Inhibition of renal arachidonic acid ω-hydroxylase activity with ABT reduces blood pressure in the SHR

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Su, Ping, K. Maya Kaushal, and Deanna L. Kroetz. Inhibition of renal arachidonic acid ω-hydroxylase activity with ABT reduces blood pressure in the SHR. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R426–R438, 1998.—The mechanism-based cytochrome P-450 (CYP)-inhibitor 1-aminobenzotriazole (ABT) was characterized as an inhibitor of renal arachidonic acid metabolism and administered to spontaneously hypertensive rats (SHRs) to determine the effect of reduced eicosanoid production on mean arterial pressure (MAP). A single intraperitoneal dose of ABT to Sprague-Dawley rats caused a dose-dependent loss of renal CYP content, arachidonic acid metabolism, and CYP4A protein. In the cortex and outer medulla, ABT showed a high degree of selectivity for the CYP4A enzymes, reflected by the potent inhibition of 19- and 20-hydroxyeicosatetraenoic acid (19- and 20-HETE) formation. A 50 mg/kg dose of ABT reduced cortical 20-HETE formation to 16.1 ± 0.82% of control and outer medullary 20-HETE formation to 23.8 ± 0.45% of control. In contrast, there was no inhibition of renal epoxygenase activity at this dose. Renal CYP content, arachidonic acid ω- and (ω-1)-hydroxylase activity, and CYP4A protein levels gradually return to control levels by 72 h after a single dose of ABT. Cortical 20-HETE formation recovered from 17.9 ± 3.15% of control at 6 h to 84.8 ± 4.67% of control at 72 h after ABT administration. A single injection of ABT to 7-wk-old SHRs caused an acute reduction in MAP, which remained suppressed for at least 12 h. The effect was maximal within 4 h and averaged 17–23 mmHg during the 4- to 12-h period after administration. 20-HETE formation was inhibited 85% in the cortex and 70–80% in the outer medulla during this period when MAP was reduced. A structurally related ABT analog 1-hydroxybenzotriazole had no effect on blood pressure or renal arachidonic acid metabolism. These results identify ABT as a selective inhibitor of renal CYP4A activity and provide further support for a role for 20-HETE in the regulation of blood pressure.

cytochrome P-450 4A; 20-hydroxyeicosatetraenoic acid; renal eicosanoids

INCREASINGLY RECOGNIZED as autocrine and paracrine mediators of renal function and vascular tone are cytochrome P-450 (CYP)-derived eicosanoids. Major products of renal CYP metabolism of arachidonic acid include the ω- and (ω-1)-hydroxylated metabolites, 20- and 19-hydroxyeicosatetraenoic acid (20- and 19-HETE), respectively, and regio- and stereoisomeric epoxyeicosatetraenoic acids (EETs) (15). 20-HETE is a major metabolite in renal microsomal preparations from rat, rabbit, and human and has been implicated in a number of functions related to vascular tone and ion transport. Canine renal arteries and proximal and distal portions of rat afferent arterioles are constricted by 20-HETE in a dose-dependent manner (10, 14). This vasoconstrictive effect is mediated, at least in part, by inhibition of the opening of a large-conductance, calcium-activated potassium channel, which leads to depolarization of the arteriolar vascular smooth muscle (36). Proximal tubular Na+K+-ATPase and the Na+K+-2Cl cotransporter in the medullary thick ascending limb of the kidney are both inhibited by 20-HETE, resulting in natriuresis and diuresis (1, 23). 20-HETE has also been implicated in the autoregulation of renal blood flow and tubuloglomerular feedback (37, 38). The effects of 19-HETE on renal vascular tone and ion transport contrast with those of 20-HETE, as 19(S)-HETE stimulates Na+K+-ATPase and 19(R)-HETE is a modest vasodilator (6, 14). Epoxy metabolites of arachidonic acid are produced in similar quantities as 20-HETE in rat renal microsomal preparations and are also implicated in the regulation of ion transport and vascular tone (15). Both 5,6- and 11,12-EET as well as 11,12-dihydroxyeicosatrienoic acid inhibit Na+K+-ATPase activity in rat proximal convoluted tubules, presumably leading to natriuresis (23). In a rat juxtamedullary nephron preparation superfusion with 11,12- or 14,15-EET caused dose-dependent vasodilation, whereas 5,6-EET caused vasoconstriction (9). EET-induced vasodilation is associated with an increased open-state probability of a calcium-activated potassium channel and hyperpolarization of the vascular smooth muscle (3). These effects have led to the proposal that EETs are endothelial-derived hyperpolarizing factors, although this may be species and tissue specific. The effects of CYP eicosanoids on renal function and vascular tone suggest that these metabolites play an important role in the regulation of blood pressure. However, the contrasting effects of these eicosanoids on renal vascular tone and tubular ion secretion make it difficult to predict their role in vivo in regulating blood pressure. The vasoconstrictive effects of 20-HETE on the vasculature would be expected to be prohypertensive, whereas the inhibition of renal tubular Na+ reabsorption would be antihypertensive. In the spontaneously hypertensive rat (SHR), renal arachidonic acid ω-hydroxylation is increased relative to age-matched normotensive Wistar-Kyoto (WKY) rats (8, 13, 22). Increased 20-HETE formation in SHR renal microsomes is maximal in young animals (8, 13, 22) and is accompanied by a decreased diameter of interlobular arteries and afferent arterioles (8). These differences in arachidonic acid metabolism between WKY and SHR renal microsomes appear to be specific because 19-HETE formation shows similar changes as 20-HETE, whereas no changes in epoxygenase activity have been reported (22). Members of the CYP4A enzyme family
are responsible for arachidonic acid \(\omega\)- and \((\omega-1)\)-hydroxylation and are expressed at high levels in the rat kidney (15). Recently, we showed that increased expression of the CYP4A3 and CYP4A8 genes and CYP4A immunoreactive proteins account for the increased arachidonic acid \(\omega\)- and \((\omega-1)\)-hydroxylation in the young SHR kidney, suggesting that altered CYP4A expression in the prehypertensive SHR kidney may contribute to the changes in renal function during this period (13).

Limited information is available about the in vivo role of the CYP eicosanoids in the SHR. Administration of the heme oxygenase inducers stannous chloride and heme arginate to 7-wk-old SHRs significantly inhibited CYP-mediated arachidonic acid metabolism in renal microsomes and reduced the blood pressure to levels found in age-matched WKY rats (16, 31). In one case the decrease in CYP metabolism was shown to be specific for \(\omega\)- and \((\omega-1)\)-hydroxylation, thus implicating these pathways in blood pressure control (16). However, the interpretation of these studies is limited because heme oxygenase inducers also affect the production of carbon monoxide and may interact with nitric oxide synthase, distinct systems that are known to modulate vascular tone. 20-HETE has also been implicated in the regulation of blood pressure in the Dahl salt-sensitive model of hypertension, although its proposed mechanism is distinct from that in the SHR. A decreased formation of 20-HETE in outer medullary microsomes of prehypertensive Dahl salt-sensitive rats relative to the salt-resistant controls is associated with an elevated loop chloride transport in these animals (35).

1-Aminobenzotriazole (ABT) is a mechanism-based CYP inhibitor. Inactivation of CYP enzymes by ABT requires catalytic formation of benzoyl, which then alkylates the prosthetic heme group (27). ABT has a broad substrate specificity and in vivo inactivates 70–80% of the total hepatic CYP content of phenobarbital-induced rats (27). ABT also inactivates CYP protein in pulmonary and renal microsomes (17, 20). Inhibition of the hepatic CYP4A enzymes by ABT has been characterized both in vitro and in vivo using lauric acid as a substrate. Preincubation of rat hepatic microsomes or treatment of rat hepatocytes with ABT results in a time- and concentration-dependent inhibition of lauric acid \(\omega\)- and \((\omega-1)\)-hydroxylation (11, 28). A single intraperitoneal injection of ABT to Sprague-Dawley rats inhibits lauric acid \(\omega\)- and \((\omega-1)\)-hydroxylation more than 70% and requires at least 5 days for complete recovery of this activity (26). Similar information regarding the inhibition of renal CYP4A enzymes by ABT is not available. ABT is particularly useful to probe the physiological roles of CYP enzymes because it is water soluble and is relatively nontoxic on chronic administration (18).

The ability to examine the in vivo significance of CYP catalyzed arachidonic acid metabolism in blood pressure regulation in the SHR has been hindered by the lack of inhibitors with in vivo activity. We report here the identification of ABT as an inhibitor of renal CYP arachidonic acid metabolism. ABT shows selectivity in the kidney for the \(\omega\)- and \((\omega-1)\)-hydroxylation of arachidonic acid and causes a loss of CYP4A apoprotein. Inhibition of renal 20-HETE formation by ABT is associated with a decrease in mean arterial pressure (MAP), providing evidence for the involvement of this eicosanoid in the regulation of blood pressure.

**METHODS**

**Materials.** Intramedic polyethylene tubing (PE-10) and Tygon tubing were purchased from Clay Adams ( Parsippany, NJ). Ketamine HCl was from Aveco (Fort Dodge, IA), and acepromazine maleate and xylazine were from Butler (Kansas City, MO and Columbus, OH). ABT and 1-hydroxybenzotriazole were obtained from Sigma Chemical (St. Louis, MO). Radiolabeled arachidonic acid was purchased from Amersham Life Science (Arlington Heights, IL) or from NEN Life Science Products (Boston, MA). HPLC solvents and ScintiVerse LC were from Fisher Scientific (Pittsburgh, PA). All other reagents were of the highest grade available and were purchased from Fisher Scientific or Sigma Chemical.

Animal surgery and treatment. Male SHRs weighing 110–150 g at 6 wk of age (Charles River Laboratories, Wilmington, MA) or male Sprague-Dawley rats weighing 170–200 g (Simonsen Laboratories, Gilroy, CA) were maintained under controlled housing conditions of light (6 AM–6 PM) and temperature (22°C) and received standard laboratory chow and water ad libitum. Rats were allowed at least 3 days to become acclimated to the housing conditions before use in experiments, and blood pressure and arachidonic acid metabolism were measured at 7 wk of age. All animal protocols were approved by the University of California San Francisco Committee on Animal Research and followed the National Institutes of Health Guide for the Care and Use of Experimental Animals.

Blood pressure was measured in freely moving rats through a PE-10 Tygon catheter. While rats were under anesthesia with a mixture of ketamine-xylazine-acepromazine (44:2.5:0.75 mg/kg ip), the PE-10 catheter (3.5–4.0 cm) was inserted into the abdominal aorta via a femoral artery. The Tygon portion of the catheter was carefully tunneled under the skin and positioned to exit in the intrascapular region. Rats were allowed at least 2–3 days until MAP was stable. The patency of the catheter was maintained by daily flushes with heparinized saline and by filling with a heparinized dextrose solution. The arterial catheter was connected directly to a transducer (Micro-Med, Louisville, KY), and MAP was recorded for 30–60 min. The mean value over this collection was calculated and recorded as MAP for a given time point. Baseline MAP was measured before treatment with ABT or 1-hydroxybenzotriazole. In some cases, rats were housed in metabolic cages for 48 h. Urine was collected for 24 h before administration of the test compound and during the first 24 h after a dose for determination of ion excretion, diuresis, and creatinine clearance. For creatinine clearance determinations, a single blood sample was collected at the midpoint of the urine collection interval from the arterial catheter.

ABT and 1-hydroxybenzotriazole were administered intraperitoneally, and rats were killed at various times after treatment. Rats were anesthetized with ethyl ether, the abdominal cavity was opened, and the liver and kidneys were perfused with ice-cold saline. Liver and kidneys were removed, the kidney was dissected into cortex and outer and inner medulla, and all tissues were frozen immediately in liquid nitrogen and stored at −80°C. Urine and plasma samples were stored at −20°C.
Clinical chemistry analysis. Urinary Na$^+$ and K$^+$ levels were determined by standard flame photometry techniques, and urinary and plasma creatinine levels were determined colorimetrically by the clinical laboratory of the General Clinical Research Center at the University of California San Francisco. Creatinine clearance was calculated from the ratio of the 24-h urinary excretion rate of creatinine to the plasma concentration of creatinine at the midpoint of the urine collection period.

Preparation of microsomes and CYP determination. Microsomes were prepared from liver, renal cortex, or outer medulla samples from a single animal as described previously (13). Microsomal protein concentrations were measured by the Pierce BCA protein assay (Pierce Chemical, Rockford, IL) with BSA as the standard. Total CYP content in hepatic or renal cortical microsomes was measured from the reduced carbon monoxide difference spectra on an Aminco DW-2000 UV-VIS spectrophotometer as described by Omura and Sato (24).

Microsomal arachidonic acid metabolism. Renal cortical and hepatic arachidonic acid metabolism was measured in incubations containing [1$^{-}$C]arachidonic acid (10 µM, 0.2 µCi), microsomal protein (0.5 mg/ml), KCl (150 mM), MgCl$ _2$ (10 mM), sodium isocitrate (8 mM), and isocitrate dehydrogenase (0.5 U) in Tris-HCl buffer (50 mM, pH 7.5). The mixtures were preincubated for 5 min at 37°C, and the reaction was started by addition of NADPH (1 mM). The incubation was carried out for 30 min at 37°C, and the reaction was terminated by acidifying to pH 3.5 with HCl. For cortical microsomes, arachidonic acid metabolism, 15 µM [1$^{-}$C]arachidonic acid (0.2 µCi) and 0.25 mg/ml microsomal protein were used, and the reaction was carried out for 45 min. Arachidonic acid and its metabolites were extracted twice with ethyl acetate, and the combined organic phase was washed once with double-distilled water. After evaporation of organic solvent under nitrogen, the dry residue was stored at −80°C until HPLC analysis.

HPLC analysis of arachidonic acid metabolites. A reverse-phase HPLC system consisting of a Shimadzu SLC-6A controller, two LC-6A pumps, and a Radiomatic 525TR flow scintillation analyzer with FLO-One software (Packard Instrument, Downers, IL) was used for the separation and quantification of arachidonic acid metabolites. Metabolites were separated on an Alltima C18 5 µm column (250 × 4 mm) with an Alltima C18 guard column and in-line filter (Alltech Associates, Deerfield, IL) exactly as described previously (13). Epoxygenase activity is reported as the sum of epoxide and dihydroxyeicosatetraenoic acid formation.

In vitro inhibition of arachidonic acid metabolism. Microsomes prepared from the renal cortex or liver of untreated Sprague-Dawley rats (5 mg/ml) were incubated with varying concentrations of ABT, 1 mM NADPH, and an isocitrate dehydrogenase generating buffer system for 30 min at 37°C. An aliquot was removed immediately after the addition of NADPH as a control, and in some cases NADPH was omitted from the incubation mixture. After inactivation with ABT the microsome samples were diluted 10-fold with reaction buffer, and arachidonic acid metabolism was measured exactly as described previously.

Immunoblotting of CYP4A proteins. Renal cortical or hepatic microsomal protein (10 µg) or renal outer medullary microsomal protein (2 µg) was electrophoretically separated on a 8% SDS-polyacrylamide gel as described by Okita et al. (21). Proteins were transferred to a nitrocellulose membrane (Trans-Blot, Bio-Rad, Hercules, CA) in 25 mM Tris-HCl, 192 mM glycine, and 20% methanol. Membranes were incubated overnight with a 500-fold dilution of goat anti-CYP4A1 serum and for 2 h with a 1,000-fold dilution of alkaline phosphatase-conjugated rabbit anti-goat IgG (liver and cortex samples) or a 7,500-fold dilution of horseradish peroxidase-conjugated rabbit anti-goat IgG (outer medulla samples). Immunoreactive proteins were detected using an alkaline phosphatase-conjugate substrate kit (Bio-Rad) or by enhanced chemiluminescence (Amersham) according to the manufacturer’s instructions.

Statistics. Values are reported as means ± SE. Significance of difference between groups was evaluated by a paired t-test or a one-way ANOVA with post hoc multiple comparisons with a modified t-test. Statistical significance was set at the level of P < 0.05.

RESULTS

Inhibition of arachidonic acid metabolism by ABT. Although ABT has been widely used as a mechanism-based CYP inhibitor, its potency and selectivity for arachidonic acid metabolism were unknown. The inhibition of arachidonic acid metabolism by ABT was first measured in vitro. Renal cortical and hepatic microsomes from untreated Sprague-Dawley rats were incubated with varying concentrations of ABT in the presence of NADPH before their use in arachidonic acid metabolism determinations. As expected for a mechanism-based inhibitor, NADPH was required in the incubation mixture for inactivation of CYP enzymes. Arachidonic acid metabolism was inhibited by in vitro inactivation with ABT in both cortical and hepatic microsomes (Fig. 1). The inhibition was clearly dose dependent and showed a fair degree of selectivity for the CYP4A catalyzed formation of 19- and 20-HETE in the cortex. At a concentration of 500 µM ABT, cortical 19- and 20-HETE formation was reduced to 25–30% of control, whereas epoxygenase activity was reduced to 56% of control. In contrast, ABT showed no selectivity for the individual arachidonic acid metabolic pathways in the liver microsomes.

The effect of in vivo administration of ABT on renal and hepatic CYP content and arachidonic acid metabolism was measured 6 h after a single intraperitoneal injection to Sprague-Dawley rats. Both cortical and hepatic CYP content was decreased by ABT in a dose-dependent manner (Fig. 2). A significant loss of CYP content was evident at ABT doses greater than 10 mg/kg and was essentially maximal at 50 mg/kg. Cortical CYP content was reduced 52% and hepatic CYP content 66% at the highest dose. The hepatic CYP content was five times greater than that in the cortex.

In cortical microsomes this loss of CYP protein was accompanied by a dose-dependent and selective inhibition of 19- and 20-HETE formation (Figs. 3 and 4A). As evident from the representative HPLC chromatogram in Fig. 3, in vivo administration of ABT shows a high degree of selectivity for the CYP4A-catalyzed formation of 19- and 20-HETE in renal cortical microsomes. This is also consistent with the selectivity found for in vitro inactivation of cortical microsomes by ABT (Fig. 1). Arachidonic acid ω-hydroxylation was inhibited at a dose of ABT as low as 5 mg/kg and was reduced to 6% of control values with a 100 mg/kg dose. The inhibition of 19-HETE formation by ABT showed a similar pattern
as with 20-HETE, although the effect was less at all except the highest dose. In contrast, arachidonic acid epoxide formation was not inhibited in cortical microsomes except at an ABT dose of 100 mg/kg. At this dose epoxygenase activity was reduced to 35% of control values. A similar inhibition profile was found when arachidonic acid metabolism was measured in outer medullary microsomes (Fig. 4B). The single major metabolite in outer medulla microsomes is 20-HETE, with only minor amounts of 19-HETE and the individual epoxides. As in the cortex, both 19- and 20-HETE formations were reduced in a dose-dependent fashion in outer medulla microsomes prepared from ABT-treated rats. Maximal inhibition of 19- and 20-HETE formation in the outer medulla was less than in the cortex (60–70% inhibition), and epoxygenase activity was not inhibited by ABT. In fact, at the 100 mg/kg dose of ABT, epoxygenase activity was significantly increased in the outer medulla. The major CYP isoforms responsible for arachidonic acid metabolism are CYP4A [ω- and (ω-1)-hydroxylation], CYP2C23, CYP2E1, and CYP2J [epoxidation and (ω-1)-hydroxylation] (15, 34). Thus, at lower doses, ABT appears to show selectivity for the CYP4A enzymes in the renal cortex and outer medulla. In contrast, ABT had a nonspecific effect on hepatic arachidonic acid metabolism (Fig. 4C). Although only CYP4A enzymes were inhibited at lower doses, as evidenced by the significant inhibition of 20-HETE formation with as little as 5 mg/kg ABT, all of the metabolic pathways were equally affected above 25 mg/kg.

Western blotting of renal and hepatic microsomes with an antibody against rat CYP4A1 examined the effect of ABT on CYP4A protein levels. As shown in Fig. 5, inhibition of arachidonic acid ω- and (ω-1)-hydroxylase activity in the cortex and outer medulla was associated with loss of CYP4A immunoreactive protein. In contrast, liver CYP4A protein levels remained constant despite significant inhibition of functional activity. Two distinct protein bands were detected in both cortex and liver microsomes from control and treated animals. On the basis of the literature, the bottom band...
in the kidney samples can be identified as a doublet of CYP4A1 and CYP4A2 and the upper band as CYP4A3. In the liver, the lower band is CYP4A1 and the upper band is CYP4A3 (21). We were not able to detect hepatic CYP4A2 in our samples, which reflects its low constitutive levels in the liver and the cross-reactivity with the CYP4A1 antibody. In the cortex, all three CYP4A isoforms showed parallel changes in response to ABT.

Recovery of functional activity after ABT treatment. The duration of the inhibitory response will be an important determinant of the effectiveness of mechanism-based inhibition to probe physiological function.

**Fig. 4. Dose-dependent inhibition of renal and hepatic arachidonic acid metabolism after ABT administration.** Male Sprague-Dawley rats were administered a single intraperitoneal dose of ABT, and tissues were harvested 6 h later. The NADPH-dependent formation of 19-HETE, 20-HETE, and EETs + DHETs (epoxygenase activity) was measured in cortical (A), outer medullary (B), and hepatic (C) microsomes with [14C]arachidonic acid. Values are expressed as percent of control and reported as means ± SE from 3–6 animals per treatment group. Treatment groups were compared with 1-way ANOVA followed by multiple comparisons with modified t-test. *Significantly different from control, P < 0.05. At the 50 and 100 mg/kg doses in the hepatic microsomes, all metabolites were significantly inhibited. Cortical control rates (pmol·min⁻¹·mg protein⁻¹) were 19-HETE, 8.52 ± 1.21; 20-HETE, 38.9 ± 4.22; and epoxygenase activity, 35.2 ± 4.06. Outer medullary control rates (pmol·min⁻¹·mg protein⁻¹) were 19-HETE, 2.39 ± 0.207; 20-HETE, 14.8 ± 1.77; and epoxygenase activity, 18.7 ± 1.59. Hepatic control rates (pmol·min⁻¹·mg protein⁻¹) were 19-HETE, 14.3 ± 1.16; 20-HETE, 22.3 ± 1.57; and epoxygenase activity, 203 ± 22.8.
The recovery of CYP content, arachidonic acid metabolism, and CYP4A protein levels was followed for 5 days after a single dose of ABT to Sprague-Dawley rats. The loss of cortical and hepatic microsomal CYP 6 h after administration of ABT was similar to that seen in the dose-response study (Fig. 6). CYP content showed signs of recovery within 48 h for the cortex and within 24 h for the liver. Recovery of CYP levels was faster in the liver, returning to basal levels by 72 h after ABT administration, whereas cortical CYP levels were significantly less than control values until 96 h after a single dose of ABT.

Recovery of arachidonic acid metabolism rates paralleled the changes in CYP content. Cortical, outer medullary and hepatic arachidonic acid ω- and (ω-1)-hydroxylase activity was maximally inhibited within 6 h after the ABT dose and gradually returned to basal levels over 3–4 days (Fig. 7). For example, cortical 20-HETE formation was 17.9 ± 3.15% of control 6 h after ABT administration and returned to 84.8 ± 4.67% of control by 72 h. In the cortex epoxygenase activity was only inhibited at the 6-h time point and was minimal (26% inhibition). Treatment with ABT did not inhibit epoxygenase activity in the outer medulla. In the liver the recovery of functional activity was identical for all three pathways and was slower than recovery in the kidney. Hepatic arachidonic acid epoxide and HETE formation was inhibited 60–76% 6 h after ABT administration and returned to basal values by 96 h. Cortical and outer medullary CYP4A levels remained depressed until 72 h after a single dose of ABT (Fig. 8, A and B). Thus recovery of CYP4A protein and arachidonic acid ω- and (ω-1)-hydroxylation in the kidney showed identical patterns. In contrast, loss of CYP4A functional activity in the liver was not due to a loss of CYP4A protein, as evidenced by the constant level of CYP4A1 and CYP4A3 throughout the 120-h study period (Fig. 8C).

Effect of ABT on blood pressure. ABT was administered to 7-wk-old SHRs to measure the effect of CYP inhibition on blood pressure. MAP was measured in freely moving animals through a catheter inserted into the abdominal aorta before and for up to 3 days after a single dose of ABT. Within several hours after treatment, MAP was significantly reduced and remained suppressed for over 12 h (Fig. 9). The effect was maximal within 4 h and averaged 17–23 mmHg during the 4- to 12-h period (8–24% decrease). ABT had no effect on heart rate during this period. Renal excretions of Na⁺ and K⁺, urine volume, and creatinine clearance were measured over the 24-h periods during the control and treatment phases of the protocol (Table 1). There was a 53% decrease in the 24-h urinary excretion of Na⁺ after a single dose of ABT. This is consistent with the decreased formation of 20-HETE, which normally promotes natriuresis and diuresis by its inhibitory effects on proximal tubular and medullary thick ascend-
ing limb of Henle Na⁺ transport. The effect of ABT on renal function and ion excretion was specific for Na⁺ because urinary K⁺ or creatinine excretion, creatinine clearance, and diuresis were unchanged.

Microsomal arachidonic acid metabolism was measured in the cortex and outer medulla from these rats at various times after ABT administration. Accompanying the acute decrease in MAP was a significant decrease in arachidonic acid ω⁻ and (ω⁻1)-hydroxylase activity in the SHR kidneys (Fig. 10). 20-HETE formation was inhibited 85% in the cortex and 70–80% in the outer medulla during the 18-h period after ABT administration. In contrast, cortical epoxygenase activity was less inhibited than HETE formation and was unaffected in cortex, which gradually recovered to control levels by 72 h. There was no effect of ABT on hepatic CYP4A content.
Fig. 9. Treatment with ABT reduces blood pressure in the SHR. Mean arterial pressure (MAP) was measured through femoral catheter in male SHRs (7 wk old) for 2–3 days before and for various times after administration of single dose of ABT (50 mg/kg). Change in MAP after ABT administration was calculated and is expressed as means ± SE of 4–11 animals per time point. Control animals (time 0) were administered vehicle only. MAP was recorded over 48 h, and average change was calculated. Average ± SE MAP before ABT treatment was 127 ± 2.4 mmHg. Effect of ABT on MAP was compared with vehicle-treated controls by 1-way ANOVA followed by multiple comparisons with modified t-test. *P < 0.05.

Table 1. Effect of ABT and 1-hydroxybenzotriazole on urinary ion excretion and renal function in the SHR

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<tr>
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<th>Control</th>
<th>Drug</th>
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<tr>
<td>Na⁺ excretion, meq/24 h</td>
<td>1.75 ± 0.18</td>
<td>0.82 ± 0.094*</td>
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<tr>
<td>K⁺ excretion, meq/24 h</td>
<td>3.33 ± 0.29</td>
<td>2.87 ± 0.34</td>
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<tr>
<td>Creatinine excretion, mg/h</td>
<td>0.182 ± 0.014</td>
<td>0.180 ± 0.109</td>
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<tr>
<td>Creatinine clearance, ml/min</td>
<td>0.726 ± 0.056</td>
<td>0.773 ± 0.109</td>
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<tr>
<td>Urine volume, ml/24 h</td>
<td>8.82 ± 0.982</td>
<td>10.8 ± 1.47</td>
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<tr>
<td>1-Hydroxybenzotriazole</td>
<td>0.893 ± 0.089</td>
<td>1.27 ± 0.133</td>
</tr>
<tr>
<td>Na⁺ excretion, meq/24 h</td>
<td>2.52 ± 0.201</td>
<td>3.44 ± 0.178*</td>
</tr>
<tr>
<td>K⁺ excretion, meq/24 h</td>
<td>0.190 ± 0.011</td>
<td>0.202 ± 0.008</td>
</tr>
<tr>
<td>Urine volume, ml/24 h</td>
<td>15.2 ± 1.46</td>
<td>13.9 ± 0.625</td>
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Values are means ± SE; n = 4–8 rats/treatment group. Urine was collected for 24 h after treatment of spontaneously hypertensive rats (SHRs) with vehicle, ABT, or 1-hydroxybenzotriazole. *Significantly different from control, P < 0.05.
tion and N-glucuronidation. The half-life of ABT and its metabolites in the plasma was found to be 9 h, whereas that in the kidney was 12 h. Less than 1% of a dose of ABT was accounted for in the kidney, suggesting that concentrations of ABT in the kidney necessary for inhibition of the CYP4A enzymes will be quite low.

ABT shows a striking selectivity for the CYP4A enzymes in both the kidney and the liver. In the liver this selectivity is evident only at very low doses (5–10 mg/kg), whereas in the kidney it persists at all except the highest dose of ABT. Maximal inhibition of arachidonic acid metabolism in the kidney is greatest for the CYP4A pathways, inhibiting 85% of 20-HETE formation and >70% of 19-HETE formation. ABT has generally been described as a nonselective inhibitor of hepatic CYP metabolism, implying that CYP enzymes with various active sites can accommodate this compound (25). The present results suggest that ABT can more easily enter the CYP4A active site than that of the arachidonic acid epoxygenases CYP2E1, CYP2C23, and CYP2J. Selectivity of ABT for the lung CYP4B enzymes has also been described (12).

The selectivity of ABT for the CYP4A enzymes is of particular significance because other characterized
CYP4A inhibitors cannot be used in vivo. Substrates for the CYP4A enzymes are limited to fatty acids and prostaglandins, which are hydroxylated preferentially at the ω-position (15). The design of selective mechanism-based CYP4A inhibitors has incorporated this relatively narrow substrate specificity. Acetylenic fatty acids access the CYP4A active site and are enzymatically converted to a ketene species, which can alkylate the protein (2). These acetylenic fatty acids inhibit lauric acid ω- and (ω-1)-hydroxylation by hepatic microsomes and purified rat liver CYP4A1 and PGE1 ω-hydroxylation by CYP4A4 purified from pregnant rabbit lungs (2, 19, 29). They have also been used in situ to establish the role of 20-HETE in the regulation of renal blood flow and tubuloglomerular feedback and as a K+ channel inhibitor in rat renal arterioles (36–38). Both ABT and 10-undecynoic acid were used in primary rat hepatocytes to demonstrate that CYP4A catalyzed fatty acid metabolites mediate the induction of peroxisomal fatty acid β-oxidation and liver fatty acid binding protein by peroxisome proliferators (11). Despite the selectivity of these acetylenic fatty acid inhibitors for the CYP4A enzymes, their use is limited to in vitro or in situ experiments because of their rapid degradation in vivo by fatty acid β-oxidation (29). One exception is an elegant study in which 17-octadecynoic acid was chronically infused into the medullary interstitium of rats and selectively inhibited CYP4A activity in the outer medulla (32). The short half-life of this inhibitor is evident in this study from the lack of effect on 20-HETE formation in the neighboring cortex region. Based on our results, ABT can be administered intraperitoneally and used to selectively inhibit CYP4A enzymes in the rat kidney, providing an important tool for characterizing the in vivo effects of 20-HETE. ABT can also be given orally with a bioavailability of >70%, further increasing its usefulness (33).

The inhibition of renal arachidonic acid metabolism was distinct from that measured in hepatic microsomes in several respects. Inhibition of 20-HETE formation was more complete in the cortex and outer medulla than in the liver, whereas epoxygenase activity was more susceptible to inhibition in the liver. One possible explanation for these findings is the relative abundance of the individual CYP isoforms in the liver versus the kidney. For example, three CYP4A genes (CYP4A1, CYP4A2, and CYP4A3) are expressed in the rat liver, whereas a fourth gene (CYP4A8) is also expressed in the rat kidney (15). The relative abundance of these isoforms also differs between the two tissues. In the liver, CYP4A1 and CYP4A3 are the major constitutive enzymes, whereas CYP4A2 is highly inducible. In contrast, in the kidney CYP4A2 and CYP4A8 are constitutively expressed at levels that are two to five times higher than that of CYP4A1 and CYP4A3 in the 7-wk-old rats used in these studies (13). A greater susceptibility of the CYP4A2 and CYP4A8 isoforms to ABT inhibition would account for the increased inhibition of 19- and 20-HETE formation in the kidney. This is consistent with the recent observation that ABT has no effect on the activity of recombinant CYP4A1 protein (5). The arachidonic acid epoxygenases CYP2C23, CYP2E1, and CYP2J are expressed in both the liver and kidney, and it is not clear which isoform is the major epoxygenase in these tissues (15, 34). On the basis of the present findings, it is likely that the major renal epoxygenase is more resistant to inhibition by ABT than is the major hepatic epoxygenase.

Surprisingly, renal CYP4A protein is lost following treatment with ABT, whereas hepatic CYP4A protein levels are unchanged. ABT has previously been shown to inactivate the CYPs by alkylation of the prosthetic heme moiety (27). The loss of CYP4A apoprotein in the cortex and outer medulla may reflect the smaller heme pool in the kidney relative to the liver. Assuming an insufficient pool to supply functional heme to the CYP4A apoprotein, it would be expected that the apoprotein would then be targeted for degradation. Alternatively, in the cortex and outer medulla ABT may also inhibit CYP4A enzymes at least in part by destruction of the apoprotein itself. Two distinct mechanisms of action may account for the increased CYP4A inhibition in the kidney relative to the liver. In the kidney the susceptible isoforms may also be more abundantly expressed, thereby accounting for the increased inhibition. Determination of the exact mechanism responsible for the loss of renal CYP4A apoprotein after ABT treatment will require further experimentation with the individual CYP4A recombinant proteins.

The decrease in MAP after ABT administration is the first report demonstrating that mechanism-based inhibition of CYP4A4 enzymes affects blood pressure in the SHR. Previous attempts at trying to associate renal arachidonic acid metabolism with the regulation of blood pressure have employed the administration of heme oxygenase inducers as an indirect method of altering CYP enzymes (16, 31). Induction of heme oxygenase reduces the levels of the prosthetic heme group and will therefore act as a nonspecific inhibitor of the CYP enzymes. In addition, heme oxygenase inducers may also affect vascular tone independent of their effects on the CYP enzymes because they alter the production of carbon monoxide and may interact with nitric oxide synthase. A single dose of ABT reduced blood pressure from 10 to 36 mmHg in individual rats. MAP began to gradually decrease within 1 h after ABT treatment, and the effect was maximal by 4 h. This correlated with the rapid destruction of CYP enzymes and inhibition of arachidonic acid metabolism, which was also maximal within 4 h. The observation that a structurally similar noninhibitory ABT analog has no effect on blood pressure provides strong evidence that the decrease in blood pressure after ABT administration is associated with its potent inhibitory effects on 20-HETE formation.

A decrease in blood pressure after CYP inhibition of arachidonic acid metabolism is consistent with some of the prohypertensive properties of the CYP4A eicosanoids and with the changes in renal function, vascular tone, and CYP4A expression that occur in the SHR. For example, 20-HETE is a potent vasoconstrictor, depolarizes vascular smooth muscle cells, and inhibits Na+-K+--
An increased production of 20-HETE has been proposed to alter renal vascular tone and elevate blood pressure in the SHR, whereas a decreased production of 20-HETE such as that observed after ABT treatment would be expected to reduce the vasoconstrictive effects and lower blood pressure. In contrast, the effects of 20-HETE on Na\(^+\) transport in the renal tubule result in natriuresis and diuresis, and a dampening or elimination of these effects resulting from decreased 20-HETE formation could lead to an increase in blood pressure. Inhibition of renal 20-HETE formation by ABT produced the expected decrease in urinary Na\(^+\) excretion, but this was not associated with an increase in blood pressure as might be predicted. This suggests that the effects of 20-HETE in regulating renal vascular tone are more important in the regulation of blood pressure than its effects on tubular Na\(^+\) transport.

The importance of 20-HETE in regulating vascular tone and blood pressure is also supported by previous studies. Vascular tone is altered very early in the SHR, with basal diameters of afferent arterioles and preglomerular vasculature 18–35% smaller in the SHR than in the normotensive WKY rat (8). Addition of CYP inhibitors to the perfusate of juxtaglomerular microvascular preparations completely eliminated these differences in vascular tone, indicating that CYP metabolites of arachidonic acid are important determinants of these changes. An increased formation of 20-HETE has also been reported in renal microsomes from young SHRs relative to normotensive WKY rats (8, 13, 22). Our recent studies suggest that increased expression of CYP4A3 and CYP4A8 in the young SHR kidney is responsible for the increased 20-HETE formation (13). However, the significance of this increased CYP4A \(\omega\)-hydroxylase activity in the elevated blood pressure in the SHR is still unclear because the absolute levels of cortical activity are similar in other normotensive rat strains, including the Sprague-Dawley rats used in this study and Lewis rats (8). Despite similar levels of 20-HETE formation in the SHR and other normotensive rats, it is possible that the SHR vasculature may be more responsive to its vasoconstrictive effects, thus accounting for the differences in blood pressure between these animals. In the case of the WKY rat a difference in vascular responsiveness would be combined with a decreased production of 20-HETE.

The overall effect of the CYP eicosanoids on renal function and blood pressure will be dependent on the relative production of the individual metabolites within specific regions of the nephron. Because the CYP4A enzymes are more potently inhibited by ABT than the other arachidonic acid metabolizing CYP enzymes, the EETs become quantitatively more important metabolites in renal microsomal fractions from the treated animals. In SHR cortical microsomes the percentage of total arachidonic acid metabolism that goes through the epoxygenase pathway increases from 45% in control animals to 71–76% during the 24-h period after ABT administration. The increase in EET contribution could be completely accounted for by a decrease in the contribution of 20-HETE. Assuming that the in vitro metabolite formation reflects the in vivo formation, the antihypertensive properties of the EETs may become dominant after CYP4A inhibition. The inhibitory effects of 5,6- and 11,12-EET against Na\(^+\)-K\(^+\)-ATPase, and the vascular smooth muscle cell hyperpolarization and ensuing vasodilation by 11,12- and 14,15-EET would lead to a lowered blood pressure (9, 23).

A persistent suppression of CYP4A functional activity was not sufficient to maintain a decreased blood pressure in the SHR. A single dose of ABT inhibits functional CYP4A activity in the cortex for >72 h, whereas MAP is completely recovered 18–24 h after treatment. Several explanations could account for this observation. First, the return of blood pressure to basal levels may be a physiological response that does not involve the CYP renal eicosanoids and that could supersede any effects of these metabolites on renal function and vascular tone. Arterial blood pressure is normally tightly controlled, owing to the multiple levels of regulation (7). Pressure controls that act on neural receptors respond within seconds to changes in blood pressure, followed by activation of hormonal control systems within minutes. The kidney-fluid system is necessary for long-term control of arterial pressure and reacts within hours or days of blood pressure changes. The return of blood pressure toward control values within 12–24 h after ABT treatment suggests that the kidney-fluid system may be operating in this case.

A second possibility that could account for the discrepancy between the duration of blood pressure suppression and inhibition of 20-HETE formation is that CYP epoxygenase activity is the more important determinant of blood pressure in the SHR. Epoxygenase activity was transiently inhibited after administration of ABT, although the effect was much less than the corresponding inhibition of CYP4A activity and 20-HETE formation. It also returned to control values within 12 h, suggesting that ABT was not inhibiting the arachidonic acid epoxygenases in a mechanism-based manner. Although MAP and cortical arachidonic acid epoxygenase activity recovered in a similar time frame, a direct reduction of blood pressure by inhibition of EET formation is not supported by the biological properties of these metabolites. In general, the EETs are considered to be antihypertensive because of their vasodilatory effects and ability to inhibit Na\(^+\)-K\(^+\)-ATPase in the tubular epithelium (9, 23). One exception is the 5,6-EET metabolite, which has vasoconstrictive properties (9). It is possible that the decrease in blood pressure could be a result of the increased contribution of the antihypertensive epoxide metabolites to the overall metabolism of arachidonic acid at early times after ABT administration. However, the EETs remain the major metabolic product until 72 h after ABT administration, which is also not consistent with the return of blood pressure to basal levels.

A final explanation that can account for the discrepancy in the recovery of blood pressure and CYP4A activity is that in vitro determinations of enzyme activity do not reflect the in vivo formation of 20-HETE.
CYP4A inhibition by ABT involves destruction of the enzymes and requires synthesis of new protein for recovery. After a single dose of ABT, complete recovery of CYP4A functional activity required >72 h, indicating a slow synthesis rate of the protein. Even longer recovery periods were reported for lauric acid ω-hydroxylase activity in hepatic microsomes after a 50 mg/kg dose of ABT to Sprague-Dawley rats (26). Despite this slow recovery it is possible that sufficient activity to provide basal levels of 20-HETE exists before complete recovery of CYP4A activity. It was recently suggested that 20-HETE can also be stored in the phospholipid pool and released in response to ANG II stimulation (4). This raises the interesting possibility that 20-HETE may be available at sufficient concentrations to produce a desired effect despite very low levels of functional CYP4A protein. Quantification of endogenous levels of the CYP eicosanoids in the renal cortex and outer medulla will provide a more accurate measurement of the functional levels of these metabolites.

In summary, we have characterized the mechanism-based CYP inhibitor ABT as a selective inhibitor of the renal CYP4A enzymes. Inhibition of the CYP4A enzymes occurs rapidly and leads to loss of apoprotein and a decreased formation of 19- and 20-HETE formation in microsomes prepared from the outer medulla and cortex. Recovery of enzyme activity involves the synthesis of new protein and occurs over >3 days. Administration of ABT to SHRs results in inhibition of up to 85% of renal 20-HETE formation and is accompanied by an acute reduction in blood pressure. The effects of ABT on blood pressure appear to be specific to its CYP4A-inhibitory properties because a structurally similar analog that does not inhibit 20-HETE formation has no effect on blood pressure. Finally, the decrease in blood pressure with ABT treatment is not consistent with changes in renal tubular ion transport or gross changes in renal function, suggesting that the vasoconstrictive properties of 20-HETE are a major determinant of its effect on blood pressure.

Perspectives

The present studies were carried out in the SHR during the developmental period of hypertension. Although these results further support a role for CYP eicosanoids in the regulation of blood pressure, the mechanism by which this occurs remains unclear. It is not known whether 20-HETE plays a role in elevating blood pressure only in rats genetically prone to develop hypertension or if it is also an important factor in normal blood pressure control. Preliminary studies suggest that inhibition of 20-HETE formation also reduces blood pressure in the normotensive Sprague-Dawley rat. The relative importance of 20-HETE in the prehypertensive stage of the disease compared with the developmental and established phases is also not known. It will be important to examine whether early modulation of the CYP4A enzymes can prevent the development of hypertension and whether it can reverse the elevated blood pressure in older animals with established disease. The vascular changes that accompany CYP inhibition also need to be characterized. Further examination of the distinct roles for 20-HETE in the regulation of blood pressure in the SHR and the Dahl salt-sensitive models of hypertension will increase our understanding of the diverse biological properties of this eicosanoid. The availability of an orally available mechanism-based CYP inhibitor with a high degree of selectivity for the renal CYP4A enzymes provides a useful tool for addressing these questions. Further characterization of the role of 20-HETE and other CYP eicosanoids in the regulation of renal function and blood pressure will provide the framework for extension of this work into understanding the role of CYP catalyzed arachidonic acid metabolism in human essential hypertension.

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