Endothelin-1 and CYP450 arachidonate metabolites interact to promote tissue injury in DOCA-salt hypertension

A. O. OYEKAN, K. McAWARD, J. CONETTA, L. ROSENFELD, AND JOHN C. McGIFF

Departments of 1Pharmacology, 2Comparative Medicine, and 3Pediatrics, New York Medical College, Valhalla, New York 10595

Oyekan, A. O., K. McAward, J. Conetta, L. Rosenfeld, and John C. McGiff. Endothelin-1 and CYP450 arachidonate metabolites interact to promote tissue injury in DOCA-salt hypertension. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R766–R775, 1999.—Inhibition of cytochrome P-450 (CYP450) enzymes with cobalt chloride (CoCl2) prevented hypertension, organ hypertrophy, and renal injury induced by DOCA and salt (1% NaCl) in uninephrectomized (UNx) rats. Systolic blood pressure (SBP) rose to 193 ± 6 mmHg by day 21 from control levels of 150 ± 7 mmHg in response to DOCA-salt treatment, a rise that was prevented by CoCl2 (24 mg·kg−1·24 h−1). The effects of DOCA-salt treatment, which increased protein excretion to 88.3 ± 6.9 mg/24 h on day 21 from 9.0 ± 1.1 mg/24 h on day 3, were prevented by CoCl2. CoCl2 also attenuated the renal and left ventricular hypertrophy and the increase in media-to-lumen ratio in hypertensive rats. DOCA-salt treatment increased excretion of endothelin (ET)-1 from 81 ± 17 to 277 ± 104 pg·100 g body wt−1·24 h−1 associated with a fourfold increase in 20-hydroxyeicosatetraenoic acid (20-HETE) excretion from 3.0 ± 1.1 to 12.2 ± 1.9 ng·100 g body wt−1·24 h−1 (days 3 vs. 21). CoCl2 blunted these increases by 58 and 72%, respectively. In aortic rings pulsed with [3H]thymidine, ET-1 increased its incorporation. Dibromododec-11-enoic acid, an inhibitor of 20-HETE synthesis, attenuated ET-1-induced increases in [3H]thymidine incorporation. We distinguished effects of CoCl2 acting via CO generation vs. suppression of CYP450-arachidonic acid metabolism by treating UNx-salt-DOCA rats with 1-aminobenzotriazole (ABT), which suppresses CYP450 enzyme activity, and compared these results to those produced by CoCl2. ABT reduced hypertension, as did CoCl2. Unlike CoCl2, ABT did not prevent organ hypertrophy and proteinuria, suggesting that these effects were partially related to CO formation. Blockade of the ETA receptor with BMS-182874 reduced SBP, organ hypertrophy, and proteinuria, indicating the importance of ET-initiated abnormalities to the progression of lesions in UNx-salt-DOCA.

The cytochrome P-450 (CYP450) monooxygenase system has the ability to convert arachidonic acid (AA) to specific classes of metabolites: the CYP2C family catalyzes transformation of AA to the epoxides, the 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs); the CYP2E family to hydroxyeicosatetraenoic acids (HETEs), including the subterminal HETEs, 16-, 17-, 18-, and 19-; and the CYP4A family/subfamily to the ω-hydroxylase product 20-HETE from AA (32). Of the CYP450-AA products, 20-HETE, the principal product of renal CYP450 AA metabolism (6), generated by the vasculature and key segments of the nephron, has the greatest potential for modifying renal function and influencing blood pressure. Altered synthesis of 20-HETE has been reported to contribute to elevation of blood pressure in the spontaneously hypertensive rat (SHR) (32), in aortic coarctation (7), and in the Dahl salt-sensitive rat (18). A CYP450-derived AA product, most likely 20-HETE, has been identified as a major component in the renal vasoconstrictor action of endothelin (ET)-1 in the rat (25). ET-1 increased 20-HETE release from the kidney (25), whereas inhibition of synthesis of CYP450-derived AA products greatly reduced the ability of ET-1 to increase renal vascular resistance and reduce glomerular filtration rate (GFR) (26). Despite reversal by CYP450 inhibitors of the negative effects of ET-1 on GFR, the natriuretic-diuretic response to ET-1 was blunted, suggesting that a CYP450 AA metabolite also acted as a second messenger for the tubular action as well as the renal vasoconstrictor effect of the peptide. Thus inhibition of CYP450 AA metabolism markedly attenuated the diuretic-natriuretic response to ET-1 in the face of substantial elevations of GFR, on the order of 20–50% above pretreatment levels. Furthermore, 20-HETE is the only CYP450-AA product that has a biological profile, vasoconstriction and natriuresis, similar to that of ET-1. On the basis of these findings, we proposed that 20-HETE mediates the renal vascular and tubular actions of ET-1 (25, 26).

Given the evidence cited above for a CYP450-AA product acting as a second messenger for the renal vascular and tubular responses to exogenous ET-1, we have examined in the present study the consequences of endogenous ET interactions with CYP450-AA products. The uninephrectomized (UNx) rat given excess salt and treated with DOCA (UNx-salt-DOCA) is characterized by enhanced activity of both the ET and CYP450 systems (3, 13–15). Because production of renal CYP450-derived AA metabolites is increased in response to salt loading (4, 19) and treatment with DOCA (4), these AA metabolites may participate in the vascular and renal responses in this experimental model of volume-dependent hypertension. Moreover, renal microsomal CYP450-linked enzymatic activity can be induced in the contralateral kidney after uninephrectomy (35). Thus the triad of uninephrectomy, salt loading, and mineralocorticoid treatment is a powerful combination of stimuli for activating CYP450 enzymes. Furthermore, in DOCA-salt hypertensive rats, the content of immunoreactive ET-1 and ET-1 mRNA was increased in the aorta and mesenteric arteries (14, 15).
indicating that increased levels of ET-1 may be an additional factor in activating CYP450 enzymes that produce AA metabolites (25, 26). The DOCA-salt model of hypertension has been characterized by marked hypertrophy of the heart and kidneys, ascribed to the mitogenic effects of ET-1 (33). Because 20-HETE can also act as a mitogen (16), a possible linkage of 20-HETE acting as a second messenger to the growth-promoting effects of ET-1 on the cardiovascular system was examined.

The purpose of this study, therefore, was to address in DOCA-salt-treated UNx rats the pathophysiologica role of CYP450-derived AA products relative to the deterioration of renal function and to the morphological changes in the heart and kidneys. We used CoCl2 and 1-aminobenzotriazole (ABT), agents that have been shown to suppress CYP450-dependent AA metabolism in the rat (21, 25–27). However, CoCl2 not only diminishes CYP450 enzyme activity (8) but also results in generating carbon monoxide (CO), which is a vasodepressor (28) and may contribute to the ameliorative effects of CoCl2 on the pathophysiological changes produced by UNx-salt-DOCA. To distinguish effects of CoCl2 acting via CO production vs. suppression of CYP450 AA metabolism, we treated UNx-salt-DOCA rats with ABT, which reduces CYP450 enzyme activity, and compared these results with those produced by CoCl2, which reduces CYP450 enzyme activity and increases CO formation. This comparison helped identify a potential contribution of CO generation produced by CoCl2 to reduction of organ hypertrophy and reversal of renal injury. Moreover, the important contribution of ET-1 to the cardiorenal changes produced by UNx-salt-DOCA was assessed by treating UNx-salt-DOCA rats with an ET receptor antagonist, BMS-182874. Blood pressure, organ hypertrophy, and proteinuria were reduced by ET receptor blockade, indicating the contribution of ET-initiated abnormalities to the progression of lesions in this hypertensive model.

MATERIALS AND METHODS

Experimental animals. Two groups of rats were used. For induction of hypertension, 5- to 6-wk-old male Sprague-Dawley rats (140–160 g; Charles River Laboratories, Wilmington, MA) were used. For [3H]thymidine incorporation studies, 12-wk-old rats were used.

Chemicals. CoCl2 (Sigma, St Louis, MO) was dissolved in normal saline (0.9% NaCl). BSA (Sigma) was dissolved in distilled water. DOCA (Sigma) was used as 25-mg slow-release pellets formed in our laboratories. ET-1 (Peninsula Laboratories, Belmont, CA) was stored in 0.1% acetic acid at −20°C. ABT (Sigma) was dissolved in dimethyl sulfoxide (final concentration 9%); BMS-182874 was dissolved in 0.1 M NaHCO3 and pH was adjusted to 7.4. Tin mesoporphyrin (SnMPP; Porphyrin Products, Logan, UT) was dissolved in 10 mg/ml Na2C2O4 and stored at 4°C protected from light. 12,12-dibromododec-11-enoic acid (DBDD) (gift from Dr. Camille Falck, University of Texas South Western Medical Center) was stored in ethanol at −70°C. Perclopac acid was obtained from Sigma. [3H]thymidine (Amersham) was supplied in an aqueous solution containing 10% ethanol. Organ culture media and fetal bovine serum (FBS) were purchased from GIBCO BRL (Gaithersburg, MD).

Experimental protocol. All procedures were in accordance with institutional guidelines for animal research. Rats were randomly allocated to groups (n = 4–6 per group). With rats under halothane (2%) anesthesia, a left dorsal incision was made, the left kidney was removed, and a 25-mg DOCA pellet or a placebo pellet was subcutaneously implanted in the neck. On recovery, the rats were fed a standard laboratory chow (Purina Laboratories, Boundbrook, NJ) and given a 1% NaCl drinking solution ad libitum. UNx rats served as controls and were maintained on the same rat chow and given 1% NaCl solution as were the rats treated with DOCA. In another group of sham-nephrectomized rats (n = 4 or 5), a laparotomy was performed but the kidneys, although located, were not removed. Rats were individually housed in metabolic cages for collection of urine for the measurement of excretion of protein, ET-1, and 20-HETE. In the first set of experiments, rats were maintained for 3 wk after surgery. DOCA rats were treated every other day for 3 wk starting on the day of surgery with CoCl2 (24 mg/kg ip) or its vehicle (normal saline 0.9% NaCl), 1 ml/kg ip. In another group of rats (n = 4), the effect of CoCl2 alone was evaluated by treating normal rats with the same dose of CoCl2 for 15 days. This dose of CoCl2 inhibited renal microsomal CYP450 4A-dependent metabolism and CYP450-mediated renal vascular response in the rat (27).

For rats in the first set of experiments, systolic blood pressure (SBP) of awake rats was measured once weekly for 3 wk by tail-cuff plethysmography using a Natsume KN-210 manometer-tachometer system (Peninsula Laboratories). The rats were fatigued at 37°C for 10 min and allowed to rest quietly in a Lucite chamber before tail-cuff plethysmography. The average of 10 pressure readings was recorded. Body weights were also measured once weekly at the time of blood pressure determination. The rats were killed 21 days after the operation. Under pentobarbital sodium anesthesia (50 mg/kg ip), the right kidney pressurized to 90–110 mmHg was perfused in situ for 10–15 min with Krebs physiological solution (pH 7.2) and preserved in neutral-buffered formaldehyde (10% Formalin) for later histological sectioning and examination. The heart was removed and weighed; left and right ventricles were then dissected and each weighed.

In the second set of experiments, in which rats were maintained for 18 days, after surgery we evaluated the specificity of effects of CoCl2 by assigning additional groups of UNx-salt-DOCA rats to these treatment groups: 1) SnMPP, a competitive inhibitor of heme oxygenase (HO) (300 µmol/kg ip, every other day), n = 6; 2) ABT, a suicide substrate inhibitor of CYP450 (50 mg/kg ip, every other day), n = 5; 3) BMS-182874, an ET receptor antagonist (40 mg·kg−1·day−1, orally by gavage), n = 5.

At the end of 18 days, mean arterial blood pressure (MBP) was measured directly with pressure transducers (Statham P23 Db) through a catheter (PE-50) inserted into the carotid artery of the rats in groups 1-3 above. With rats under pentobarbital sodium anesthesia (50 mg/kg ip), the right kidney and cardiac left ventricle were removed, blotted dry, and weighed. Protein as well as ET-1 and 20-HETE excretion were also determined in the urine collected for 24 h on day 18 in rats placed in metabolic cages.

Histological examination. After alcohol dehydration, kidney slices (1 mm thick) from DOCA-treated rats or UNx controls were embedded in paraffin blocks, and histological sections (2–4 µm thick) from each were stained with hematoxylin and eosin. Cross-sectional areas of the media and lumen of small renal arteries (200–300 µm) were measured in a single-blind fashion by area morphometry system using a microscope connected through a videocamera (Sony, Tokyo, J apan) to an image analysis processor (Natchet 1500; Natchet, Kyoto, Japan).
ET-1 and CYP450 products

Palo Alto, CA), at a mass-to-charge ratio of 393, which was identified using a mass spectrometer (HP 5989A; Hewlett Packard, Palo Alto, CA, USA). Duplication of the cross-sectional area of media by that of the lumen, media thickness of blood vessels was obtained from media cross-sectional area. Duplicate measurements of three to five vessels were averaged to obtain one value for each section. Two to three sections were examined per kidney.

Urinary protein was measured with the Pierce BCA protein assay kit (Pierce Chemical) following the protocol recommended by the manufacturer. BSA was used as the standard.

Measurement of urinary excretion of ET-1. The method we described in a previous study (29) was used to quantify the excretion of immunoreactive ET-1 in the urine. Briefly, thawed urine samples collected into 10% potassium-[EDTA] (10:1) were passed through prewashed Sep-Pak C18 cartridges (Waters, Milpore, Milford, MA) and eluted with methanol-water-trifluoroacetic acid (160:19:1) to remove heparin and other cross-reacting substances. After lyophilization, the samples were reconstituted in RIA buffer (0.1 M phosphate, 0.5 M NaCl, 0.1% BSA, 0.02% NaN₃, 0.1% Triton X-100, and 0.1% phenol red, pH 7.6). Aliquots of the samples were assayed in duplicate. Immunoreactive ET-1 was measured by RIA (Peninsula Laboratories). The final pellet was counted and data analyzed by a gamma-counter (Beckman LS1801). The RIA was sensitive to 1.0 pg/tube. Intra- and interassay coefficients of variance were 4.7 and 12%, respectively. The polyclonal antibody in these kits had 17% cross-reactivity against human Big ET and 7% cross-reactivity against ET-2 and ET-3 (data provided by Peninsula Laboratories).

Measurement of urinary excretion of 20-HETE. The amount of 20-HETE excreted in urine was determined by gas chromatography/mass spectrometry (GC/MS) by a modification of the method described previously (24). Briefly, urine samples were acidified with 10% formic acid and supplemented with 20,20-dideutero-20-HETE as internal standard (1 ng/mL). The urine samples were subjected to a two-stage purification process involving thin-layer and high-performance liquid chromatography. After extraction with ethyl acetate and evaporation of the organic extracts to dryness, metabolites were purified initially by thin-layer chromatography using the A9 solvent system (ethyl acetate-isooctane-acetic acid-water, 55:25:10:50). After extraction of scraped silica columns corresponding to HETE and evaporation to dryness, metabolites were further purified by reverse-phase HPLC using Ultra- sphere C18 column (250 × 4.6 mm, Beckman). Samples were chromatographed using a linear solvent gradient of water in acetonitrile (37.5% to 0%, containing 0.01% acetic acid) at 1.875%/min and at a solvent flow rate of 1 ml/min. Fractions were collected every minute and separate fractions containing 20-HETE were evaporated to dryness and dissolved in 100 µl acetonitrile. For GC/MS analyses, samples were treated with pentafluorobenzylbromide in the presence of N,N-diisopropyl ethylamine and then derivatized with bis(trimethylsilyl)-fluoroacetamide and pyridine. Selected ion monitoring was used to record ion abundances using a mass spectrometer (HP 5989A; Hewlett Packard, Palo Alto, CA), at a mass-to-charge ratio of 393, which corresponds to 20-HETE.

Tissue isolation and organ culture. After pentobarbitone anaesthesia (60 mg/kg ip) in male adult Sprague-Dawley rats (302 ± 7 g), the thoracic aorta was carefully removed, taking care not to damage the endothelium because removal of endothelial cells increases serum-induced growth responses in intact vessel preparations (9). Once removed, the aorta and adhering periadventitial fat were placed on a gauze pad and bathed in warmed serum-free medium (SFM, described below) in a 60-mm cell culture dish where the periadventitial fat was carefully cut away. A 3-mm section from both ends of the aorta was removed to eliminate areas of possible injury incurred by handling the aorta with forceps. The aorta was cut into 2- to 3-mm rings and transferred to fresh SFM for measurement of DNA synthesis.

[3H]Thymidine uptake. DNA synthesis was measured by determination of [3H]thymidine incorporation as described by Yao and Wang (39). Aortic rings were placed in 5 ml SFM consisting of DMEM and F-12 nutrient mixture (1:1), glutamine (200 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), insulin (5 µg/ml), and transferrin (5 µg/ml). The rings were then placed in a humidified incubator with a 95% air-5% CO₂ atmosphere at 37°C for 16 h for [3H]thymidine uptake study. 20-HETE (0.1, 1, and 10 µM), ET-1 (1 nM), or FBS (20%) were then added in the absence or presence of DBDD (2 µM). Sixteen hours after agonist treatment, 5 ml SFM containing 2 µCi/ml [3H]thymidine (Amersham) were added. Two hours later, the aortic rings were washed with phosphate-buffered saline three times and homogenized in 0.2 M perchloric acid (PCA). After centrifugation at 1,000 g for 10 min, the pellet was washed with 0.2 M PCA three times. The pellet was then resuspended and heated in 0.5 M PCA for 20 min at 90°C to solubilize the DNA. After cooling and centrifugation at 1,000 g for 10 min, the supernatant was used for DNA determinations. [3H]thymidine incorporation into DNA was assessed with a liquid scintillation system (LS1801, Beckman Instruments). All counts were normalized to micrograms DNA content of individual rings and results were expressed as a percentage of SFM control.

Statistical analysis. Results are expressed as means ± SE. Statistical significance was determined with ANOVA and Duncan’s multiple range test. Differences were considered statistically significant if P ≤ 0.05.

RESULTS

Blood pressures and body and heart weights. Figure 1 illustrates the results for SBP in salt-drinking, UNx

Fig. 1. Systolic blood pressure of uninephrectomized rats drinking salt (1% NaCl) (UNx-salt), treated with DOCA alone (UNx-salt-DOCA) or treated with DOCA and CoCl₂ (UNx-salt-DOCA+CoCl₂). Vehicle-treated rats received normal saline (1 ml/kg ip), and CoCl₂-treated rats received CoCl₂ at 24 mg·kg⁻¹·24 h⁻¹ ip. DOCA was given as 25-mg pellets implanted subcutaneously. Data are presented as means ± SE (n = 5 per group). *P < 0.05 vs. day 3 values. #P < 0.05 vs. UNx-salt-DOCA.
rats with DOCA (UNx-salt-DOCA), or without DOCA (UNx-salt), as affected by CoCl₂ treatment after 21 days. SBP rose in UNx-salt-DOCA rats as expected. As early as day 8 of treatment, the increase in SBP was significantly higher in UNx-salt-DOCA rats relative to UNx-salt controls, an increase prevented by CoCl₂. By day 21, SBP rose to 193 ± 6 mmHg (P < 0.05; n = 5) in UNx-salt-DOCA-treated rats; CoCl₂ reduced the blood pressure in UNx-salt-DOCA rats (P < 0.05; n = 5) to 157 ± 7 mmHg, a level not significantly different from those observed in UNx-salt controls. The percentage increases in SBP between days 3 and 21 in the three groups of rats amounted to 93 ± 6, 43 ± 7, and 28 ± 9% in UNx-salt-DOCA, CoCl₂-treated UNx-salt-DOCA, and UNx-salt control rats, respectively (Fig. 1). In sham-nephrectomized rats not receiving salt, SBP increased from 108 ± 4 mmHg on day 1 to 122 ± 3 mmHg on day 21 (P < 0.05; n = 4). In normal littermates of UNx rats also not receiving salt, treated with CoCl₂ for 15 days, SBP was not significantly different between days 1 and 15 (day 1, 111 ± 4 mmHg; day 15, 103 ± 5 mmHg; n = 4). In Table 1, values are shown for MBP, organ weights, and protein excretion after 18 days in DOCA hypertensive rats treated with CoCl₂ and/or SnMPP, ABT, and BMS-182874. MBP increased from 105 ± 2 mmHg in UNx-salt (n = 4) rats to 143 ± 3 mmHg (P < 0.05) in UNx-salt-DOCA (n = 5) rats. The increase in MBP was either prevented or markedly blunted in response to treatment with CoCl₂, ABT, and BMS-182874. However, SnMPP alone was not effective on MBP but on coadministration with CoCl₂ antagonized the reduction in MBP by CoCl₂.

Body weight before the start of drug treatment and implantation of DOCA pellets was not significantly different among the different groups of rats to be treated for 21 days. On day 3, the group mean values of rats ages 5 to 6 wk ranged between 151 and 156 g. Values of body weight on day 21 are shown in Table 2.

Table 1. Mean blood pressure, weights of the left ventricle and kidney, and protein excretion measured 18 days postsurgically

<table>
<thead>
<tr>
<th></th>
<th>UNx-Salt</th>
<th>UNx-Salt-DOCA</th>
<th>UNx-Salt-DOCA + CoCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP, mmHg</td>
<td>100 ± 2</td>
<td>105 ± 2</td>
<td>143 ± 3</td>
</tr>
<tr>
<td>LV weight, g/100 g body wt</td>
<td>0.19 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.31 ± 0.01*</td>
</tr>
<tr>
<td>Kidney weight, g/100 g body wt</td>
<td>0.42 ± 0.01</td>
<td>0.63 ± 0.03*</td>
<td>0.87 ± 0.04*</td>
</tr>
<tr>
<td>Protein excretion, mg/100 g body wt⁻¹, 24 h⁻¹</td>
<td>8.9 ± 1.4</td>
<td>10.9 ± 2.1</td>
<td>54.7 ± 4.5*</td>
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</tbody>
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Table 2. Body, cardiac, and kidney weights of uninephrectomized or vehicle-treated or CoCl₂-treated DOCA-salt hypertensive rats at death (21 days)

<table>
<thead>
<tr>
<th></th>
<th>UNx-Salt</th>
<th>UNx-Salt-DOCA</th>
<th>UNx-Salt-DOCA + CoCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>343.2 ± 13.6</td>
<td>255.5 ± 15.1*</td>
<td>239.1 ± 18.9*</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.22 ± 0.09</td>
<td>1.42 ± 0.07*</td>
<td>1.21 ± 0.09</td>
</tr>
<tr>
<td>LV weight, g</td>
<td>0.84 ± 0.06</td>
<td>1.02 ± 0.06*</td>
<td>0.81 ± 0.08*</td>
</tr>
<tr>
<td>RV weight, g</td>
<td>0.19 ± 0.01</td>
<td>0.21 ± 0.02</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>3.7 ± 0.2</td>
<td>4.5 ± 0.3*</td>
<td>2.8 ± 0.4†</td>
</tr>
</tbody>
</table>

Values are means ± SE. RV, right ventricle. *P < 0.05 vs. UNx-salt; †P < 0.05 vs. UNx-salt-DOCA.

The mean body weight of CoCl₂-treated UNx-salt-DOCA rats was not significantly different from that of UNx-salt-DOCA rats. However, both groups of rats weighed less than UNx-salt control rats and sham-nephrectomized rats receiving salt.

Relative to UNx-salt control rats, heart weights normalized for body weights were greater in vehicle-treated UNx-salt-DOCA hypertensive rats (P < 0.05; n = 5; Fig. 2A). CoCl₂ attenuated the increase in heart weight induced by DOCA-salt treatment (P < 0.05; n = 5). The changes in heart weight (Table 2) were paralleled by increased left ventricular (LV) weight (Fig. 2B) but not right ventricular weight. CoCl₂ attenu-
ated LV hypertrophy in DOCA-salt hypertensive rats. Heart weights were not different between UNx-salt and sham-nephrectomized rats (Table 2), nor were heart weights of either of these groups different from heart weights of normal rats treated with CoCl₂ (1.18 ± 0.06 mg).

In rats treated with DOCA and salt for 18 days (Table 1), SnMPP when coadministered with CoCl₂ abolished the effect of CoCl₂ in reducing LV hypertrophy, increasing LV weight from 0.22 ± 0.02 g/100 g body wt in CoCl₂-treated DOCA hypertensive rats to 0.36 ± 0.01 g/100 g body wt, a value greater than that seen in UNx-salt-DOCA hypertensive rats (0.31 ± 0.01 g/100 g body wt). Moreover, SnMPP did not prevent the increase in LV weight produced by UNx-salt-DOCA hypertensive rats and appeared to augment it (Table 1). ABT, the suicide substrate inhibitor of CYP450 enzymes, also increased kidney weight to 1.02 ± 0.02 g/100 g body wt (P < 0.05; n = 5), whereas BMS-182874, an ETA-receptor antagonist, attenuated the increase in LV weight in UNx-salt-DOCA hypertensive rats to 0.24 ± 0.02 g/100 g body wt (P < 0.05).

Kidney weights and morphological characteristics of glomerular blood vessels. Right kidney weights were twofold greater in UNx-salt rats (3.7 ± 0.2 g) than the sham-nephrectomized rats (1.9 ± 0.4 g, P < 0.05) in rats treated for 21 days (Table 2 and Fig. 3A). By contrast, kidney weights in CoCl₂-treated rats (1.7 ± 0.2 g) were not different from those of sham-nephrectomized rats (data not shown). Kidney weights and kidney weights normalized for body weight (kidney weight-to-body weight ratio) were greater in vehicle-treated UNx-salt-DOCA hypertensive rats compared with UNx-salt control rats (P < 0.05; n = 5) (Table 2 and Fig. 3A). In UNx-salt-DOCA rats treated with CoCl₂ (UNx-salt-DOCA+CoCl₂), the increase in kidney weight was blunted (P < 0.05; n = 5), there being no significant difference in kidney weight between UNx-salt-DOCA+CoCl₂ and UNx-salt control rats (Table 2 and 3A).

In UNx-salt-DOCA hypertensive rats studied after 18 days of treatment with CoCl₂ and/or SnMPP, SnMPP abolished the reduction in kidney weights by CoCl₂ (P < 0.05). However, SnMPP further increased kidney weight in UNx-salt-DOCA hypertensive rats from 0.87 ± 0.04 to 1.08 ± 0.03 g/100 g body wt (P < 0.05). ABT also caused an increase in kidney weight (from 0.87 ± 0.04 to 1.02 ± 0.02 g/100 g body wt; P < 0.05). On the other hand, BMS-182874 reduced kidney weight in UNx-salt-DOCA hypertensive rats to 0.75 ± 0.03 g/100 g body wt (P < 0.05).

Morphometric analysis of renal cortical vessels showed that the renal small arteries of vehicle-treated UNx-salt-DOCA hypertensive rats exhibited smaller internal diameters and greater media thickness, media cross-sectional areas, and media-to-lumen ratios compared with UNx-salt rats (Fig. 3B, Table 3). CoCl₂ reduced by ~30% (P < 0.05; n = 5) the increased media-to-lumen ratio induced by DOCA-salt treatment and increased the internal diameter (Fig. 3B and Table 3). Nonetheless, media thickness and cross-sectional media area were higher in UNx-salt-DOCA+CoCl₂ than those of UNx-salt control rats (P < 0.05; n = 5) (Table 3).

Urinary protein excretion. As shown in Fig. 4, urinary protein excretion did not change over the 3 wk of the study in salt-drinking UNx-salt control rats (P > 0.05; n = 5) and remained at levels similar to that of littersmates that underwent sham nephrectomy (11.4 ± 2.3 mg·100 g body wt⁻¹·24 h⁻¹). By contrast, urinary protein excretion rose steadily in UNx-salt-DOCA rats, reaching significant levels by day 15 and rising to 88.3 ± 6.9 mg·100 g body wt⁻¹·24 h⁻¹ on day 21, representing a 10-fold increase over the day 3 level. CoCl₂ markedly inhibited protein excretion in UNx-salt-DOCA rats (P < 0.01; n = 5), attaining a value of 21.2 ± 3.3 mg·100 g body wt⁻¹·24 h⁻¹ on day 21, just slightly higher than that observed in UNx-salt rats (13.7 ± 2.6 mg·100 g body wt⁻¹·24 h⁻¹).

Table 3. Internal diameter, cross-sectional area, thickness, and media-to-lumen ratio of renal arteries of salt-drinking, uninephrectomized control rats or those treated with DOCA alone or DOCA and CoCl₂

<table>
<thead>
<tr>
<th></th>
<th>UNx-Salt</th>
<th>UNx-Salt-DOCA</th>
<th>UNx-Salt-DOCA + CoCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal diameter, µm</td>
<td>182.4 ± 33.1</td>
<td>85.7 ± 14.1*</td>
<td>127.9 ± 16.6†</td>
</tr>
<tr>
<td>Media area, µm²</td>
<td>8,657 ± 626</td>
<td>14,475 ± 3,123*</td>
<td>14,146 ± 1,976*</td>
</tr>
<tr>
<td>Media thickness, µm</td>
<td>18.5 ± 2.9</td>
<td>30.3 ± 2.3*</td>
<td>28.1 ± 3.4*</td>
</tr>
<tr>
<td>Media-to-lumen ratio</td>
<td>0.21 ± 0.03</td>
<td>0.56 ± 0.09*</td>
<td>0.39 ± 0.06†</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. UNx-salt; †P < 0.05 vs. UNx-salt-DOCA.
DISCUSSION

Blood pressure and protein excretion increased progressively over the 21-day period of the study in UNx rats receiving excess salt and treated with DOCA. DOCA and salt treatment resulted in renal injury, as reflected in urinary excretion of protein. Organ and vascular hypertrophy were also induced in UNx-salt-DOCA rats, confirming the reports that in this model of
hypertension marked hypertrophy stands in contrast to more limited hypertrophy in other models (33). A novel finding in the present study was the protection afforded by CoCl\textsubscript{2} against renal injury induced by DOCA-salt treatment in UNx rats, raising the possibility that CYP450 AA metabolites are mediators of the injury and hypertrophy that occur with induction of hypertension by DOCA-salt. CoCl\textsubscript{2}, like SnCl\textsubscript{2}, induces HO, increased activity of which accelerates heme degradation, including that associated with CYP450, thereby impairing the ability of the CYP450 system to metabolize AA (8, 32). Treatment with either CoCl\textsubscript{2} or SnCl\textsubscript{2} has been shown to normalize blood pressure in the SHR both in the short and long term (10, 32). Moreover, the decline in blood pressure in the SHR was accompanied by natriuresis, suggesting restoration to normal of the relationship between blood pressure and sodium excretion (32). In the present study, CoCl\textsubscript{2} treatment lowered blood pressure, reduced proteinuria, attenuated hypertrophy of the kidney and left ventricle, and diminished the increased media-to-lumen ratio of small renal arteries produced by DOCA-salt treatment. Questions concerning the specificity of CoCl\textsubscript{2} as an inhibitor of CYP450 AA metabolism have been addressed in our two previous studies (25, 26). In these studies, CoCl\textsubscript{2} had effects indistinguishable from those of DBDD, a mechanism-based inhibitor of \textit{\omega}-hydroxylase (38). The renal excretory and hemodynamic responses to ET-1 were shown to be dependent on 20-HETE acting as a second messenger and were prevented by either CoCl\textsubscript{2} or DBDD, each of which decreased 20-HETE levels intrarenally. The ameliorative effects of CoCl\textsubscript{2} on renal function were associated with decreased urinary excretion of both 20-HETE and ET-1. It should be noted that urinary excretion of 20-HETE and ET-1 as an index of changes in their production within the kidney may not reflect early changes in their intrarenal levels such as those generated by the renal vasculature, particularly the microvasculature, which is a major site of ET-1 and 20-HETE synthesis. Thus, on day 15, blood pressure and protein excretion were elevated in the absence of significant increases in excretion of 20-HETE and ET-1 (Fig. 5).

Recent studies support ET-1 as a key mediator in DOCA-salt hypertension. Plasma ET-1 was demonstrated to be increased (30); mRNA for ET-1 was also increased severalfold in the vasculature, as was histochemical staining for ET-1 (14, 15). Despite these

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Fig. 6. Urinary excretion of immunoreactive ET-1 and 20-HETE on day 18 in rats that underwent sham uninephrectomy (Sham), uninephrectomy and salt treatment (UNx-salt), or uninephrectomy, DOCA, and salt treatment (UNx-salt-DOCA) and additional treatment with 9% DMSO (Vehicle), CoCl\textsubscript{2} (24 mg·kg\textsuperscript{-1}·24 h\textsuperscript{-1}), 1-aminobenzotriazole (ABT, 50 mg·kg\textsuperscript{-1}·24 h\textsuperscript{-1}), and BMS-182874 (40 mg·kg\textsuperscript{-1}·24 h\textsuperscript{-1}). RIA and GC/MS were used to quantitate ET-1 and 20-HETE levels, respectively, as described in MATERIALS AND METHODS. Data are presented as means ± SE. *P < 0.05 vs. UNx-salt. #P < 0.05 vs. +Vehicle.

Fig. 7. A: effects of 20-HETE (0.1, 1, and 10 µM), ET-1 (1 nM), or the combination of ET-1 (1 nM) and 20-HETE (1 µM) on \textsuperscript{[3]H}thymidine incorporation in cultured rat aortic rings. Data are expressed as percent of serum-free medium (SFM) control. *P < 0.05 vs. SFM. B: effects of 12,12-dibromodec-11-enoic acid (DBDD; 2 µM) on increases in \textsuperscript{[3]H}thymidine incorporation in cultured rat aortic rings induced by FBS (20%), ET-1 (1 nM), or 20-HETE (1 µM). DBDD inhibited FBS- and ET-1-induced increases in \textsuperscript{[3]H}thymidine incorporation. *P < 0.05 vs. control.
changes, results obtained using ET\textsubscript{A} or ET\textsubscript{B} and ET\textsubscript{B}-receptor antagonists in DOCA hypertensive rats demonstrated only moderate lowering of blood pressure (1, 33), raising questions as to whether ET-1 is essential to the development of hypertension in DOCA-salt-treated rats. In most studies, plasma circulating levels of ET-1 in control and DOCA-salt hypertensive rats were similar (14, 15), possibly because ET-1 is secreted contraluminally. In the present study, urinary excretion of ET-1 increased markedly in response to DOCA-salt treatment, in agreement with studies that demonstrated increased formation of ET-1 in DOCA-salt hypertension (14, 15). BMS-182874, an ET\textsubscript{A} receptor antagonist, reduced ET-1 excretion and prevented the increase in blood pressure, organ hypertrophy, and proteinuria, supporting a major role for ETs in this model of hypertension (Fig. 5).

Urinary excretion of 20-HETE also increased in step with that of ET-1, suggesting that endogenous production of 20-HETE is related to ET-1 production. This finding is in keeping with our observation that 20-HETE may contribute to ET-1-induced effects on renal function as release of 20-HETE into the renal effluents increased severalfold after injection of ET-1 into the rat isolated perfused kidney (25). Inhibition of CYP450 AA metabolism reduced urinary excretion of 20-HETE by >70% in response to challenge with ET-1 (26). The parallel increases in the urinary excretion of ET-1 and 20-HETE especially at the established phase of the hypertension in DOCA-salt hypertensive rats suggest the importance of interactions of the ET and CYP450 systems. An ET-CYP450 linkage is strengthened by the observation that ET receptor antagonists with BMS-182874 reduced the excretion of 20-HETE. The reduction by CoCl\textsubscript{2} and ABT of urinary excretion of 20-HETE is consistent with the known properties of these agents to suppress the activities of CYP450 enzymes (21, 32). However, the concomitant reduction of ET-1 excretion by CoCl\textsubscript{2} was unexpected, as this has not been reported to be an effect of CoCl\textsubscript{2}. Levels of ET-1 in the body are a net effect of synthesis of ET-1 from Big ET-1 through the action of ET-converting enzyme and degradation of the peptide by membrane metalloendopeptidase I, which is expressed on the external surface of the plasma membrane and is particularly abundant in the kidney (36). Decreased ET-1 production may reflect either decreased synthesis or increased degradation. In the isolated perfused kidney of the rat, CoCl\textsubscript{2} inhibited the vasoconstrictor response to Big ET-1, suggesting that CoCl\textsubscript{2} possesses a phosphoramidon-like activity to inhibit ET-converting enzyme in the rat (unpublished observations).

In addition, CoCl\textsubscript{2} will diminish the activity of antihypertensive CYP450-AA products, the EETs, as well as decreasing the formation of 20-HETE, a prohypertensive eicosanoid. Increased formation of the epoxide metabolites of AA prevented salt-induced elevation of blood pressure while inhibition of ET formation rendered the rat salt sensitive, facilitating the development of hypertension (19) in the face of salt loading. Because synthesis of EETs, in addition to 20-HETE, will be diminished by CoCl\textsubscript{2}, changes in the levels of antihypertensive EETs will, presumably, affect the onset of the pressor response to DOCA-salt as well as modify the response to CoCl\textsubscript{2}. Synthesis of EETs, therefore, would tend to reduce the magnitude and delay the onset of hypertension in DOCA-salt-treated rats and may be responsible for the slow onset of hypertension in UNx-DOCA-salt rats.

CoCl\textsubscript{2} induction of vascular HO activity will also increase production of CO, which subserves a vasodilator function in rats (12, 28). Like NO, CO stimulates guanylate cyclase and dilates blood vessels. The contribution of CO to the beneficial effects of CoCl\textsubscript{2} treatment on cardiovascular and renal function in UNx-salt-DOCA derived from induction of HO. Moreover, because SnMPP, by itself, intensified the organ hypertrophy and proteinuria (compare in Table 1, UNx-salt-DOCA vs. SnMPP), inhibition of the constitutive HO (HO-2), in addition to the CoCl\textsubscript{2}-inducible form (HO-1), appears likely. However, the results obtained with SnMPP do not allow us to distinguish which one of the dual effects of CoCl\textsubscript{2}, generation of CO vs. decreased production of AA products of CYP450 origin, is mainly responsible for the beneficial actions of CoCl\textsubscript{2} in reversing the negative impact of UNx-salt-DOCA treatment. An answer to this question may be provided by comparing the effects obtained with ABT, an inhibitor of CYP450 enzymes, to those obtained with CoCl\textsubscript{2} treatment. ABT reduced the hypertensive response as did CoCl\textsubscript{2} but unlike CoCl\textsubscript{2} was without effect on organ hypertrophy and proteinuria in UNx-salt-DOCA rats, suggesting that the ability of CoCl\textsubscript{2} to reverse the latter was related, at least in part, to the generation of CO whereas the antihypertensive response to CoCl\textsubscript{2} reflected inhibition of CYP450 AA metabolism.

To understand the complex role of 20-HETE in regulating blood pressure, additional factors need to be considered because 20-HETE can act in antihypertensive as well as prohypertensive mechanisms in the kidney. The opposite effects of 20-HETE on blood pressure are based on the generation of 20-HETE by different isoforms of \omega-hydroxylase (4A1, 2, 3, and 8) located in different structures; viz., 4A1 and 3 in the renal tubules vs. 4A2 in renal microvessels (22). Moreover, the \omega-hydroxylase isoforms are subject to different regulatory factors. For example, angiotensin does not stimulate 20-HETE production in the proximal tubules (24) but does stimulate such production in the medullary thick ascending limb (mTAL) (17). The 4A2 isoform of \omega-hydroxylase is partially regulated by testosterone in renal microvessels (11), in which site 20-HETE can be released by angiotensin II but not by ET-1 (Dr. M. A.
Carroll, personal communication). Additionally, the diverse biological properties of 20-HETE on both ion movement and vasomotion (20) and the structures where \( \omega \)-hydroxylase isoforms are localized determine the outcome of 20-HETE production on blood pressure. For example, increased production of 20-HETE by the renal vasculature contributes to the elevation of blood pressure in the SHR and to the renal vasoconstrictor effect of hyperchloremia. Indeed, a selective increase of 20-HETE by preglomerular microvessels can account for renal vasoconstriction and salt and water retention, characteristic features of hypertension (20). In contrast, decreased synthesis of 20-HETE in the mTAL facilitates chloride and sodium reabsorption at this tubular site and, thereby, elevates blood pressure as 20-HETE modulates the activity of the cotransporter Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\), which is primarily responsible for reabsorption of chloride and accompanying sodium by the mTAL (20). The primary deficiency in the Dahl salt-sensitive hypertensive rat has been reported as a deficit in 20-HETE formation by the mTAL that, when corrected, results in normalization of blood pressure (18). Therefore, when establishing cause-and-effect relationships for AA metabolites of CYP450 in the control of blood pressure, caution is required. Nonetheless, certain relationships are becoming clear; namely, elevated blood pressure of itself does not increase 20-HETE production in the SHR as this eicosanoid achieves its highest levels in the developmental stages between weeks 5 and 9 and then subsides to much lower levels when hypertension becomes established (23). Moreover, the highest levels of 20-HETE have been recorded in patients with advanced liver disease and incipient hepatorenal syndrome, a condition associated with lowering of blood pressure (31).

In addition to ETs, several products of CYP450 AA metabolism can act as mitogens. Lin et al. (16) demonstrated that 20-HETE, produced in abundance by renal proximal tubules, is a potent mitogen and acts as a mediator of the growth-promoting response of the proximal tubules to epidermal growth factor (EGF). Uddin et al. (37) reported that norepinephrine-induced mitogen signaling in vascular smooth muscle involves 20-HETE activation of mitogen-activated protein kinase. The observation that both HETEs and epoxides possess mitogenic effects, mediating the cellular growth effects of EGF in the proximal tubules (2, 16) and mesangial cells (34), raises the possibility that both groups of metabolites contribute to the hypertrophy in DOCA-salt UNx rats. In these studies, 20-HETE and 5,6- and 14,15-EETs acted as mitogens (34). In the present study, 20-HETE was shown to increase thymidine incorporation in rat aortic rings. Moreover, FBS- and ET-1-induced thymidine incorporation were each inhibited by DBDD, a mechanism-based inhibitor of \( \omega \)-hydroxylase, the enzyme that generates 20-HETE, suggesting that the eicosanoid mediates the mitogenic effects of ET-1 and serum on aortic rings. The differential potency of 20-HETE (\( \mu \)M) vs. ET (nM) in promoting thymidine incorporation in the rat aorta can be attributed to the avid acylation of 20-HETE by phospholipids (5). However, the present findings do not allow the general conclusion that 20-HETE is the mitogen responsible for cardiac and renal hypertrophy and increased media-to-lumen ratio of renal arteries for the following reasons: 1) CoCl\(_2\) is a nonspecific inhibitor of CYP450 AA metabolism inhibiting production of all CYP450-AA products; 2) several CYP450-AA products have mitogenic properties and may respond to ET-1 as does 20-HETE; 3) production of 20-HETE by the heart is uncertain, although it is a major product of the renal vasculature and tubules (20); 4) ET-1 may act directly or through other second messengers in addition to stimulating CYP450 AA metabolism; and 5) induction of HO by CoCl\(_2\) promotes CO production that has the potential of interacting with a variety of heme-related prosthetic groups such as guanylate cyclase (28) that could influence cell hypertrophy/hyperplasia.

In conclusion, interactions of AA products of the CYP450 enzyme system with ETs are proposed to contribute to the pathological changes in DOCA-salt-UNx rats. The ability of CoCl\(_2\) treatment to prevent hypertension, to diminish proteinuria, and to reduce renal and cardiac hypertrophy in UNx-salt-DOCA rats supports the proposal that increased activity of the CYP450 system, possibly induced by ETs, contributes to cardiovascular and renal injury in this experimental model. An additional mechanism, increased production of CO in response to CoCl\(_2\), contributes to the beneficial effects of CoCl\(_2\) on organ hypertrophy and renal injury that characterize the UNx-salt-DOCA hypertensive rat. Finally, prevention by DBDD, an inhibitor of \( \omega \)-hydroxylase, of the mitogenic effects of ET-1 in aortic rings suggests that 20-HETE is a potential mediator of some of the mitogenic effects of ETs.

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