Cytochrome P450 metabolites of arachidonic acid may be important mediators in angiotensin II-induced vasoconstriction in the rat mesentery in vivo

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ABSTRACT

We have investigated the role of cytochrome P450 (CYP-450) metabolites of arachidonic acid in the modulation of vascular reactivity to angiotensin II in vivo using an in situ blood-perfused mesenteric preparation in anaesthetized spontaneously hypertensive rats (SHR). Miconazole, a non-selective inhibitor of CYP-450 that inhibits both hydroxylation and epoxidation, substantially suppressed mesenteric vasoconstrictor responses to angiotensin II in SHR, but had no effect on responses to noradrenaline or sympathetic nerve stimulation. In normotensive Wistar–Kyoto (WKY) rats, miconazole caused only a modest suppression of vasoconstrictor responses to angiotensin II. N-Methylsulphonyl-12,12-dibromododec-11-enamide (DDMS), a new selective inhibitor of CYP-450 ω-hydroxylase activity, decreased mean intra-arterial blood pressure and significantly attenuated mesenteric angiotensin II-induced vasoconstrictor responses in SHR. Isolated mesenteric vessels were able to metabolize 14C-labelled arachidonic acid to hydroxyeicosatetraenoic acids (HETEs) in vitro, and this was substantially inhibited by DDMS. The results from the present studies combined with the existing evidence that angiotensin II stimulates the release of 20-HETE, a CYP-450 metabolite of arachidonic acid, suggest that CYP-450-derived HETEs may be important mediators in angiotensin II-induced vasoconstriction. However, the development of more sensitive assays for the detection in vivo of 20-HETE in mesenteric vessels would be required to confirm these findings.

INTRODUCTION

Phospholipases liberate arachidonic acid from membrane-bound phospholipids in vascular tissues and other cell types [1]. In response to a variety of physiological and pathophysiological stimuli, free arachidonic acid can then be metabolized by three enzyme systems, i.e. cyclo-oxygenase, lipoxygenase and cytochrome P450 (CYP-450), to biologically active metabolites. Of these, the cyclo-oxygenase and lipoxygenase systems have been

Key words: cytochrome P450, spontaneously hypertensive rat, vasoconstriction.

Abbreviations: AUC, area under the curve; CYP-450, cytochrome P450; DDMS, N-methylsulphonyl-12,12-dibromododec-11-enamide; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; SHR, spontaneously hypertensive rats; WKY rats, Wistar–Kyoto rats.

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most studied, and their physiological role has been established [2]. The metabolism of arachidonic acid by CYP-450 in the vasculature can be divided into two broad categories [1]: that by epoxygenases, which catalyse the formation of epoxyeicosatrienoic acids (EETs), and that by hydroxylases, which produce hydroxyeicosatetraenoic acids (HETEs), such as 20-HETE, the product of hydroxylation at the ω position of the fatty acid. In vitro 20-HETE is a vasoconstrictor of renal arterioles, and in vivo it can affect renal vascular resistance and autoregulation of blood flow [3]. In the kidney, some EETs (11,12- and 14,15-EET) cause vasodilation, while others (5,6-EET) can cause vasoconstriction [4]. EET-induced vasodilation is associated with an increased open state of calcium-activated potassium channels and hyperpolarization of the vascular smooth muscle, effects which have led to the proposal that EETs may be endothelial-derived hyperpolarizing factors [5].

Evidence suggests that CYP-450-dependent HETEs and EETs influence vascular tone [6] and may be important in the progression of hypertension in animal models [7,8]. Inhibition of renal ω-hydroxylation with aminobenzotriazole lowers blood pressure in spontaneously hypertensive rats (SHR) [9]. Since relatively little is known about the role of CYP-450 arachidonic acid metabolites in vascular beds such as the mesentery, and since there is some evidence that vasoconstrictor HETEs are stimulated by vasoactive peptides such as angiotensin II [10], we have investigated the role of CYP-450 in the modulation of vasoconstrictor responses to angiotensin II using an *in situ* blood-perfused mesenteric preparation in anaesthetized SHR. Non-selective inhibition of CYP-450 was achieved using miconazole [11], and *N*-methylsulphonyl-12,12-dibromododec-11-enamide (DDMS) was used as a selective inhibitor of ω-hydroxylation [12].

**METHODS**

**Evaluation of DDMS as a selective ω-hydroxylase inhibitor**

Rat renal microsomes were isolated using the method of Capdevila et al. [13]. The incubation buffer consisted of Tris/HCl (0.05 M, pH 7.5), MgCl₂ (10 mM), KCl (0.15 M) and indomethacin (20 μM), with δ,δ-isocitric acid (8 mM) and isocitrate dehydrogenase (0.5 i.u./ml) added as an NADPH-regenerating system. Microsomes (0.5–0.75 mg of protein/ml) were preincubated in the above buffer with either miconazole (0–50 μM) or DDMS (0–50 μM) for 5 min in open tubes at 37 °C. [1-¹⁴C]Arachidonic acid (7 μM) and NADPH (1 mM) were then added to the tubes, followed by incubation for 15 min; formic acid (0.2 mM) was added to stop the reaction. The reaction products were then extracted with an equal volume of ethyl acetate and dried by evaporation under nitrogen. The residue was reconstituted in 40 μl of methanol. HPLC separation was performed on a LiChrospher 100 RP-18 column (Hewlett Packard; 5 μm particle size; 125 mm × 4mm) with a linear gradient elution from acetoniitrile/water (2:3, v/v, containing 0.5% acetic acid) to 100% acetoniitrile over a 20 min period, at a flow rate of 1 ml/min. Fractions were collected every 30 s for the duration of the run. Scintillation fluid was added to each fraction and radioactivity was quantified using a Wallac 1410 liquid scintillation counter. The identity of each metabolite was confirmed by co-migration with authentic standards. Results are expressed as a percentage of control activity (mean ± S.E.M.).

**Vascular reactivity in the mesenteric circulation**

Female SHR (*n* = 10) and normotensive Wistar–Kyoto (WKY) rats (*n* = 8) aged 13–15 weeks were anaesthetized with *α*-chloralose (150 mg/kg, subcutaneous) and heparinized (heparin 100 units/kg, intravenous), and the blood-perfused mesenteric preparation used to assess mesenteric vascular reactivity *in vivo* as described previously [14]. Briefly, the left femoral artery and vein were cannulated for mean blood pressure measurement and normal saline supplementation (0.1 ml/min) respectively. The superior mesenteric artery and carotid artery were isolated, and after complete haemostasis by electrical cautery, 1500 units/kg heparin was administered into the femoral vein. The carotid artery was cannulated and the perfusion line filled with blood. The superior mesenteric artery was then cannulated and perfusion was begun from the carotid to the mesenteric artery using a roller pump at constant flow rate of 2 ml/min. Central nervous system control of the mesenteric preparation was abolished by severing the mesenteric artery proximal to the cannulation point, and the surrounding tissue was also severed. Bipolar platinum electrodes were placed peri-arterially approx. 2 mm distal to the cannulation point. The ischaemic time of the mesentery was of the order of 1–2 min. Femoral artery mean blood pressure and mesenteric perfusion pressure were monitored and recorded with a Mac-Lab polygraph. In this constant-flow system, changes in perfusion pressure were indicative of alterations in vascular resistance. The preparation was allowed to equilibrate for 30 min before examination of vascular reactivity.

Miconazole was administered to inhibit both epoxygenases and hydroxylases. DDMS was used to selectively inhibit hydroxylases. Vasoconstrictor drugs were introduced at the inlet of the mesenteric circulation. In the first experiment, dose-dependent mesenteric vasoconstrictor responses to angiotensin II (10–300 ng), noradrenaline
(100–560 ng) and electrical stimulation (15 V, 1 ms, 3–9 Hz, 1 min duration) were determined before and 30 min after addition of miconazole (25 mg/kg, intravenous, femoral vein). In the second experiment dose-dependent responses to angiotensin II were characterized before and 30 min after addition of DDMS (10 mg/kg, intravenous).

Metabolism of arachidonic acid by the isolated mesenteric artery
Mesenteric arteries from five SHR were carefully dissected to remove all adherent fat and tissue. Vessels were homogenized in Tris buffer and incubated with [14C]arachidonic acid as described above for renal microsomes. Products were recovered into ethyl acetate and separated by reverse-phase HPLC, with fractions collected every 30 s for counting of radioactivity. Tissue homogenate was solubilized by incubation with a solution containing 0.5 M NaOH and 2% sodium deoxycholate prior to protein determination by the Lowry assay.

Statistical analysis
Area under the curve (AUC) was calculated for dose-dependent responses to angiotensin and noradrenaline, and for frequency-dependent responses to electrical stimulation. Comparisons of intra-arterial mean blood pressure, basal mesenteric perfusion pressure and mesenteric vascular reactivity before and after treatment with miconazole or DDMS were carried out by paired t-test. Analysis of conversion of arachidonic acid into 20-HETE by renal microsomes with and without DDMS was carried out using Student’s unpaired t-test. A P value of <0.05 was considered to be significant.

RESULTS
Microsomal preparations contain epoxide hydrolase, and EETs are converted into their corresponding metabolites, dihydroxyeicosatrienoic acids (DHETs). Epoxigenase activity was considered as the sum of EET and DHET formation. Miconazole (0–50 μM) dose-dependently inhibited the production of 20-HETE and the synthesis of EETs and DHETs by renal microsomes (Figure 1a). Miconazole was particularly potent at inhibiting the epoxygenase products, with an IC50 of less than 2 μM. In contrast, DDMS (0–50 μM) selectively and dose-dependently inhibited 20-HETE production, without significant effects on the synthesis of EETs and DHETs by renal microsomes (Figure 1b). The IC50 for DDMS for inhibition of HETEs was 2 μM; this is in substantial agreement with values published recently for this inhibitor [12].

Miconazole substantially suppressed mesenteric vasoconstrictor responses to angiotensin II in SHR (before miconazole, AUC = 198 ± 11; after miconazole, AUC = 95 ± 14; P < 0.01, n = 10) (Figure 2a), without having significant effects on mean intra-arterial blood pressure (before miconazole, 142 ± 13 mmHg; after miconazole, 141 ± 14 mmHg, n = 5) or on mesenteric vasocostructor responses to noradrenaline (Figure 2b) or electrical stimulation (Figure 2c). DDMS decreased mean blood pressure (before DDMS, 134 ± 11 mmHg; after DDMS, 107 ± 18 mmHg; P < 0.05) and significantly attenuated mesenteric responses to angiotensin II (P < 0.05) (Figure 3) in SHR. There was no effect of either miconazole or DDMS on basal perfusion pressure. Miconazole also decreased mesenteric vasoconstrictor responses to angiotensin II in WKY rats (before miconazole, AUC = 132 ± 12; after miconazole, AUC = 102 ± 6; P < 0.05, n = 8) (Figure 4), although the effect was much less pronounced than that seen in SHR (Figure 2a).

Labelled arachidonic acid was metabolized by isolated mesenteric arteries obtained from SHR to products which co-eluted on reverse-phase HPLC with 20-HETE.© 2000 The Biochemical Society and the Medical Research Society
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Figure 2 Mesenteric vascular reactivity in SHR before and after treatment with miconazole
(a) Angiotensin II-induced mesenteric vasoconstrictor responses were significantly decreased after (●, n = 10) compared with before (■, n = 10) miconazole (P < 0.01). (b, c) Mesenteric vasoconstrictor responses to electrical and noradrenaline stimulation did not decrease significantly after (●, n = 8) compared with before (■, n = 8) miconazole.

Figure 3 Effect of DDMS on mesenteric vasoconstrictor responses to angiotensin II in SHR
Responses were measured before (■) and after (●) treatment with DDMS (n = 5, P < 0.05).

Figure 4 Effect of miconazole on mesenteric vasoconstrictor responses to angiotensin II in WKY rats
Responses were measured before (■) and after (●) treatment with miconazole (n = 8, P < 0.05).

DISCUSSION
To our knowledge, this is the first study showing that inhibitors of CYP-450 enzymes reduce mesenteric vasoconstrictor responses to angiotensin II in SHR in vivo. Our in vitro experiments using renal microsomes confirmed that miconazole is a non-selective inhibitor of CYP-450, and that it inhibited the production of both HETEs and EETs. In contrast, DDMS selectively inhibits...
the production of HETEs without affecting the synthesis of EETs. Although the metabolism of arachidonic acid by CYP-450 in mesenteric vessels is poorly understood, we have presented evidence here that mesenteric vessels can metabolize arachidonic acid in vitro to HETE and EET products. However, the relative contributions of EETs and HETEs to tone in this vascular bed is not known. The recent availability of more specific inhibitors of CYP-450-catalysed reactions should provide useful tools for evaluating the role of CYP-derived arachidonic acid metabolites on vascular function and blood pressure [12,15].

In the present study we used DDMS, a selective, mechanism-based inhibitor of CYP-450 enzymes of the 4A family that catalyse ω-hydroxylation of long-chain fatty acids. We administered DDMS at a dose of 10 mg/kg; although we did not measure plasma concentrations, a similar dose has been shown previously to inhibit 20-HETE in the rat and have similar effects on mean blood pressure [15]. Brand-Schieber et al. [16] have also reported previously that a similar dose of DDMS reduces the urinary excretion of 20-HETE. In view of the similar effects of miconazole and DDMS in reducing vasoconstrictor responses to angiotensin II, it is likely that HETEs are partly responsible for mediating angiotensin II-induced vasoconstriction in vivo. The differential effects of miconazole and DDMS on systemic blood pressure suggest that the balance of HETEs and EETs plays a role in blood pressure regulation. Miconazole causes non-selective inhibition of CYP-450 enzymes, resulting in a relatively smaller disturbance of the balance of vasoconstrictor HETEs and potentially vasodilator EETs, and therefore of systemic blood pressure. In contrast, sole inhibition of the production of vasoconstrictor HETEs by DDMS induces vaso-
dilatation and a decrease in systemic blood pressure. However, we cannot rule out the possibility that inhibition of the CYP pathway shunts arachidonic acid metabolism down an alternative pathway leading, for example, to the formation of vasodilator prostanooids.

CYP-450 inhibition by miconazole appeared to specifically suppress angiotensin-induced vasoconstriction in the present study. As miconazole did not significantly affect vasoconstriction induced by noradrenaline or electrical stimulation, its effect is unlikely to be due to non-specific membrane hyperpolarization or vascular smooth muscle toxicity. Previous studies have shown that angiotensin II can stimulate 20-HETE release in isolated perfused rat kidneys [10] and enhances 20-HETE synthesis by renal microvessels [17]. The present findings suggest that HETEs, particularly 20-HETE, are important mediators in angiotensin II-induced vasoconstriction in this major vascular bed. Lange et al. [18] reported that 20-HETE elicits cat cerebral vasoconstriction by inhibition of whole-cell K⁺ currents in smooth muscle in which protein kinase C is an integral part in the signal transduction pathway. Therefore 20-HETE may act as a mediator for the intracellular actions of angiotensin II in vascular tissue by regulation of calcium-activated potassium channels. Furthermore, Saïto et al. [19] reported that 12S-HETE, a lipoxygenase metabolite of arachidonic acids, restored attenuated angiotensin II-stimulated increases in cytosolic calcium in cultured rat vascular smooth muscle cells pretreated with lipoxygenase inhibitors, suggesting that 12S-HETE is an important mediator of angiotensin II-induced calcium influx. It is possible that HETEs derived from lipoxygenase and those derived from CYP-450 ω-hydroxylase may share biological properties in influencing angiotensin II-stimulated calcium influx in rat smooth muscle cells [19,20].

SHR have been used widely as an animal model for essential hypertension, and there is evidence that an increased CYP-450-dependent metabolism of arachidonic acid may contribute to the development of hypertension in this model [4–6]. In normotensive WKY rats the mesenteric vasoconstrictor responses to angiotensin II were also decreased following miconazole treatment, although the effect was much less marked than that observed with SHR. These data lend support to the proposal that 20-HETE has a key role in the vasoconstrictor responses to angiotensin in SHR.

In summary, the present studies demonstrate that drugs capable of causing acute inhibition of 20-HETE synthesis reduce mesenteric vasoconstrictor responses to angiotensin II. This suggests that CYP-450 metabolites of arachidonic acid play an important role in the regulation of vascular tone by modulation of vascular reactivity to angiotensin II. Whether the modulating action of 20-HETE is a generalized phenomenon in various vascular beds as well as in different forms of
hypertension remains to be established, and the development of more sensitive assays for measuring 20-HETE in vivo will be important for confirming these observations. Further investigation of this novel intermediary pathway of the vasoconstrictor effects of angiotensin II in human hypertension may offer the opportunity for a new approach to drug therapy.

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REFERENCES


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