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# Functional response of the rat kidney to inhibition of nitric oxide synthesis: role of cytochrome P450-derived arachidonate metabolites

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**1** We tested the hypothesis that nitric oxide (NO) exerts a tonic inhibitory influence on cytochrome P450 (CYP450)-dependent metabolism of arachidonic acid (AA).

**2** $N^{\omega}$-nitro-L-Arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase (NOS), increased mean blood pressure (MBP), from $91\pm6$ to $137\pm5$ mmHg, renal vascular resistance (RVR), from $9.9\pm0.6$ to $27.4\pm2.5$ mmHg ml$^{-1}$ min$^{-1}$, and reduced renal blood flow (RBF), from $9.8\pm0.7$ to $6.5\pm0.6$ ml min$^{-1}$) and GFR from $1.2\pm0.2$ to $0.6\pm0.2$ ml 100 g$^{-1}$ min$^{-1}$) accompanied by diuresis (UV, $1.7\pm0.3$ to $4.3\pm0.8$ $\mu$l 100 g$^{-1}$ min$^{-1}$), and natriuresis ($U_{Na}V$, $0.36\pm0.04$ to $1.25\pm0.032$ $\mu$mol 100 g$^{-1}$ min$^{-1}$).

**3** 12, 12 dibromododec-enoic acid (DBDD), an inhibitor of $\omega$ hydroxylase, blunted L-NAME-induced changes in MBP, RVR, UV and $U_{Na}V$ by $63\pm8$, $70\pm5$, $45\pm8$ and $42\pm9$%, respectively, and fully reversed the reduction in GFR by L-NAME. Clotrimazole, an inhibitor of the epoxygenase pathway of CYP450-dependent AA metabolism, was without effect.

**4** BMS182874 (5-dimethylamino)-N-(3,4-dimethyl-5-isoxazolyl)-1-naphthalenesulfonamide), an endothelin $(ET)_A$ receptor antagonist, also blunted the increases in MBP and RVR and the diuresis/natriuresis elicited by L-NAME without affecting GFR.

**5** Indomethacin blunted L-NAME-induced increases in RVR, UV and $U_{Na}V$. BMS180291 (1S-(1$\alpha$,2$\alpha$,3$\alpha$.4$\alpha$)]-2-[[3-[4-[(pentylamino)carbonyl]-2-oxazolyl]-7-oxabicyclo[2.2.1]hept-2-yl]methyl]benzenepropanoic acid), an endoperoxide receptor antagonist, attenuated the pressor and renal haemodynamic but not the renal tubular effects of L-NAME.

**6** In conclusion, the renal functional effects of the CYP450-derived mediator(s) expressed after inhibition of NOS with L-NAME were prevented by inhibiting either CYP450 $\omega$ hydroxylase or cyclo-oxygenase or by antagonizing either $ET_A$ or endoperoxide receptors. 20-hydroxyeicosatetraenoic acid (20-HETE) fulfils the salient properties of this mediator.

**Keywords:** Nitric oxide; 20-HETE; CYP450; renal function; cyclo-oxygenase; L-NAME

## Introduction

Nitric oxide (NO) has a number of effects that impact on the regulation of renal function and blood pressure (Baylis *et al*., 1990) as it has been implicated in renal autoregulation (Deng & Baylis, 1993), tubuloglomerular feedback (Thorup & Persson, 1994), and pressure natriuresis (Johnson & Freeman, 1992; Hu & Manning, 1995). The influence of NO production on physiological processes has been assessed by administration of L-arginine analogues such as $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME), which competitively inhibit NO synthase (NOS). Acute administration of L-NAME increased arterial pressure (see Sventek *et al*., 1997) accompanied by decreased GFR and renal blood flow (RBF) (Deng & Baylis, 1993; Ziyyat *et al*., 1996; Baylis *et al*., 1990), and increased excretion of salt and water (Baylis *et al*., 1990; Johnson & Freeman, 1992).

*A priori*, a potential exists for interactions between NO and cytochrome P450 (CYP450) mono-oxygenase-dependent generation of lipid mediators. NO has been reported to inhibit CYP450 enzymes of the 1A, 2B1 (Wink *et al*., 1993), 3C (Khatsenko *et al*., 1993) and 4A (Alonso-Galicia *et al*, 1997) families, probably, by forming iron-nitrosyl complexes at the catalytic heme binding sites of these enzymes. NO suppressed CYP450-dependent oxygenation reactions in the hepatic (Khatsenko *et al*., 1993) and renal microsomes (Alonso-Galicia *et al*., 1997), decreased CYP450 activity and content (Khatsenko *et al*., 1993), and inhibited CYP450-dependent production of arachidonic acid (AA) metabolites that dilate the renal vasculature (Oyekan, 1994). These interactions have functional implications for the kidney and other organs involved in the regulation of blood pressure. CYP450-AA metabolites are produced in the renal and systemic vasculatures; a role in cardiovascular and renal function was suggested on the basis of their properties which include: vasoactivity, inhibition of $Na^+$ transport and modulation of cell growth (McGiff, 1991).

In the light of these observations, the present study was designed to test the hypothesis that NO is an endogenous regulator of CYP450-AA metabolism. If NO tonically inhibits CYP450 enzyme activity, inhibition of NOS would be expected to increase formation of CYP450-AA metabolites which should contribute to the renal haemodynamic and excretory effects following withdrawal of NO.

We were guided in developing an experimental design by studies that have addressed the contribution of CYP450-AA products to the renal functional responses to those vasoactive peptides that have been shown to stimulate CYP450-AA metabolism. For example, both the renal haemodynamic and the tubular effects of endothelin-1 (ET-1) could be accounted for by peptide-induced release of 20-HETE. 20-HETE, the principle product of renal CYP450-AA metabolism (McGiff,

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1991), is involved in the regulation of salt and water excretion and renal haemodynamics (Escalante *et al*., 1991; Takahashi *et al*., 1990) and acts as a second messenger for the renal effects of ET-1 (Oyekan & McGiff, 1998). Moreover, as endothelins have been reported to be primarily responsible for the acute pressor response to inhibition of NOS (Richard *et al*., 1995; Banting *et al*., 1996), blockade of ET receptors should also attenuate the acute renal functional response to L-NAME, which proved to be the case. Finally, as 20-HETE can be metabolized by cyclo-oxygenase (COX) (Escalante *et al*., 1989; Schwartzman *et al*., 1989), forming vasoconstrictor analogues of thromboxane (20-OH-$TxA_2$) and prostaglandin (PG) endoperoxides (20-OH-$PGG_2$ and 20-OH-$PGH_2$) that increase RVR and depress GFR in the rat, it should also be possible to modify L-NAME-induced renal functional effects, either by inhibiting COX or by blocking the $PGH_2/TxA_2$ receptor.

Based on the above analysis, we modified, at several key sites, the renal functional effects of increased production of CYP450-AA metabolites consequent to inhibition of NO formation. To achieve this objective, we selected interventions that deleted/suppressed selectively, each of the several postulated components of the heightened CYP450 response to NOS inhibition: (1) blockade of endothelin receptors; (2) inhibition of synthesis of CYP450-AA metabolites; (3) prevention of metabolism of CYP450-AA products by inhibiting COX; and (4) blockade of the $PGH_2/TxA_2$ receptor. The working hypothesis in the present study that provides the rationale for the pharmacological interventions targeted at several sites in the sequential transformation of 20-HETE is shown in Figure 1.

## Methods

L-NAME (Sigma, St Louis, MO, U.S.A.), BMS 180291 (1S-(1α,2α,3α.4α)] - 2 - [[3-[4-[(pentylamino)carbonyl]-2-oxazolyl]-7-oxabicyclo[2.2.1]hept-2-yl]methyl]benzenepropanoic acid), and chlorisondamine (Ecolid, Ciba Pharmaceutical Products, Summit, NJ, U.S.A.) were dissolved in normal saline. Indomethacin (Sigma, St Louis, MO, U.S.A.) was dissolved in 50% ethanol, clotrimazole (Sigma, St Louis, MO, U.S.A.) in sesame oil (Sigma, St Louis, MO, U.S.A.) and BMS182874 (5-dimethylamino)-N-(3,4-dimethyl-5-isoxazolyl)-1-naphthalene-sulfonamide) in 0.1 M $NaHCO_3$. 12, 12 dibromododec-enoic acid (DBDD) was a gift from Dr Camille Falck, University of Texas South Western Medical Center and was stored in ethanol at −20°C.

The experiments were performed on male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA, U.S.A.; body weight 297 ± 5 g). The animals were maintained on standard rat food (Purina Chow) and were allowed *ad libitum* access to water and food until the beginning of the experiments. [Experimental protocols for these studies in rats was approved by Institutional Animal Care and Use Committee (IACUC)].

### *Physiological measurements*

Clearance studies were performed in rats that were anaesthetized with an intraperitoneal injection of Inactin (100 mg $kg^{-1}$; Research Biochemicals International, Natick, MA, U.S.A.) and placed on a heated platform to maintain body temperature at 37°C. The urinary bladder was exposed by an abdominal incision and a cannula (PE 205) was placed to drain urine. Dead space was minimized by a ligature which functionally excluded a great part of the bladder. A tracheostomy (PE 250) was performed for spontaneous ventilation, and cannulae (PE 50) were placed in the right carotid artery to monitor blood pressure and right femoral artery to collect blood samples. A tail vein was also cannulated with a 23G butterfly needle (Abbott Hospitals Inc., IL, U.S.A.) for infusion or administration of drugs. A left laparotomy was performed and electromagnetic flow probes (Carolina Medical Electronics, NC, U.S.A.) placed over the left renal artery to measure RBF. A constant infusion of saline (2 ml $h^{-1}$) was administered throughout the experiment to maintain a euvolemic condition. Inulin (10 mg $ml^{-1}$) was included in the solution for measurement of GFR. Mean arterial blood pressure (MBP) was measured with a pressure transducer (Statham model P231D; Statham, Oxnard, CA, U.S.A.) and recorded on a polygraph (Grass model 7D, Grass Instruments, Quincy, MA, U.S.A.). RVR was estimated from the MBP and RBF values using the formula: (MBP-5)/(RBF); 5 mmHg was used as an estimate of renal venous pressure.

Following surgery, each rat received the ganglionic blocking agent, chlorisondamine, at a dose of 5 mg $kg^{-1}$ (s.c.) to block cardiovascular reflexes. Injections were divided between two doses given 10 min apart. The rats were allowed to equilibrate for at least 45 min or until urine flow was steady. After the postsurgical equilibration period and a 30 min control period

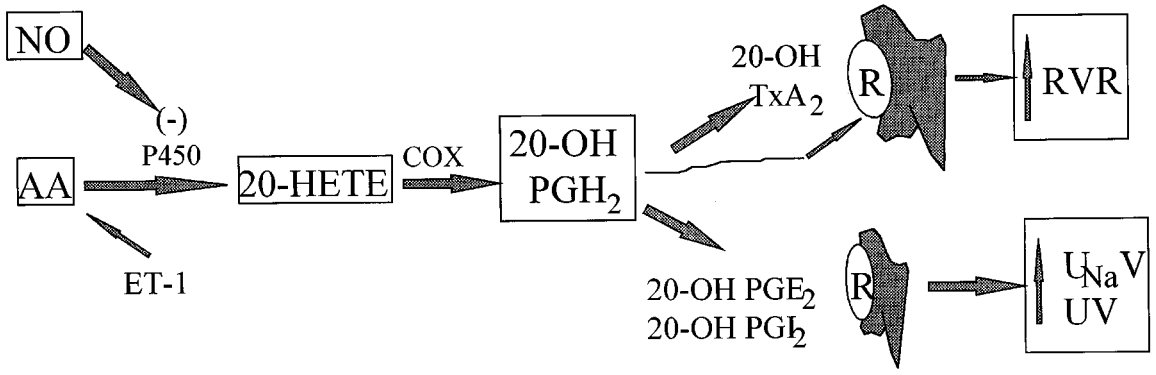


**Figure 1** Schematic showing the interrelationships among NO, ET-1 and 20-HETE and the targets for interrupting 20-HETE-mediated changes in renal function. Inhibition of NO synthesis by L-NAME facilitates ET-1 stimulation of phospholipase, releasing arachidonic acid (AA) which is metabolized by ω-hydroxylase (CYP450) forming 20-HETE. Transformation of 20-HETE by cyclo-oxygenase (COX) yields the endoperoxide analogue (20-OH $PGH_2$), the precursor of 20-OH $TxA_2$, 20-OH $PGE_2$ and 20-OH $PGI_2$. These analogues can increase renal vascular resistance (RVR) through stimulation of the $PGH_2/TxA_2$ receptor or produce natriuresis/diuresis through stimulation of $PGE_2/PGI_2$ receptors.

following the administration of 50% ethanol or 0.1 M $NaHCO_3$, the vehicles for inhibitors, L-NAME was administered. The effects of L-NAME on blood pressure and renal function were studied in seven groups of five to six rats each. Each group received an intravenous injection of L-NAME (10 mg $kg^{-1}$). This dose was selected on the basis of preliminary data indicating that, of the doses of L-NAME evaluated, 2.5, 5, 10 and 25 mg $kg^{-1}$, the 10 mg $kg^{-1}$ dose evoked the maximal increase in RVR. A time control group ($n=4$) was treated with an identical injection of normal saline (1 ml $kg^{-1}$) not containing L-NAME. In all cases, changes in MBP and RBF were continuously monitored. Urine samples were also collected. 400 $\mu$l of arterial blood was collected into 3.5% sodium citrate during the middle of a 30-min urine collection period for measurement of GFR by inulin clearance. An equal amount of normal saline was infused for volume replacement. Urine volume (UV) was measured by gravimetry and urinary sodium excretion ($U_{Na}V$) was measured with the Ciba Corning Na/K/Cl Analyzer (Model 644).

### *Experimental groups*

Renal functional response to L-NAME was determined in the absence or presence of inhibitors of CYP450 mono-oxygenase or COX and antagonists of $PGH_2/TxA_2$ or ET receptors. Experiments were performed on seven groups of rats as follows:

*Group I.* Time control ($n=4$). In this group, baseline measurements were performed (0–30 min) and repeated during an experimental period (30–60 min) following the administration of normal saline.

*Group II.* Renal response to L-NAME was determined in rats that received a bolus dose of L-NAME (10 mg $kg^{-1}$ (i.v.) given slowly over 1 min) after the baseline period (0–30 min).

In the rats in Groups III and IV, there were three clearance periods; a baseline period (0–30 min) during which neither inhibitor nor vehicle was administered (pre-vehicle or pre-inhibitor), another period (30–60 min, pre-L-NAME) during which an inhibitor or its vehicle alone was administered, and an experimental clearance period (60–90 min) during which L-NAME was administered (as in Group II) concurrently with an inhibitor or its vehicle. Rats received inhibitors or their respective vehicles at indicated times before the administration of L-NAME.

*Group III.* Rats in this group received DBDD, a mechanism-based inhibitor of CYP-dependent $\omega/\omega$-1 hydroxylase (2.5 mg $kg^{-1}$ $h^{-1}$, i.v.; $n=8$) or its vehicle (50% ethanol, $n=4$). DBDD infusion was initiated 30 min before the administration of L-NAME.

*Group IV.* Rats in this group received indomethacin (5 mg $kg^{-1}$ (i.v.) 30 min before L-NAME administration, $n=5$) to inhibit COX. Control rats received 50% ethanol (vehicle; 1 ml $kg^{-1}$, i.v.).

*Group V.* In this group ($n=5$), epoxygenase activity was blocked with clotrimazole (80 mg $kg^{-1}$ (i.p.) for 2 days; Makita *et al.*, 1994). Control rats received sesame oil (1 ml $kg^{-1}$, i.p., $n=4$). In both groups of rats, renal clearances were conducted under basal condition (0–30 min; pre-L-NAME) and 30 min following the administration of L-NAME.

In Groups VI and VII, receptor antagonists or their respective vehicles were given to the rats at indicated times before administering L-NAME. In these cases, only two clearance periods were obtained, a baseline period (0–30 min; pre-L-NAME) and an experimental period (30–60 min) during which responses to L-NAME were evaluated.

*Group VI.* In these rats, the $PGH_2/TxA_2$ receptor was antagonized with the orally-active, highly selective antagonist, BMS180291 (25 mg $kg^{-1}$ (p.o.) by gavage; $n=5$) alone or in combination with DBDD ($n=4$). BMS180291 was given before anaesthesia and L-NAME was injected 1 h thereafter. Control rats ($n=4$) received 0.9% NaCl (1 ml $kg^{-1}$, p.o.; Vehicle).

*Group VII.* Rats in this group received BMS182874 (40 mg $kg^{-1}$, orally), an $ET_A$ receptor antagonist (Stein *et al.*, 1994) alone ($n=8$) or in combination with DBDD (2.5 mg kg $h^{-1}$, i.v.; $n=5$). BMS182874 was administered before anaesthesia 2–3 h before L-NAME. Control rats ($n=6$) received 0.1 M $NaHCO_3$ orally and 50% ethanol i.v., the respective vehicles for BMS182874 and DBDD.

In all experiments, renal functional responses to L-NAME were evaluated, measurements being performed during the 30–60 min period following the stabilization or baseline period. Baseline, as well as L-NAME-induced changes in UV, $U_{Na}V$, GFR, RBF and renal vascular resistance were evaluated. Independent effects of the various inhibitors were evaluated by comparing renal function during the baseline period with renal function during the administration of the respective vehicles. The effects of the inhibitors on L-NAME induced changes in renal function were evaluated by comparing renal effects to L-NAME in the presence of an inhibitor or its vehicle.

### *Data analysis*

MBP and RBF responses were recorded as changes ($\Delta$) relative to baseline (pre-L-NAME values). Values of GFR, UV and $U_{Na}V$ are presented as absolute values. All data are expressed as means $\pm$ s.e.mean. Data were analysed by determining areas under the curve for changes in MBP or RVR versus time. This was followed by the use of randomized block analysis of variance followed by a Newman-Keuls multiple range test for comparisons within the groups and unpaired *t*-test for comparisons between groups. In all cases, $P<0.05$ was considered significant.

## Results

Values of MBP, RBF and RVR obtained after chlorisondamine administration were $91\pm6$ mmHg, $9.8\pm0.7$ ml $min^{-1}$ and $9.9\pm0.6$ mmHg $ml^{-1}$ $min^{-1}$ ($n=26$). Injection of L-NAME produced a rapid and pronounced increase in MBP, a reduction in RBF and a consequent increase in RVR that were sustained for at least 30 min (Figure 2a,b). The peak changes in these parameters in response to L-NAME (10 mg $kg^{-1}$) were; $46\pm5$ mmHg, $-3.3\pm0.6$ ml $min^{-1}$, and $17.5\pm2.5$ mmHg $ml^{-1}$ $min^{-1}$, respectively. L-NAME also reduced GFR (from $1.2\pm0.2$ to $0.6\pm0.2$ $ml^{-1}$ $min^{-1}$; $P<0.05$) (Figure 2c). D-NAME (10 mg $kg^{-1}$), which is the optical isomer of L-NAME and is not an inhibitor of NOS, did not show any pressor activity nor did it affect RBF. Accompanying L-NAME-induced changes in systemic and renal haemodynamics were increases in UV (from $1.7\pm0.3$ to $4.3\pm0.8$ $\mu$l 100 $g^{-1}$ $min^{-1}$; $P<0.05$) and $U_{Na}V$ (from $0.36\pm0.04$ to $1.25\pm0.32$ $\mu$mol 100 $g^{-1}$ $min^{-1}$; $P<0.05$) (Figure 2d). In time controls, there were no differences in MBP, RBF, UV and $U_{Na}V$ for the 60 min following the administration of normal saline. In rats treated with 50% ethanol, the vehicle for DBDD and indomethacin, values of MBP, RBF, UV and $U_{Na}V$ under basal conditions or following L-NAME injection were not different from rats that received normal saline. Hence, data presented in Figures 2 and 4

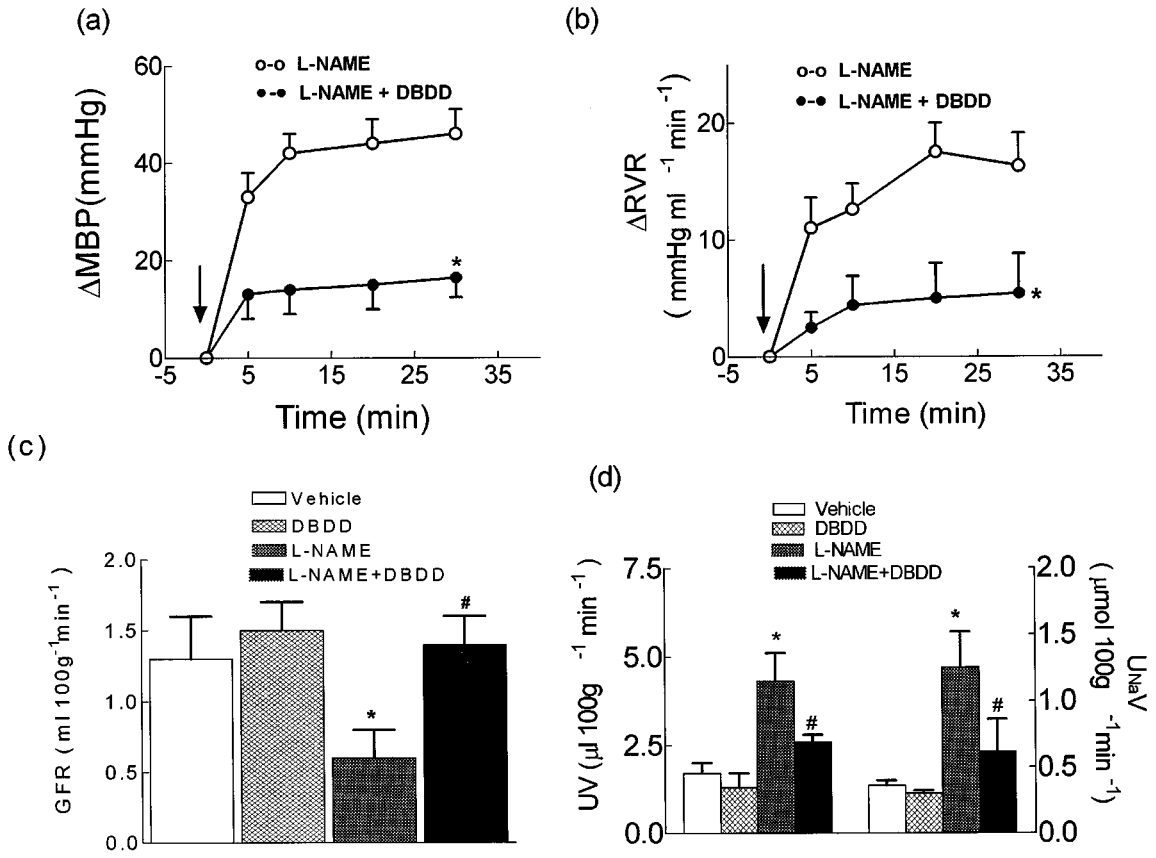


**Figure 2** Effects of L-NAME (10 mg kg$^{-1}$, i.v.) on changes ($\Delta$) in mean blood pressure (MBP), (a) renal vascular resistance (RVR), (b) GFR, (c) urine volume (UV) and sodium excretion ($U_{Na}V$) (d) in rats treated with DBDD 2.5 mg kg$^{-1}$ h$^{-1}$, i.v. ($n=8$) or 0.9% NaCl or 50% ethanol (Vehicle; $n=26$). Data presented for L-NAME in a and b represent pooled data from rats treated with normal saline ($n=26$) or 50% ethanol. L-NAME + DBDD represent data from DBDD-treated rats. L-NAME was given at time 0 min (see arrow). In c and d, values of GFR, UV and $U_{Na}V$ as affected by either 0.9% NaCl, 50% ethanol (Vehicle) or L-NAME with DBDD (L-NAME + DBDD) or without DBDD (L-NAME). Results are presented as means ± s.e.mean. *$P<0.05$ vs Vehicle; #$P<0.05$ vs L-NAME.

represent pooled data from rats treated with normal saline or 50% ethanol.

### *Effect of inhibition of CYP450 on renal responses to L-NAME*

Figure 2 shows that administration of DBDD, an inhibitor of CYP450 $\omega$-hydroxylase given 30 min before L-NAME greatly attenuated the pressor, renal haemodynamic and tubular responses to L-NAME. All responses were analysed by determining the areas under the curve of MBP and RBF vs time. Except for a slight increase in RBF (from 9.8 ± 0.7 to 11.3 ± 1.3 ml min$^{-1}$; $P>0.05$), DBDD was without effect on basal haemodynamics for the 30 min of the control period. However, DBDD attenuated L-NAME-induced reduction in RBF by 47 ± 6% ($P<0.05$); blunted the increase in MBP by L-NAME (63 ± 8%, $P<0.05$, Figure 2a) and consequently reduced L-NAME-induced increase in RVR by 70 ± 5% ($P<0.05$; Figure 2b). DBDD was without significant effect on GFR (1.5 ± 0.2 vs 1.3 ± 0.3 ml min$^{-1}$; Figure 2c); but L-NAME-induced reduction in GFR was abolished such that there was no significant difference between GFR in vehicle (50% ethanol)-treated (1.3 ± 0.3 ml 100 g$^{-1}$ min$^{-1}$, $n=4$) and DBDD-treated ($n=8$) rats (1.4 ± 0.2 ml 100 g$^{-1}$ min$^{-1}$) (Figure 2c). Accompanying these haemodynamic changes, DBDD attenuated L-NAME-induced increases in UV (from 4.3 ± 0.8 to 2.6 ± 0.2 $\mu$l 100 g$^{-1}$ min$^{-1}$; $P<0.05$) and $U_{Na}V$ (from 1.25 ± 0.32 to 0.62 ± 0.28 $\mu$mol 100 g$^{-1}$ min$^{-1}$; $P<0.05$) (Figure 2d).

In rats treated with clotrimazole ($n=5$) to block CYP450-dependent epoxygenase activity (Makita *et al.*, 1994), there were no differences in baseline renal parameters when compared to vehicle-(sesame oil)-treated ($n=4$) rats. Thus, baseline MBP and RBF were 84 ± 8 mmHg and 8.5 ± 0.8 ml min$^{-1}$, respectively in vehicle-treated rats and 95 ± 7 mmHg and 7.5 ± 0.6 ml min$^{-1}$, respectively in clotrimazole-treated rats. Clotrimazole did not affect the renal functional responses to L-NAME. For example, L-NAME-induced increase in RVR peaked at 24.7 ± 8.9 and 30.3 ± 10.9 mmHg ml$^{-1}$ min$^{-1}$ in vehicle- and clotrimazole-treated rats, respectively. Neither did clotrimazole modify the changes in GFR induced by L-NAME (L-NAME + Clotrimazole, 0.7 ± 0.2 ml min$^{-1}$; L-NAME + Vehicle, 0.5 ± 0.2 ml min$^{-1}$). Similarly, L-NAME-induced increases in UV and $U_{Na}V$ were not different between vehicle- and clotrimazole-treated rats.

### *$ET_A$ receptor antagonism and renal effects of L-NAME*

As L-NAME increased plasma ET-1 (Richard *et al.*, 1995) and ET-1 stimulated 20-HETE production in the isolated perfused rat kidney (Oyekan *et al.*, 1997), L-NAME-induced changes in renal function were evaluated in the presence of BMS182874, an $ET_A$ receptor antagonist (Stein *et al.*, 1994), alone or in combination with DBDD. MBP and RBF in rats treated with vehicle (50% ethanol i.v. and 0.1 M $NaHCO_3$, p.o.; $n=7$) were 87 ± 4 mmHg and 8.9 ± 0.3 ml min$^{-1}$, respectively. These values were not significantly different from those obtained in

rats treated with BMS182874 alone ($n=8$) or those treated with BMS182874 and DBDD ($n=5$) in which MBP and RBF were $76\pm7$ mmHg and $9.3\pm0.9$ ml min$^{-1}$, and $77\pm4$ mmHg and $10.8\pm0.8$ ml min$^{-1}$, respectively. RVR was not significantly different between the two groups of rats treated with BMS182874 ($8.3\pm0.4$ mmHg$^{-1}$ ml$^{-1}$ min$^{-1}$ (BMS182874) vs $7.5\pm0.8$ mmHg$^{-1}$ ml$^{-1}$ min$^{-1}$ (BMS182874+DBDD). UV was significantly lower in rats treated with BMS182874 or BMS182874+DBDD, $0.7\pm0.1$ or $0.6\pm0.2$ $\mu$l$^{-1}$ 100 g$^{-1}$ min$^{-1}$, respectively, compared with vehicle-treated rats ($1.2\pm0.3$ $\mu$l$^{-1}$ 100 g$^{-1}$ min$^{-1}$; $P<0.05$). As expected, BMS182874 blunted the increase in RVR produced by ET-1 (0.5 $\mu$g kg$^{-1}$, i.v.) by $67\pm5\%$ ($n=3$; $P<0.05$). BMS182874 also attenuated L-NAME-induced increases in MBP (Figure 3a) and RVR (Figure 3b) by $28\pm4\%$ and $34\pm6\%$ ($P<0.05$), respectively and blunted L-NAME-induced reduction in RBF by $56\pm6\%$ ($P<0.05$). However, BMS182874 did not affect the reduction in GFR by L-NAME (Figure 3c) but did attenuate the increases in UV and $U_{Na}V$ (Figure 3d). Coadministration of DBDD did not alter the effects of BMS182874 on the increases elicited by L-NAME on UV, $U_{Na}V$, and MBP. However, the reduction in GFR by L-NAME which was not significantly affected by BMS182874 alone was restored by combined administration of DBDD and BMS182874 (Figure 3c). In addition, DBDD further enhanced the blunting effect of BMS 182874 on L-NAME-induced reduction in RBF ($\Delta-2.7\pm0.3$ ml min$^{-1}$; Vehicle) from a nadir of $\Delta-1.9\pm0.1$ (BMS182874) to $\Delta-0.9\pm0.2$ (BMS182874+DBDD) ml min$^{-1}$ ($P<0.05$), thus exacerbating the reduction in RVR by BMS182874 by $55\pm6\%$ ($P<0.05$). When compared with rats treated with DBDD (Figure 2), the effects of combined treatment with BMS182874 and DBDD on systemic and renal hemodynamics were not greater than that obtained with DBDD alone.

### *Effect of COX blockade on L-NAME-induced changes in renal function*

As the renal vasoconstrictor effect of 20-HETE in the rat is COX-dependent (Askari *et al.*, 1997), the effect of indomethacin on the renal response to L-NAME was evaluated. Indomethacin (5 mg kg$^{-1}$; Indo) had no effect on either MBP or RBF and was without significant effect on L-NAME-induced increases in MBP (Figure 4a). Indomethacin blunted L-NAME-induced reduction in RBF by $54\pm8\%$ ($P<0.05$) and attenuated the peak increase in RVR (at 20 min) elicited by L-NAME from $17.5\pm2.5$ in vehicle-treated rats to $9.4\pm1.7$ mmHg ml$^{-1}$ min$^{-1}$ ($P<0.05$), (Figure 4b). Indomethacin partially reversed L-NAME-induced reduction in GFR though this was not significant (Figure 4c). Indomethacin reduced basal UV (from $1.7\pm0.3$ to $0.9\pm0.2$ $\mu$l 100 g$^{-1}$ min$^{-1}$; $P<0.05$) and $U_{Na}V$ (from $0.36\pm0.08$ to $0.20\pm0.04$ $\mu$mol 100 g$^{-1}$ min$^{-1}$; $P<0.05$), and abolished L-NAME-induced increases in UV and $U_{Na}V$ ($P<0.05$), (Figure 4d).

### *Effect of $PGH_2/TxA_2$ receptor antagonism on renal response to L-NAME*

Our recent observation that the renal vasoconstrictor action of 20-HETE is prostaglandin endoperoxide-dependent (Askari *et al.*, 1997) as well as COX-dependent (Figure 5), prompted examining the effect of BMS180291, a $PGH_2/TxA_2$ receptor

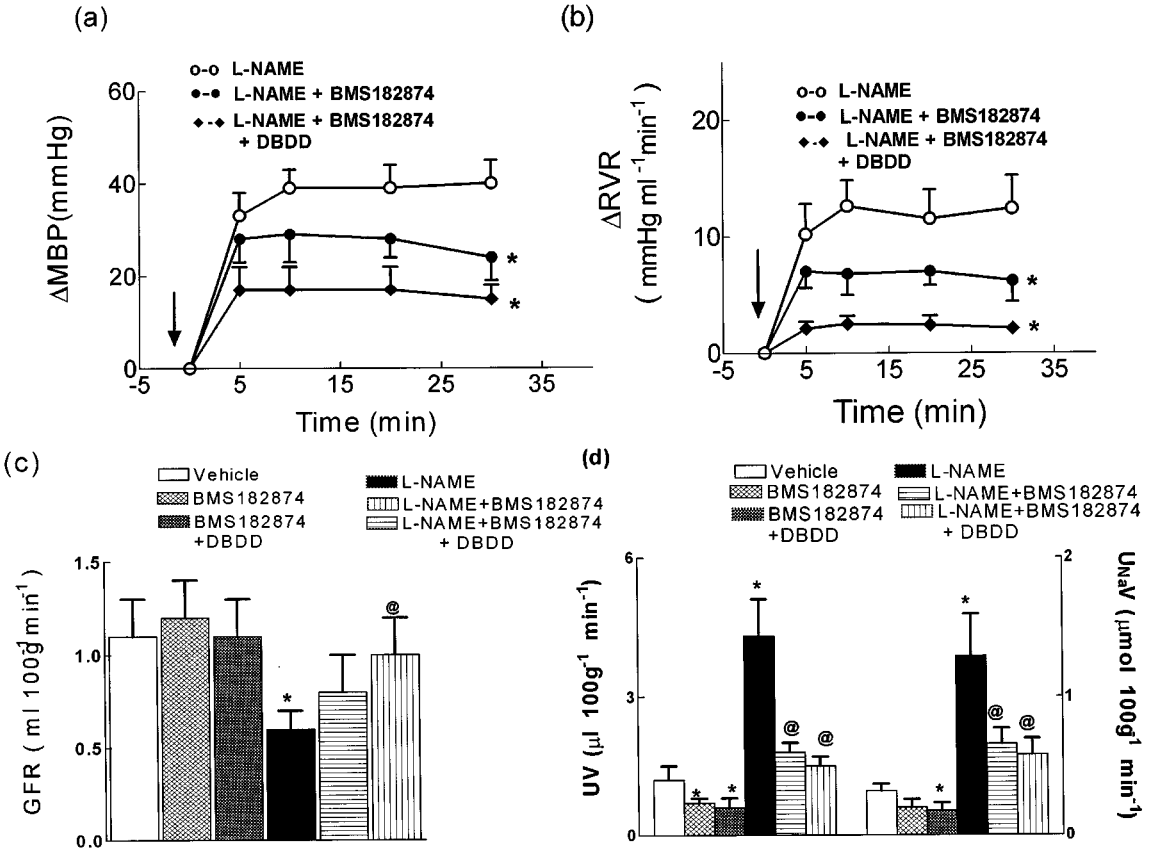


**Figure 3** Effects of BMS182874, an $ET_A$ receptor antagonist on changes ($\Delta$) in MBP (a) and RVR (b) GFR (c) and UV and $U_{Na}V$ (d) in response to bolus intravenous dose of L-NAME (10 mg kg$^{-1}$) in anaesthetized rats treated with 0.1 M $NaHCO_3$ (Vehicle) or BMS182874. Control rats ($n=4$) received 0.1 M $NaHCO_3$ (Vehicle, 1 ml kg$^{-1}$ orally). Arrow (a and b) denotes point of injection of L-NAME (0 min). Data are presented as means$\pm$s.e.mean. *$P<0.05$ vs Vehicle; #$P<0.05$ vs L-NAME.

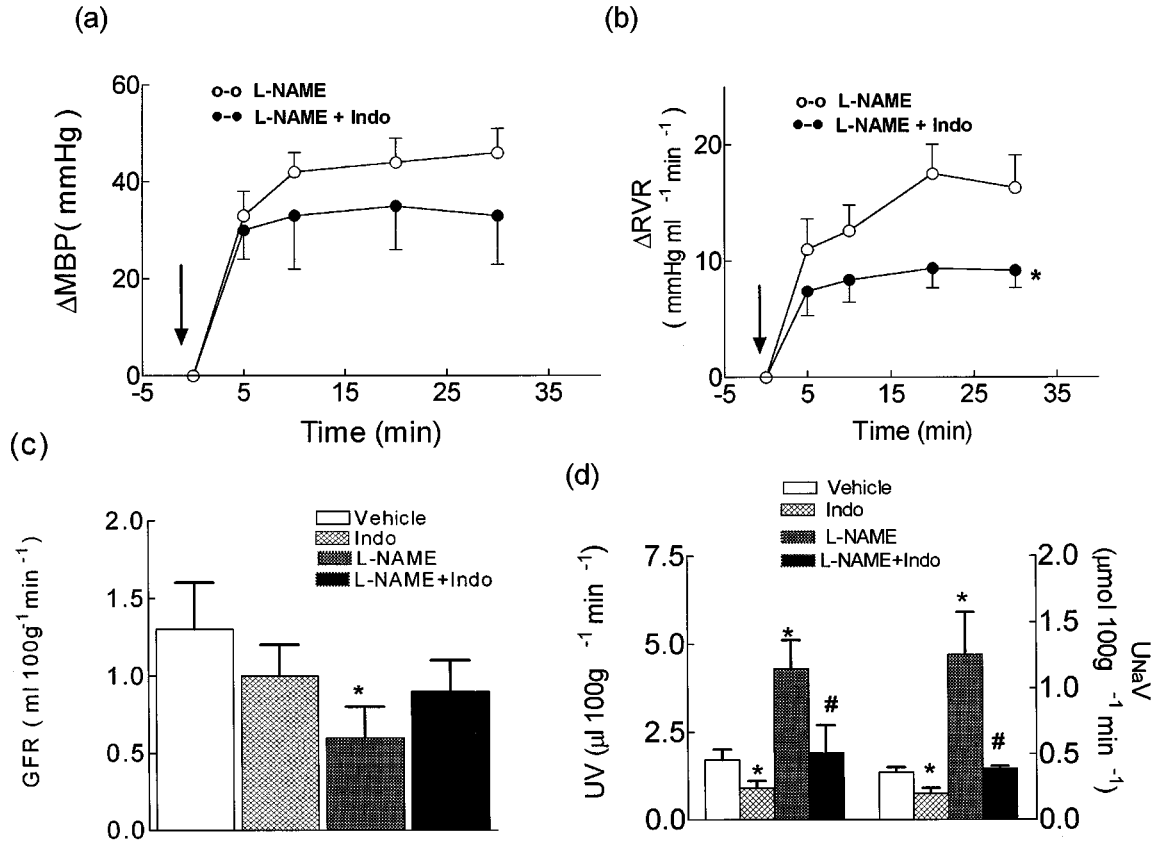


**Figure 4** Changes (Δ) in MBP (a) and RVR (b) GFR (c) and in UV and $U_{Na}V$ (d) in response to L-NAME (10 mg $kg^{-1}$, i.v.) in rats pretreated with indomethacin (Indo; 5 mg $kg^{-1}$, i.v., $n=5$; L-NAME + Indo) or receiving 50% ethanol (Vehicle). Arrow denotes point of injection of L-NAME (0 min). Results are presented as means ± s.e.mean. *$P<0.05$ vs Vehicle; #$P<0.05$ vs L-NAME.

antagonist, on L-NAME-induced changes in renal function. BMS180291 did not affect MBP and RBF; these values were: 94 ± 9 mmHg and 8.7 ± 0.4 ml $min^{-1}$, respectively ($n=5$); not significantly different from those in rats treated with vehicle ($n=4$); 93 ± 8 mmHg and 9.6 ± 0.6 ml $min^{-1}$, respectively. UV was similar in rats treated with BMS180291 (1.4 ± 0.4 μl 100 $g^{-1}$ $min^{-1}$) or its vehicle (1.8 ± 0.3 μl 100 $g^{-1}$ $min^{-1}$) (Figure 5d). BMS180291 (25 mg $kg^{-1}$) abolished the increase in RVR (18.3 ± 3.6 mmHg $ml^{-1}$ $min^{-1}$) produced by U46619 (1 μg $kg^{-1}$, i.v.), the $PGH_2/TxA_2$-mimetic and attenuated L-NAME-induced increases in MBP and RVR by 46 ± 8% ($P<0.05$; Figure 5a) and 65 ± 7% ($P<0.05$; Figure 5b), respectively. In contrast, BMS180291 did not affect the reduction in GFR (Figure 5c), nor the increases in UV and $U_{Na}V$ induced by L-NAME (Figure 5d). However, addition of DBDD ($n=4$), to those rats receiving BMS180291 abolished the increases in UV and $U_{Na}V$ elicited by L-NAME (Figure 5d).

## Discussion

There is abundant evidence to support the concept that tonically generated NO plays a major modulatory role in the regulation of vasomotor tone and fluid and electrolyte balance (Baylis *et al.*, 1990; Deng & Baylis, 1993). The diverse actions of NO and the effects of NO on the multiple cell types in the kidney has led to assigning a pivotal role for NO in the complex integrated functions of the kidney. Acute systemic NOS inhibition resulted in vasoconstriction and produced dose-dependent increases in arterial pressure, indicating that constitutive release of NO maintains the vasculature in a partially dilated state (Vallance *et al.*, 1989). The vasoconstriction by NOS inhibition, results not simply from withdrawal of an active NO vasodilator stimulus, but from amplification of underlying vasoconstrictor systems, including the renin-angiotensin (Gruetter *et al.*, 1988) and sympathetic nervous systems (Topouzis *et al.*, 1991), and endothelins (Richard *et al.*, 1995) which are kept in abeyance by the tonic modulatory activity of NO. In particular, for the present study, endothelins make a significant contribution to the acute pressor response produced by inhibition of NOS (Banting *et al.*, 1996; Richard *et al.*, 1995; Thompson *et al.*, 1995).

### *CYP450-AA metabolic activity: Inhibition by NO*

In the present study, we have tested the hypothesis that NO exerts a braking action on the vasoconstrictor activity of products of CYP450-AA metabolism. Hence, after elimination of endogenous NO by administration of L-NAME, the tonic inhibitory effect of NO on CYP450-AA metabolism was removed allowing full expression of the renal functional effects of CYP450-AA products. These products must arise from CYP450 mono-oxygenase activity as their effects were abolished by DBDD, a mechanism-based inhibitor of CYP450 ω-hydroxylase activity. Further, these CYP450-AA products must be subjected to transformation by COX as their renal functional effects were attenuated by indomethacin. The CYP450-AA product that best fits the biological profile of the mediator of renal functional responses to NOS inhibition is 20-HETE which, alone of the CYP450-AA metabolites, constricts the rat renal vasculature while promoting salt and water excretion (Takahashi *et al.*, 1990). Moreover, 20-HETE can be converted by COX to prostaglandin analogues that

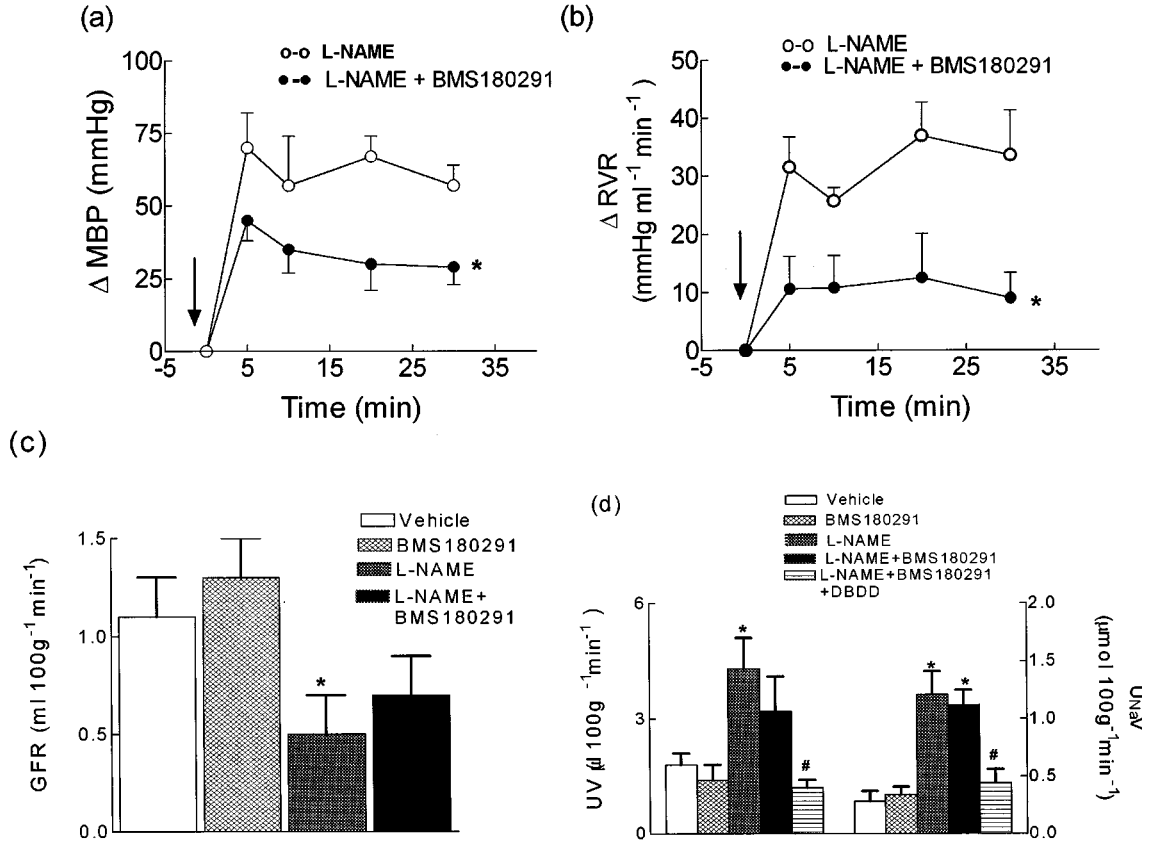


**Figure 5** Effects of L-NAME (10 mg $kg^{-1}$, i.v.) on changes (Δ) in MBP (a) and RVR (b), and on GFR (c), UV and $U_{Na}V$ (d) in rats receiving either vehicle (L-NAME) or BMS180291 (25 mg $kg^{-1}$, orally, $n=5$) alone (L-NAME + BMS180291) or in combination with DBDD 2.5 mg $kg^{-1}$ $h^{-1}$ (L-NAME + BMS180291 + DBDD; $n=4$). Arrow (a and b) denotes point of injection of L-NAME (0 min). Data are presented as means ± s.e.mean. *$P<0.05$ vs Vehicle; #$P<0.05$ vs L-NAME.

constrict blood vessels (Escalante *et al.*, 1989). L-NAME-induced increases in MBP and RVR and decreases in RBF and GFR, as well as natriuresis, were all attenuated by DBDD, the selective, mechanism-based inhibitor of P450 enzymes of the 4A family that catalyze ω-hydroxylation of AA to form 20-HETE. The ability of DBDD, the inhibitor of ω-hydroxylase, to suppress L-NAME responses (Figure 2) is in agreement with the study of Alonso-Galicia *et al.* (1997) who reported that inhibition of 20-HETE production attenuated the renal haemodynamic response to L-NAME. L-NAME also produced diuresis-natriuresis, findings similar to those of Johnson & Freeman (1992) in conscious rats and Ziyyat *et al.* (1996) in the rat isolated kidney. These findings support the interpretation that suppression of NO formation favours functional expression of a CYP450-AA product(s) that promotes salt and water excretion and constricts the renal vasculature. Of the CYP450-AA products, two have been shown to have diuretic-natriuretic capabilities, 20-HETE, which inhibits the $Na^+$-$K^+$-$2Cl^-$ cotransporter and $Na^+$-$K^+$-ATPase (Escalante *et al.*, 1991; Ribeiro *et al.*, 1994), and the epoxide, 5-,6-EET, which inhibits $Na^+$ reabsorption in the proximal (Romero *et al.*, 1990) and distal tubules (Jacobson *et al.*, 1984). As clotrimazole, the epoxygenase inhibitor, was without effect on the diuresis-natriuresis produced by L-NAME, an AA epoxide metabolite of the CYP450 system was eliminated as a mediator of the excretory response to inhibition of NOS. Further, 5-6-EET dilates blood vessels (McGiff, 1991), a property inconsistent with the marked renal vasoconstriction associated with expression of the activity of the putative CYP450-AA products unmasked by L-NAME.

### *Endothelins and CYP450-AA products*

Of the possible pressor systems evaluated as contributors to the L-NAME response, the endothelin system has been studied in the greatest detail. NO prevents, while inhibition of NOS promotes, the expression and production of ET-1 in cultured human endothelial cells (Kourembanas *et al.*, 1993) and in the isolated porcine aorta (Boulanger & Luscher, 1990). Goligorsky *et al.* (1994) demonstrated that NO directly modulates ET-1 gene expression, production and receptor binding, displacing bound ET-1 from its receptor in Chinese hamster ovary cells and interfering with postreceptor pathways for $Ca^{2+}$ mobilization. These *in vitro* data were complemented by an *in vivo* study showing that administration of L-NAME increased plasma levels of ET-1 (Richard *et al.*, 1995). In addition, in studies using ET-1 receptor antagonists, the endothelin system was demonstrated to contribute to the elevation of blood pressure in response to inhibition of NO production, especially in the acute phase of blockade of NOS (Banting *et al.*, 1996; and references 12, 13 and 14, therein). However, other studies suggest only a minor role (Morreau *et al.*, 1997) or no role (Sventek *et al.*, 1997) for ET following acute or chronic NOS blockade. In the present study, BMS182784, the orally active $ET_A$ receptor antagonist (Stein *et al.*, 1994), attenuated the increases in MBP and RVR induced by L-NAME, findings in agreement with the studies of Thompson *et al.* (1995) that underscore the contribution made by ET to the L-NAME-induced acute pressor response. Relevant to the present study, the effects of ET-1 on the rat kidney have been linked to a CYP450-AA metabolite, most

likely 20-HETE, which, we have proposed, acts as a second messenger for ET-1, constricting the renal vasculature and promoting salt and water excretion (Oyekan *et al*., 1997; Oyekan & McGiff, 1998). We have provided compelling evidence to support this proposal linking renal functional effects of ET-1 to 20-HETE (Oyekan *et al*., 1997; Oyekan & McGiff, 1998): (1) DBDD and $CaCl_2$, each suppressing CYP450 but by radically different mechanisms, had similar inhibitory effects on the renal functional response to ET-1. (2) Low dose ET-1 increased by 4 fold renal efflux of 20-HETE. (3) Inhibition of CYP450 AA metabolism greatly reduced the renal vasoconstrictor and natriuretic actions of ET-1. Finally, ET-1 and 20-HETE have similar renal effects: each constricts renal blood vessels while promoting salt and water excretion. If ET-1 acts *via* the same pathway as 20-HETE, then combined use of DBDD (to inhibit 20-HETE synthesis) and BMS182874 (to block $ET_A$ receptors) should have a similar dampening effect on L-NAME-evoked reductions in renal blood flow and sodium excretion as either agent given alone. This proved to be the case with respect to attenuating L-NAME-induced changes in arterial pressure, UV and $U_{Na}V$ as inhibition by DBDD + BMS182874 given together were indistinguishable from the effects produced by either agent given alone. However, reduction of GFR and RBF produced by L-NAME were reversed to a lesser degree by BMS than by BMS + DBDD, perhaps, reflecting stimulation by ET-1 of vasoconstrictor prostanoids, $TxA_2$ and $PGH_2$, which have been shown to be increased by ET-1 (Asano *et al*., 1994). Further, activation of $ET_B$ receptors, which were not blocked, can contribute to the renal vasoconstriction induced by ET-1 (Pollock & Opgenorth, 1994)

We have shown that the diuretic-natriuretic responses to ET-1 can be attenuated by inhibiting CYP450 AA metabolism, specifically formation of 20-HETE (Oyekan & McGiff, 1998). The renal tubular effects of ET-1 were independent of elevations in renal perfusion pressure as subpressor doses of ET-1 produced a dose-dependent natriuresis despite a progressive decline in GFR. These findings are in agreement with those of Harris *et al*. (1991) who observed a potent natriuretic action of ET that was often masked by intense renal vasoconstriction. However, under the present experimental conditions, a significant, if not dominant, component of the natriuresis produced by L-NAME resulted from the marked and sustained elevation in renal perfusion pressure produced by inhibiting NOS (Johnson & Freeman, 1992).

### *CYP450-AA products: Transformation by COX*

$PGH_2/TxA_2$ receptor blockade with BMS180291 (Figure 5) and inhibition of COX with indomethacin (Figure 4) both attenuated L-NAME-induced increases in RVR. The ability of indomethacin to attenuate the renal vasoconstrictor response produced by inhibition of NOS with L-NAME, presumably, resulted from prevention of 20-HETE transformation by COX to $PGG_2$, $PGH_2$ and $TxA_2$ analogues of 20-HETE that mediate the contractile response of the aorta to 20-HETE (Escalante *et al*., 1989). Further, 20-HETE-induced vasoconstriction in the rat kidney has been shown to be COX-dependent and inhibitable by either indomethacin or $PGH_2/TxA_2$ receptor blockade (Askari *et al*., 1997), supporting the interpretation that COX-related transformation of 20-HETE contributes to the renal vasoconstriction that follows suppression of NO.

If the CYP450-AA metabolite that is transformed by COX is responsible for both the renal vascular and tubular effects produced by L-NAME, then COX inhibition with indomethacin and $PGH_2/TxA_2$ receptor blockade with BMS180291 should have identical effects. Although each intervention reduced RVR, they differed in their effects on UV and $U_{Na}V$, suggesting that more than one product of CYP450-AA transformation by COX was involved. Whereas indomethacin suppressed the diuretic-natriuretic response to L-NAME, presumably mediated by prostaglandin analogues of 20-HETE, blocking the $PGH_2/TxA_2$ receptor with BMS180291 was without effect on the renal excretory response to L-NAME. As both $PGE_2$ and $PGI_2$ elicit diuresis and natriuresis (Bolger *et al*., 1978), a 20-HETE analogue of either $PGI_2$ or $PGE_2$ may mediate the renal excretory response to L-NAME. Blockade of the $TXA_2/PGH_2$ receptor with BMS180291 would not be expected to prevent the action of either a $PGI_2$ or $PGE_2$-like analogue of 20-HETE which proved to be the case. However, subsequent inhibition of 20-HETE synthesis with DBDD in rats that had received BMS180291 did prevent the diuresis-natriuresis produced by L-NAME, confirming the essential contribution of a CYP450-AA product to the renal functional effects of L-NAME. These findings indicate that the mediator of the diuretic-natriuretic effect arose from the CYP450 pathway and was subsequently transformed by COX. This CYP450-AA product has as its precursor most likely 20-HETE, a substrate for COX transformation, giving rise to prostaglandin analogues that can affect renal function similarly to L-NAME-induced inhibition of NOS (Ziyyat *et al*., 1996; Takahashi *et al*., 1990). Thus, both vasodilator-diuretic (20-OH $PGE_2$, 20-OH $PGI_2$) and vasoconstrictor (20-OH $TxA_2$ and 20-OH $PGH_2$) prostaglandin analogues of 20-HETE can be generated. We suggest that these separate pathways (20-OH-$PGH_2 \rightarrow$20-OH $TxA_2$ vs 20-OH $PGH_2 \rightarrow$20-OH $PGE_2$/20-OH $PGI_2$) for metabolizing 20-HETE *via* COX constitute the basis for the differences observed in the effects of COX inhibition vs blockade of $TxA_2/PGH_2$ receptors on sodium excretion (Figure 1). It is possible, based on this analysis, to construct a paradigm to account for the ability of either DBDD, $ET_A$ receptor blockade, indomethacin or an antagonist of the $TxA_2/PGH_2$ receptor to affect the renal functional effects of L-NAME acting *via* increased production of one or more CYP450 products that augment RVR, UV and $U_{Na}V$ and depress GFR (Figure 1).

In conclusion, we have presented evidence that inhibition of endogenous NOS, uncovers a major vasoconstrictor system operating through one or more CYP450-dependent AA metabolites that contribute to the renal haemodynamic and excretory effects that follow suppression of NOS. This conclusion is supported by the ability of DBDD, a selective inhibitor of $\omega$-hydroxylase, to inhibit the renal function response to L-NAME. Further, ET-1, which stimulates CYP450-AA metabolism, has effects on renal function similar to those of L-NAME-induced inhibition of NO formation (King *et al*., 1989). The effects of both ET-1 and L-NAME are best explained by increased production of 20-HETE. Finally, the demonstration that COX inhibition and $PGH_2/TxA_2$ receptor blockade antagonized the renal functional effects of L-NAME provides additional evidence that 20-HETE is involved in the renal functional response to NOS inhibition as 20-HETE has been shown to be transformed *via* COX to biologically active prostaglandin analogues (Schwartzman *et al*., 1989). These findings, it should be noted, are applicable only to the acute phase of the response to NOS inhibition in the anaesthetized rat; the chronic phase may reflect the operation of a different set of pressor systems.

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