruno A., John C. McGiff, and Adebayo O. Oyekan. Role of cytochrome P-450 arachidonate metabolites in endothelin signaling in rat proximal tubule. Am J Physiol Renal Physiol 282: F144–F150, 2002. First published August 21, 2001; 10.1152/ajprenal.00064.2001.—We examined the rat proximal tubule (PT) response to endothelin-1 (ET-1) in terms of 20-hydroxyecosatetraenoic acid (HETE) dependency. Arachidonic acid (AA) (1 μM) decreased ouabain-sensitive 86Rb uptake from 2.1 ± 0.1 to 0.3 ± 0.08 ng Rb·10 μg protein⁻¹·2 min⁻¹ (P < 0.05); 20-HETE (1 μM) had similar effects. Dibromododecenoic acid (DBDD) (2 μM), an inhibitor of ω-hydroxylase, abolished the inhibitory action of AA on 86Rb uptake whereas the PT response to 20-HETE was unaffected. ET-1 at 0.1, 1, 10, and 100 nM reduced 86Rb uptake from 2.8 ± 0.3 in control PTs to 2.4 ± 0.2, 1.7 ± 0.1, 0.67 ± 0.08, and 0.1 ± 0.03 ng Rb·10 μg protein⁻¹·2 min⁻¹, respectively, DBDD (2 μM) abolished the inhibitory effect of ET-1 on 86Rb uptake as did BMS182874 (1 μM), an ET₁-selective receptor antagonist. ET-1 (100 nM) significantly increased PT 20-HETE release by ~50%, an effect prevented by DBDD. N⁶-nitro-L-arginine-methyl ester (l-NAME), given for 4 days to inhibit nitric oxide synthase (NOS), increased arterial pressure from 92 ± 12 to 140 ± 8 mmHg and increased endogenous release of 20-HETE from isolated PTs (measured by gas chromatography/mass spectrometry). In l-NAME-treated PTs, but not in control PTs, 0.1 μM AA inhibited ouabain-sensitive 86Rb uptake by >40%; the response to AA was attenuated by DBDD. We conclude that, in the PTs, 1) 20-HETE is a second messenger for ET-1 and 2) conversion of AA to 20-HETE is augmented when NOS is inhibited.

20-hydroxyecosatetraenoic acid; arachidonic acid metabolites; endothelin-1; nitric oxide

The renal functional effects of endothelins have been related to eicosanoid-dependent mechanisms served by cytochrome P-450 (CYP450)-derived arachidonate metabolites (22, 20, 11). A CYP450 arachidonate metabolite, likely 20-hydroxyecosatetraenoic acid (20-HETE) and its metabolites via cyclooxygenase (COX), the prostanoid analogs of 20-HETE, contribute substantially to the renal vasoconstrictor and diuretic actions of endothelin-1 (ET-1) (22). The diuretic response to ET-1 is independent of the pressor action of the peptide and is abrogated by inhibition of CYP450 arachidonic acid (AA) metabolism (21), suggesting that ET-1 acts on tubular function via a CYP450-dependent AA metabolite. The similarity of the unique renal functional effects of ET-1 and 20-HETE, namely, diuresis despite renal vasoconstriction and depression of glomerular filtration rate (GFR), is in keeping with the proposal that 20-HETE and its metabolites via COX act as second messengers for the renal vascular and tubular actions of ET-1 (22, 17). Furthermore, ET-1 increases renal efflux of 20-HETE (20).

The proximal tubule (PT) represents an ideal site to study eicosanoid-dependent mechanisms, particularly those involving CYP450 products that are activated by endothelins: 1) they are endowed with the highest renal activity of ω-hydroxylase, the enzyme responsible for generating 20-HETE (19); 2) COX activity of the PT is absent or negligible (31), thereby eliminating or minimizing 20-HETE metabolism by COX, which can complicate interpretation of 20-HETE-ET-1 interactions; and 3) nitric oxide synthase (NOS) is present in PT (35, 26); this affords the opportunity to study the modulatory influence of NO on 20-HETE-ET-1 interactions, which has been reported to be considerable in the rat kidney (22, 23).

We found that 20-HETE functions as a second messenger, mediating the effects of ET-1 on ion transport in PTs, and inhibition of NOS greatly enhances AA conversion to 20-HETE with attendant augmented effects on PT transport.

MATERIALS AND METHODS

Materials. ET-1 (Peninsula Laboratories, Belmont, CA) was dissolved in 0.1% acetic acid; BMS182874 (5-dimethylaminomethyl-N-(3,4-dimethyl-5-isoxazolyl)-1-naphthalene sulfonamide was dissolved in 0.1 M NaHCO₃, N⁶-nitro-L-argininemethyl ester (l-NAME; Sigma), amiloride (Sigma), and ouabain (Sigma) were dissolved in distilled water. SigmaCote (Sigma) was dissolved in Hanks’ solution and Percoll (Atlanta Biologicals, Norcross, GA) was dissolved in Tyrode saline.

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solution. Iron oxide was suspended in Tyrode solution. Dibromododec-11-enioic acid (DBDD) and 20-HETE (gifts from Dr. Camille Falck, University of Texas Southwestern Medical Center, Dallas, TX) were stored in ethanol at −20°C. Sodium arachidonate (Nuchek, Elysisan, MN) was dissolved in distilled water in a stock solution (1 mg/ml) and stored under nitrogen at −70°C. 86Rb (Amersham International) was supplied in aqueous solution in a stock concentration of 37 MBq (1 μCi/ml).

Isolation of PTs. Male Sprague-Dawley rats (180–198 gm) were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Kidneys were perfused in situ with 10 ml ice-cold Tyrode solution containing 1 mg/ml iron oxide. The renal cortex was separated and minced (1 mm³) with a surgical blade (size 12). The minced cortical tissue was subjected to enzyme digestion at 37°C in oxygenated (95% O₂-5% CO₂) Tyrode solution containing collagenase (200 U/ml), hyaluronidase (200 U/ml), soybean trypsin inhibitor (0.5 mg/ml), glucose (10 mM), sodium succinate (1 mM), albumin (10 mg/ml), and l-alanine (5 mM). Every 5 min, the supernate of the incubation solution was drawn off and a fresh digestion solution added. A sample of the tissue was removed and examined under a low-power microscope to determine the extent of digestion. After repeating this process 3–4 times, a side-pull magnet (Perspective Biosystems, Framingham, MA) was used to separate the tubule from the iron-containing vascular tissue. Tubular fractions were gently layered onto a 35% Percoll solution and centrifuged for 10 min at 13,000 g. The bottom layer, which contained PTs, was removed for the experiments. The purity of the preparation was 90–95% as confirmed by light microscopy. Protein concentration was determined in PT suspensions using a protein assay kit (Bio-Rad Chemical Division, Richmond, CA).

86Rb uptake. 86Rb uptake was determined using the method we described previously (6). Briefly, freshly isolated PTs were preincubated on ice for 20 min in K⁺-free (substitution of NaCl for KCl on a mole-for-mole basis) Hanks’ buffered saline solution (HBSS), pH 7.4, with 5.5 mM glucose as the sole substrate. In preliminary experiments (n = 6), optimal incubation time was established for 86Rb uptake by determining uptake in PTs (100 μg protein) incubated in KCl solution (final K⁺ concentration, 5 mM) containing 86Rb (0.7 μCi/ml) in a shaking water bath (37°C) for different times: 0, 0.5, 1, 2, 5, 10, and 15 min. Isotope uptake was terminated by the addition of 100 μl of a stop solution (Sigma) to cell suspension, which was pelleted (13,000 g × 30 s) immediately. Specific activity of 86Rb in the pellet was determined using a scintillation counter (Beckman LS 1301). 86Rb uptake by PTs was calculated from standards and expressed as ng Rb·10 μg protein⁻¹·2 min⁻¹. Because of interexperimental variations, 86Rb uptake was performed in the presence of ouabain (1 mM) in each experiment to evaluate ouabain-sensitive 86Rb uptake, which was calculated by subtracting 86Rb uptake in the presence of 1 mM ouabain from that in its absence. Comparability of data was assessed by evaluating the degree of ouabain inhibition of 86Rb uptake (positive control).

The ion transport mechanisms were evaluated by determining 86Rb uptake in PTs pretreated for 10 min with ouabain (1 mM; n = 5), the Na⁺/K⁺-ATPase inhibitor, or amiloride (1 mM; n = 5), an inhibitor of the Na⁺/H⁺ exchanger (Fig. 1). In the first series of experiments, 86Rb uptake was determined in PTs exposed to AA (0.1 and 1 μM), ET-1 (1, 10, and 100 nM), 20-HETE (1 μM), or vehicle (control). These agents were added to the PT suspension during the last 2 min of the 20-min preincubation in K⁺-free HBSS. To examine a role for CYP450-AA metabolites in the ET-1 effect on 86Rb uptake by PTs, 2 μM DBDD, an inhibitor of CYP450-AA metabolism that is highly selective for ω-hydroxylase (34), was added to the PT cell suspension 5 min before the cells were exposed to either AA, 20-HETE, or ET-1. The concentration of DBDD selected, 2 μM, was based on the reported IC₅₀ of DBDD (34) as well as the ability of DBDD to produce substantial inhibition of ω-hydroxylase in rat kidney (20). The receptors involved in ET-1 effects were evaluated by determining 86Rb uptake in response to a submaximal concentration of ET-1 (10⁻⁸ M) in PTs pretreated with BMS182874 (1 μM), the ETA-selective antagonist (33). We also tested the effects of inhibition of NO changes on ion transport in PTs induced by CYP450 arachidonate metabolites. Rats (n = 5) were treated with the NOS inhibitor L-NAME, (50 mg/day in drinking water for 4 days). Blood samples were taken and nitrates were measured by HPLC (4). Blood pressure was measured through a catheter placed in the carotid artery and connected to a pressure transducer (model P231D; Statham, Oxnard, CA). PTs were isolated from both control and L-NAME-treated rats as above and divided into two, one for evaluation of 86Rb uptake in response to AA (0.1 and 1.0 μM). The effect of AA on ion transport in PTs was compared with control and L-NAME-treated rats. The other batch of PTs was used to measure endogenous production of 20-HETE.

PT production of 20-HETE. The amount of 20-HETE produced in isolated PTs was determined by gas chromatography/mass spectrometry (GC/MS) using the methods previously described (20). Fifty micrograms of PT tissue protein from control- or L-NAME-treated rats was incubated in PBS, pH 7.4, containing CaCl₂ (1.2 mM), glucose (11 mM), NADPH (1 mM), and phenanthroline (1.2 mM) in the presence or absence of ET-1 (1, 10, and 100 nM) for 10 min at 37°C. Reaction was terminated by the addition of 500 μl ethanol containing 0.5 ng of [20,20-² H]20-HETE as an internal standard. After total lipid extraction with ethyl acetate, the final dried extract was subjected to GC/MS analysis to determine the amount of 20-HETE released into the medium. Data were expressed as nanograms of 20-HETE released per micrograms of PT protein.

Data analysis. All data are expressed as means ± SE. Statistical significance was determined by ANOVA, followed by a modified Student’s t-test for specific comparison. In all cases, P < 0.05 was considered significant.

RESULTS

Ion transport in freshly isolated PTs. In preliminary experiments, we established that 86Rb uptake increased in a time-dependent manner and reached saturation after 10 min. Ouabain (1 mM) and amiloride (1 mM) inhibited 86Rb uptake by 68 ± 7 and 74 ± 4%;
Effects of AA and 20-HETE on ion transport in PTs. To examine the participation of a transport mechanism related to CYP450-AA metabolism in PT, the effect of AA (1 μM) on 86Rb uptake by PTs was determined in the absence or presence of DBDD (2 μM), an inhibitor of CYP450-dependent AA metabolism (35). AA decreased 86Rb uptake from 2.1 ± 0.1 to 0.3 ± 0.08 ng Rb/10 μg protein/min (n = 5) as did 20-HETE (1 μM; n = 5) (Fig. 2). Coincubation with DBDD (2 μM) abolished the inhibitory effect of AA on 86Rb uptake by PTs, suggesting that a CYP450-AA product inhibited 86Rb uptake. DBDD did not affect the response to 20-HETE.

Effects of ET-1 on ion transport, prevention by ETₐ receptor antagonism, and the role of 20-HETE. ET-1 decreased 86Rb uptake in freshly isolated PTs in a dose-dependent manner (Fig. 3). ET-1 at 0.1, 1, 10, and 100 nM reduced 86Rb uptake from 2.8 ± 0.3 in control PTs to 2.4 ± 0.2, 1.7 ± 0.1, 0.67 ± 0.08, and 0.1 ± 0.03 ng 86Rb/10 μg protein/min, respectively. BMS182874 (1 μM), an ETₐ-selective receptor antagonist (33), abolished the decrease in PT 86Rb uptake produced by ET-1 (10 nM) (Fig. 4). DBDD (2 μM), which was without effect on basal 86Rb uptake, abolished the inhibitory effect of 10 nM ET-1 on 86Rb uptake, suggesting that the effects of ET-1 were mediated by a CYP450-AA metabolite (Fig. 5). PT production of 20-HETE was evaluated in preparations to which ET-1 was added (Fig. 6). Basal production of 20-HETE was 0.83 ± 0.22 ng/μg protein and was decreased by DBDD (2 μM) to 0.45 ± 0.05 ng/μg protein (P < 0.05). Incubation of PTs with ET-1 significantly increased 20-HETE release only in response to 100 nM ET-1; viz, to 1.21 ± 0.22 ng/μg protein (P < 0.05), an effect that was prevented by DBDD.

Effects of L-NAME treatment on AA-induced inhibition of 86Rb uptake by PTs. L-NAME treatment for 4 days increased carotid arterial blood pressure from 92 ± 12 to 140 ± 8 mmHg (P < 0.05) and decreased plasma nitrite concentration from 275 ± 66 to 150 ± 50 μM (P < 0.05) for control (n = 5) and L-NAME-treated (n = 5) rats, respectively. Concomitant with these changes, L-NAME treatment increased endogenous production of 20-HETE in isolated PTs to 2.4 ± 0.5 from 1.4 ± 0.2 ng/μg protein in control rats (P < 0.05) (Fig. 7). DBDD (2 μM) inhibited 20-HETE production in PTs obtained from both control and L-NAME-treated rats. L-NAME potentiated the inhibitory effect of AA on ouabain-sensitive 86Rb uptake; viz, in PTs from L-NAME-treated rats, AA (0.1 μM) inhibited 86Rb uptake by ~40% in contrast to no effect at the same concentration of AA in vehicle-treated rats, and AA (1 μM) inhibited ouabain-sensitive AA uptake by 55% before L-NAME treatment and 78% after (P < 0.05) (Fig. 8). DBDD (2 μM) attenuated the inhibitory effect.
likely 20-HETE, mediated the responses to AA. In rats treated with a CYP450-AA metabolite, 20-HETE, the responses to AA were attenuated, suggesting that a CYP450-AA metabolite, likely 20-HETE, mediated the responses to AA.

**DISCUSSION**

The CYP450 monoxygenases, which can be activated by ET-1 (22), generate AA metabolites acting on the renal vasculature and tubules to regulate volume and composition of body fluids (10, 17). As the renal effects of ET-1 resemble those of 20-HETE and are unique (21), we hypothesized that a significant component of the natriuretic-diuretic action of ET-1 was mediated by 20-HETE as a result of inhibition of ion transport by the eicosanoid in the PTs. We also postulated that inhibition of NOS would potentiate 20-HETE-mediated effects on PT ion transport in view of the tonic inhibitory action exerted by NO on 20-HETE-activated ion transport in rat PTs via a CYP450-dependent eicosanoid mechanism involving 20-HETE as a substrate of NOS (34), attenuated ET-1-induced reduction of 86Rb uptake, and 20-HETE mimicked ET-1 inhibition of 86Rb uptake; 2) elimination of the inhibitory effect of NO on 20-HETE-converted AA conversion by NO synthase to 20-HETE (1, 22) and, thereby, potentiated CYP450-dependent AA effects on PT ion transport (Fig. 8); and 3) ET-1 has the capacity to release 20-HETE from PTs (Fig. 6). It should be noted that the potency of ET-1 for releasing 20-HETE is greatly underestimated because of the rapid disposition of newly synthesized 20-HETE by metabolism, conjugation, and incorporation into phospholipids (17).

CYP450-derived eicosanoids, the HETEs and epoxyeicosatrienoic acids, affect ion transport and water movement and are released in response to hormones that affect tubular function (17); they act as second messengers that affect tubular function through several mechanisms: 1) inhibition of Na+/K+-ATPase (30, 3); 2) activation of the Na+/H+ exchanger (9); 3) inhibition of arginine vasopressin-stimulated water reabsorption in the collecting duct (10); and 4) facilitation of calcium entry associated with inhibition of Na+ transport in PTs in response to high-dose angiotensin II (28).

The principal renal CYP450 eicosanoid, 20-HETE, is generated by key nephron segments, the mTAL (3) and PTs (19), the latter having the highest activity of 20-HETE synthase and 20-HETE production. PTs in response to high-dose angiotensin II (28).

The 20-HETE production of 0.1 and 1.0 μM AA on 86Rb uptake in 1-NAME-treated rats, suggesting that a CYP450-AA metabolite, likely 20-HETE, mediated the responses to AA.
to the renal actions of ET-1 was demonstrated in two recent studies: 1) the renal vasoconstrictor and diuretic-natriuretic response to ET-1 in the euolemic anesthetized rat was blunted by inhibiting 20-HETE production (21); and 2) the precipitous decline in GFR, pressor response, and diuresis-natriuresis, evoked by inhibiting NO production with l-NAME, was attenuated by either blockade of endothelin receptors or inhibiting synthesis of 20-HETE (22).

Involvement of endotoxins in the regulation of PT transport is supported by several studies: 1) endotoxins are synthesized by PTs (14); 2) endotoxin binding sites have been identified in PTs (15); 3) ET-1 affects bulk flow in isolated perfused straight segments of PTs (7); and 4) ET-1 inhibits PT Na−K−ATPase, a principal determinant of transcellular Na movement in the nephron (8). Furthermore, ET-1 effects on tubular ion transport have been related to eicosanoid-dependent mechanisms, which is not unexpected, in view of the ability of endotoxins to stimulate phospholipases, releasing AA for conversion to eicosanoids (2, 8).

For example, ET-1 activates prostaglandin-dependent mechanisms in the collecting duct to promote diuresis-natriuresis (36). More to the point, ET-1 has been suggested to stimulate AA metabolism via both lipoxygenases and cyclooxygenases in PTs (7), producing eicosanoids that contribute to the diuresis-natriuresis evoked by the peptide. A key target for eicosanoids in both collecting ducts and PTs is Na−K−ATPase, the sodium pump, an essential component in tubular mechanisms governing renal sodium reabsorption. The sodium pump of PTs appears to be the critical point of convergence of mechanisms involving ET-1 and 20-HETE in regulating sodium reabsorption, as each inhibits PT Na−K−ATPase, ET-1 indirectly via stimulating 20-HETE synthesis, which in turn activates protein kinase C (PKC) (18) that inhibits the sodium pump. Inhibition of PT sodium reabsorption by this mechanism, involving a CYP450 AA product, likely 20-HETE, and PKC also appears to be utilized by dopamine (29) and parathyroid hormone (24), as well as by ET-1.

NO regulates the activities of both the endothelin and CYP450 systems (21). NO prevents, whereas inhibition of NO synthesis promotes, the expression and production of ET-1 in cultured endothelial cells (16). NO also inhibits hemoproteins, including CYP450 enzymes (13).

Inhibition of NOS in the anesthetized rat uncovers a major vasoconstrictor diuretic-natriuretic system operating through one or more CYP450-dependent metabolites generated by ω-hydroxylases (22). We tested the hypothesis that inhibition of NOS affects CYP450-AA metabolism in PTs by comparing the effects of AA on ion transport in rats before and after treatment with an NOS inhibitor. l-NAME-treated rats demonstrated potentiation of CYP450-dependent AA-induced reduction in 86Rb uptake (Fig. 8) associated with increased 20-HETE release by PTs (Fig. 7), indicating that a CYP450-dependent ion transport mechanism involving ω-hydroxylase was disinhibited when NO production was blocked. Thus after l-NAME treatment, a concentration of AA (0.1 μM), which had been without effect on 86Rb uptake, showed a greater than 40% inhibition of ouabain-sensitive 86Rb uptake (Fig. 8), an effect of AA attenuated by inhibition of ω-hydroxylase, indicating that conversion of AA to 20-HETE was involved. These findings are in accord with our in vivo studies regarding the effects of both l-NAME and ET-1 on sodium excretion; namely, that natriuresis was blocked by inhibition of 20-HETE synthesis and was independent of their pressor effects. These findings also complement those of earlier studies (27, 35) that recognized the potential importance of NO in modulating PT sodium reabsorption.

The present study, as well as our recent in vivo studies, provide a mechanism for the diuretic-natriuretic response to inhibition of NOS; namely, 20-HETE mediates a significant component of the tubular effects produced by suppression of NO formation as the synthesis of 20-HETE, which is subject to tonic inhibition by NO, is greatly enhanced when NO production is blocked. There are several issues that have not been resolved by the present study. The claims of previous studies for both lipoxygenase and COX representation in PTs and their activation by ET-1 was based on 1) renal clearance methods in a study that identified a lipoxygenase interacting with endotoxins (25), and 2) perfusion of the isolated straight segment in a study that indicated involvement of both COX and lipoxygenase interacting with ET-1 to affect sodium transport (7). A direct comparison of our study with the aforementioned studies is difficult, if not precluded, given marked differences in the experimental preparations and design as well as in methods. We have attempted to allay concerns regarding claims made for the in-
volvement of 20-HETE in the PT effects of ET-1 by measuring the release of 20-HETE from PTs. Moreover, we obtained evidence for the ability of DBDD to inhibit synthesis of 20-HETE. One area, namely, the positive effects of very low concentrations of ET-1 (pM) on PT sodium transport (7), was not addressed, as our intention was to focus on the natriuretic component of the PT action of ET-1, because this area of the dose-response curve to ET-1 was postulated to involve a 20-HETE dependency, which the present study supports.

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