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Contribution of cytochrome P450 4A isoforms to renal functional response to inhibition of nitric oxide production in the rat

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20-Hydroxyeicosatetraenoic acid (20-HETE), a major renal eicosanoid, regulates renal function and contributes to renal responses following withdrawal of nitric oxide (NO). However, the role of 20-HETE-synthesizing isoforms in renal function resulting from NO inhibition is unknown. The present study evaluated the role of cytochrome (CYP)4A1, -4A2 and -4A3 isoforms on renal function in the presence and absence of NO. Antisense oligonucleotides (ASODN) to CYP4A1, -4A2 and -4A3 reduced 20-HETE synthesis and downregulated the expression of CYP4A isoforms in renal microsomes. N^{ω}-L-nitromethyl arginine ester (L-NAME, 25 mg kg^{-1}), an inhibitor of NO production, increased mean arterial blood pressure (MABP, $\Delta = +18$ to 26 mmHg), reduced renal blood flow (RBF, $\Delta = -1.8$ to 2.9 ml min^{-1}), increased renal vascular resistance (RVR, $\Delta = +47$ to 54 mmHg ml^{-1} min^{-1}), reduced glomerular filtration rate (GFR), but increased sodium excretion ($U_{Na}V$). ASODN to CYP4A1 and -4A2 but not -4A3 reduced basal MABP and RVR and increased basal GFR, while ASODN to CYP4A2 significantly reduced basal $U_{Na}V$ suggesting a differential role for CYP4A isoforms in the regulation of renal function. ASODN to CYP4A2 but not -4A1 or -4A3 blunted the increase in MABP by L-NAME ($38 \pm 9\%$, $P < 0.05$). ASODN to CYP4A1, -4A2 and -4A3 attenuated the reduction in RBF and the consequent increase in RVR by L-NAME with a potency order of CYP4A2 = CYP4A1 > CYP4A3. ASODN to CYP4A1 and -4A2 but not -4A3 attenuated L-NAME-induced reduction in GFR, but ASODN to all three CYP4A isoforms blunted the L-NAME-induced increase in $U_{Na}V$ (CYP4A3 > CYP4A1 >> CYP4A2). We conclude from these data that CYP4A isoforms contribute to different extents to basal renal function. Moreover, CYP4A2 contributes greatest to haemodynamic responses while CYP4A3 contributes greatest to tubular responses following NO inhibition. We therefore propose that NO differentially regulates the function of CYP4A1, -4A2, and -4A3 isoforms in the renal vasculature and the nephron.

Many studies have clearly indicated that products of the ω/ω-1 hydroxylase pathway of cytochrome P450 (CYP)-dependent arachidonic acid (AA) metabolism are synthesized in the kidney and exert profound effects therein (McGiff & Quilley, 1999). The major ω-hydroxylation product of AA in tubular and vascular structures of the renal cortex and outer medulla of the rat is 20-hydroxy-eicosatetraenoic acid (20-HETE) (Omata *et al.* 1992*a,b*; Imig *et al.* 1996), an important regulator of renal vascular tone, tubular reabsorption and the control of arterial pressure (see McGiff & Quilley, 1999). ω-Hydroxylation of fatty acids, including AA has been characterized and shown to be catalysed by enzymes of the CYP4A family. In the rat, four isoforms have been identified: CYP4A1, -4A2, -4A3 and -4A8, and mRNA for all four have been identified in the kidney (Kimura *et al.* 1989*a,b*; Stromstedt *et al.* 1990). These isoforms, although sharing 66–98 %

homology and a common unique catalytic activity, i.e. hydroxylation at the ω-carbon, are localized to different renal structures. For example, CYP4A1, -4A3, and -4A8 are highly expressed in proximal tubules (Stromstedt *et al.* 1990; Hardwick, 1991; Omata *et al.* 1992*a,b*) and the renal microvasculature (Wang *et al.* 1999). On the other hand, CYP4A2, the constitutively expressed isoform especially in male rats (Kimura *et al.* 1989*a,b*; Sundseth & Waxman, 1993), is preferentially expressed in the outer medulla and thick ascending limb of the loop of Henle and is believed to be the major isoform in the kidney (Kimura *et al.* 1989*a,b*; Sundseth & Waxman, 1993). The differential expression of the AA ω-hydroxylating enzymes along the nephron may therefore contribute to the selective effects of 20-HETE on tubular function. The regulation of CYP4A is the subject of active interest and hypolipidaemic agents have been shown to selectively increase the expression of CYP4A1

and -4A3 isoforms (Hardwick, 1991). A potential exists also for a differential regulation of renal CYP4A by nitric oxide (NO) but this has not been actively explored. It has been demonstrated that NO inhibits CYP enzymes including the 4A family (Oyekan *et al.* 1999) by forming stable iron–nitrosyl complexes at the catalytic haeme binding site in this enzyme (Minamiyama *et al.* 1997; Mehl *et al.* 1999). This inhibition is corroborated by the observations that NO donors inhibit the synthesis of 20-HETE by renal microsomes (Alonso-Galicia *et al.* 1997; Oyekan *et al.* 1999) and that inhibition of NO production increased CYP4A expression and renal efflux of 20-HETE in the perfused rat kidney (Oyekan *et al.* 1999) and in the isolated proximal tubule of the normal rat (Escalante *et al.* 2002) and in the renal microvessels of the pregnant rat (Wang *et al.* 2002). In addition, incubation of recombinant CYP4A protein with NO donors revealed a differential formation of iron–nitrosyl complexes between different CYP4A isoforms (Wang *et al.* 2002). Since the capacity for 20-HETE production and therefore its renal effect are determined by the expression of specific CYP4A isoforms, for which expression differs between vascular and tubular sites in the kidney, we therefore hypothesize that the renal effect of NO inhibition (to increase CYP4A expression) will depend on the extent of NO regulation of specific CYP4A isoform(s). The availability of antisensense technology in the form of molecular probes has facilitated a definition of the functional role of each of the isoforms of the CYP4A family, permitting recognition of their separate and overlapping spheres of activity and, therefore, of the physiological significance of each isoform. Antisense technology has been used in other studies to demonstrate the roles of CYP4A1 *versus* -4A2 isoforms in the regulation of blood pressure in normotensive and spontaneously hypertensive rats (Wang *et al.* 1999, 2001). In the present study, we evaluated changes in renal haemodynamics and excretory function in rats that were treated with antisense oligonucleotides directed against CYP4A1, -A2 and -A3.

METHODS

Materials

N^{ω}-L-nitromethyl arginine ester (L-NAME; Sigma-Aldrich, MO, USA) and inulin (Sigma-Aldrich) were dissolved in 0.9 % NaCl. Inactin was obtained from Research Biochemicals International (Natick, MA, USA). The derivatives of the selected oligo-nucleotides (ODNs) were synthesized and purified by Gene Link (Thornwood, NY, USA).

Antisense and scrambled ODNs targeted to bases −3 to +21 of the CYP4A1 and CYP4A2 cDNAs (Kimura *et al.* 1989*a*) encompassing the ATG translation site codon were employed. The scrambled ODNs were designed such that they have the same base composition as the antisense ODNs. All ODN sequences were aligned to the DNA database (GenBank) using the MacVector Sequence Analysis Software. The following ODNs were used: antisense for CYP4A2, 5′-GCT-AAA-TAC-AGA-GAA-ACC-CAT-GGT-3′; scrambled ODN for CYP4A2, 5′-CAG-

ACC-GCA-GGA-CTA-AAT-AGA-TAT-3′; antisense for CYP4A1, 5′-CAG-TGC-AGA-GAC-GCT-CAT-GGT-3′ (21 bases) and 5′-CAG-TGC-AGA-GAC-GCT-CAT-3′ (18 bases); scrambled ODN for CYP4A1, 5′-CTG-ACC-GCA-GCA-GGA-CTT-AGA-TGG-3′. Using computer analysis, the 4A2-antisense ODN showed no homology with CYP4A1 or CYP4A8 mRNA sequences (Wang *et al.* 1999). However, it did recognize CYP4A3 mRNA; the homology between these two isoforms in their coding regions is 97 %. The 4A2-scrambled ODN contained the same base composition and computer analysis showed no sequence homology with CYP4A2 or any known CYP sequences (Wang *et al.* 1999).

Animal treatment

Experiments were conducted on male and female Sprague-Dawley rats (Harlan Sprague-Dawley, Houston, TX, USA; body weight 320 ± 8 g) according to protocols approved by the Institutional Animal Care and Use Committee. The animals were placed in a room with lighting adjusted to produce a normal day/night cycle (illuminated from 08.00 to 20.00 h). They were maintained on a standard rat food (Purina Chow) and were allowed *ad libitum* access to water and food prior to the experiments.

Under pentobarbital sodium anaesthesia (60 mg kg^{-1} I.P.), rats were chronically instrumented and a polyethylene (PE-50) catheter was placed into the left jugular vein for administration of oligonucleotides (ODNs). Recovery from anaesthesia was ascertained by full regain of righting, pain and palpable reflexes, and by full return to normal locomotor activity. The rats were allowed to recover for 24 h before commencing treatment. Antisense (AS) and scrambled ODNs were administered as the phosphorothioate derivatives described by Wang *et al.* (1999). Briefly, rats ($n = 5$–7 per group) were treated with liposome-encapsulated ASODN directed against CYP4A1, -4A2/3 (167 nmol kg^{-1} day^{-1} I.V. for 5 days). At these doses, 4A2- and 4A1-antisense ODNs decreased 20-HETE synthesis in microvessel homogenates by 40 and 50 %, respectively (Wang *et al.* 1999). Rats treated with scrambled ODNs (167 nmol kg^{-1} day^{-1} I.V. for 5 days) served as controls. For the purpose of these experiments, female rats treated with CYP4A2/3 ASODN were considered to have been treated with CYP4A3 antisense while male rats treated with CYP4A2/3 ASODN were considered to have been treated with CYP4A2. This approach was based on the close homology of CYP4A2 and -4A3 (Wang *et al.* 1999) and the known selective expression of CYP4A2 in males and its absence in females (Kimura *et al.* 1989*b*; Hardwick, 1991; Ito *et al.* 1998). Some of the rats were killed under inactin anaesthesia and the kidneys were harvested for determination of CYP4A expression and ω-hydroxylase activity. The other rats were used for haemo-dynamic studies, as detailed below.

CYP4A expression and ω-hydroxylase activity

These were evaluated as described previously (Oyekan *et al.* 1999; Wang *et al.* 1999). Briefly, microsomes (150 μg) were pre-incubated with [1-^{14}C]arachidonic acid (0.4 μCi, 7 nmol) in 100 mM phosphate buffer, pH 7.4, containing 10 mM MgCl$_2$ for 3 min at 37 °C. NADPH (1 mM) and the reaction mixture (0.4 ml final volume) were incubated together for 30 min at 37 °C. The reaction was terminated by acidification to pH 3–4 with 2 M formic acid, and metabolites were extracted with ethyl acetate. The final extract was evaporated under nitrogen, resuspended in 50 μl methanol, and subjected to reverse phase HPLC fractionation using a linear gradient ranging from acetonitrile:water:acetic acid

Table 1. Effects of administration of liposome-encapsulated scrambled (S) or antisense (AS) ODNs directed against CYP4A1 and -4A2 in male rats, and of ODNs directed against CYP4A2 in female rats (CYP4A3-S and -AS) on MABP, RBF, RVR, GFR and $U_{Na}V$

	MABP (mmHg)	RBF (ml min^{-1})	RVR (mmHg ml^{-1} min^{-1})	GFR (ml min^{-1})	$U_{Na}V$ (μmol min^{-1} (g kidney)$^{-1}$)
CYP4A1-S ($n = 7$)	142 ± 6	5.1 ± 1.9	31.4 ± 6.1	0.27 ± 0.06	0.79 ± 0.11
CYP4A1-AS ($n = 7$)	$131 \pm 5\dagger$	5.0 ± 1.9	$25.6 \pm 4.6\dagger$	0.36 ± 0.09	$0.63 \pm 0.12\dagger$
CYP4A2-S ($n = 6$)	137 ± 6	5.9 ± 2.4	27.4 ± 5.1	0.31 ± 0.07	0.87 ± 0.13
CYP4A2-AS ($n = 6$)	$125 \pm 5\dagger$	5.6 ± 2.3	24.8 ± 3.6	$0.43 \pm 0.06\dagger$	$0.56 \pm 0.15\dagger$
CYP4A3-S ($n = 5$)	130 ± 6	3.1 ± 1.4	43.6 ± 6.1	0.34 ± 0.05	0.45 ± 0.08
CYP4A3-AS ($n = 5$)	126 ± 6	3.2 ± 1.4	38.6 ± 4.7	0.33 ± 0.03	$0.36 \pm 0.09\dagger$

ODN, oligonucleotide; MABP, basal blood pressure; RBF, renal blood flow; RVR, renal vascular resistance; GFR, glomerular filtration rate; $U_{Na}V$, urinary sodium excretion. See details of treatment under Methods. *n* refers to the number of experiments. $\dagger$ *P* < 0.05 *versus* scrambled ODN-treated rats.

(50:50:0.1) to acetonitrile:acetic acid (100:0.1) at a flow rate of 1 ml min^{-1} for 30 min. The elution profile of the radioactive products was monitored by a flow detector (Packhard Instruments, Meridien, CT, USA) and the identity of each metabolite was confirmed by its comigration with an authentic standard.

Paired microsomal samples were treated with SDS reducing buffer containing 62.5 mM Tris HCl, pH 6.8, 10 % glycerol, 2 % SDS, 5 % mercaptoethanol and 0.125 % (w/v) bromophenol blue, then further incubated in boiling water for 4 min. SDS electrophoresis was performed on 8 % SDS-PAGE gel at 25 mA gel^{-1} at 4 °C for 18–20 h, followed by immunoblotting using goat anti-rat CYP4A1 antibody (Gentest, Woburn, MA, USA), a polyclonal antibody preparation that cross-reacts with all CYP4A proteins. Its sensitivity for detecting CYP4A1 protein is ~30 times higher than that of CYP4A2 protein and two times greater than that of CYP4A3. Antibody binding was demonstrated using an alkaline phosphatase detection system (Amersham International, UK). Autoradiographs were quantified by densitometric scanning using SigmaScan software (Jandel Scientific Software, Palo Alto, CA, USA).

Renal functional measurements

Clearance studies were performed on rats that were anaesthetized with inactin (100 mg kg^{-1} I.P.; Research Biochemical International) and instrumented as detailed below. Polyethylene cannulas were placed in the trachea (PE-205) to allow free breathing, in the bladder (PE-205) to facilitate voiding and in the right carotid artery (PE-50) for measuring and recording mean arterial blood pressure (MABP) by means of a pressure transducer (model BPLR, World Precision Instruments, Sarasota, FL, USA) coupled to a transbridge (model TBM-4, World Precision Instruments) and a data acquisition system (model DI-712, DataQ Instruments, Akron, OH, USA). A tail vein was also cannulated with a 23 g butterfly needle (Abbott Hospitals Inc., IL, USA) for infusion of agents. Through the tail vein, an infusion of 0.5 ml of a saline solution (0.9 % NaCl) containing 1 % inulin was administered over 2 min as a priming dose, followed by a maintenance infusion at the rate of 2 ml h^{-1}. A left laparotomy was performed and a transonic flow probe (Transonic Systems Inc., Ithaca, NY, USA) was placed over the left renal artery to measure renal blood flow (RBF). Renal vascular resistance (RVR) was calculated using a standard formula:

$$RVR = MABP - 5 \text{ mmHg}/RBF,$$

where 5 mmHg was used as an estimate of renal venous pressure.

During the experiments, the animals were placed on a heated table to maintain the body temperature at 37 °C. The experiments were started after an equilibration period of at least 60 min or until urine flow became steady. Starting from the beginning of the stabilization period, isotonic saline (0.9 % NaCl) was infused at a rate of 2 ml h^{-1}. After the stabilization period, urine was collected every 30 min. In the clearance experiments, ~400 μl of arterial blood was withdrawn from the femoral artery in the middle of each clearance period for measurements of glomerular filtration rate (GFR) by the inulin clearance method. An equal amount of normal saline was infused for volume replacement. Urinary sodium excretion ($U_{Na}V$) was determined by flame photometry (Genway FP7, Jenway Ltd, Essex, UK).

After the postsurgical equilibration/stabilization period and a 30 min baseline (basal) period, the effects of L-NAME on blood pressure and renal function were studied in six groups of five to seven rats each that were treated with CYP4A1, -A2 and -A3 antisense or scrambled ODNs. Each group of rats treated with ASODNs to CYP4A isoforms received an intravenous injection of L-NAME (25 mg kg^{-1} slow I.V.). This dose was selected on the basis of preliminary data indicating that, of the doses of L-NAME evaluated (5, 10, 25, 50 and 100 mg kg^{-1}) the 25 mg kg^{-1} dose evoked the maximal increase in RVR. During the experiment, changes in MABP and RBF were continuously monitored. At the end of each experiment the animals were killed using an overdose of inactin (250 mg kg^{-1}, I.P.).

Independent effects of the various ASODNs were evaluated by comparing renal function between rats treated with ASODNs to CYP4A1, -4A2 or -4A3, or their corresponding scrambled ODNs. The effects of ASODN on L-NAME-induced changes in renal function were evaluated by comparing renal effects of L-NAME between ASODN-treated rats and those treated with scrambled ODNs.

Data analysis

Haemodynamic responses were recorded as changes (Δ) relative to baseline (pre-L-NAME values). Values for GFR and urinary $U_{Na}V$ are presented as percentages. All data are expressed as means ± S.E.M. Haemodynamic data were analysed by determining areas under the curve for changes in MABP, RBF or RVR *versus* time. All data were subjected to the randomized block analysis of variance followed by a Newman-Keuls multiple range test. In all cases, *P* < 0.05 was considered significant.

RESULTS

Effects of CYP4A ASODN on CYP4A protein expression and ω-hydroxylase activity

Specific activity of microsomal ω-hydroxylase in whole kidneys harvested from rats treated with CYP4A1, -4A2, or -4A3 scrambled ODNs was: 82 ± 14, 48 ± 8 or 29 ± 4 pmol mg^{-1} min^{-1}, respectively. Treatment of rats for 5 days with ASODNs to CYP4A1, -4A2 or -4A3 reduced ω-hydroxylase activity to 48 ± 6, 35 ± 4 or $40 \pm 6\%$, respectively (Fig. 1A). Western blot analysis of paired samples from these rats is illustrated in Fig. 1B. In samples obtained from rats treated with ASODNs to CYP4A1, -4A2 or -4A3, the expression of the respective CYP4A isoforms was selectively reduced. However, the expression of CYP4A isoform(s) different from the one(s) targeted by a particular CYP4A isoform was also reduced though to a much smaller extent. Thus, the expression of

CYP4A1 or -4A2 was reduced by $18 \pm 4\%$ in rats treated with ASODN to CYP4A3 while the expression of CYP4A3 was reduced by 22 ± 4 or $35 \pm 5\%$ in rats treated with ASODN to CYP4A1 or -4A2, respectively.

Effects of CYP4A ASODN on basal renal function

Baseline values of MABP, RBF, RVR, GFR and $U_{Na}V$ obtained in inactin-anaesthetized rats treated with ASODNs or scrambled ODNs to CYP4A1 ($n = 7$), -4A2 ($n = 6$) and -4A3 ($n = 5$) are given in Table 1. Baseline MABP was similar in all the rats treated with scrambled ODNs to all CYP4A isoforms and ranged between 130 and 142 mmHg. RBF values were also similar in rats treated with CYP4A1 and -4A2 scrambled ODNs and ranged between 5.1 and 5.9 ml min^{-1}. However, basal RBF was lower in rats (female) treated with CYP4A3 scrambled ODN resulting in basal RVR that was markedly greater than that obtained for rats treated with scrambled ODNs to CYP4A1 and -A2.
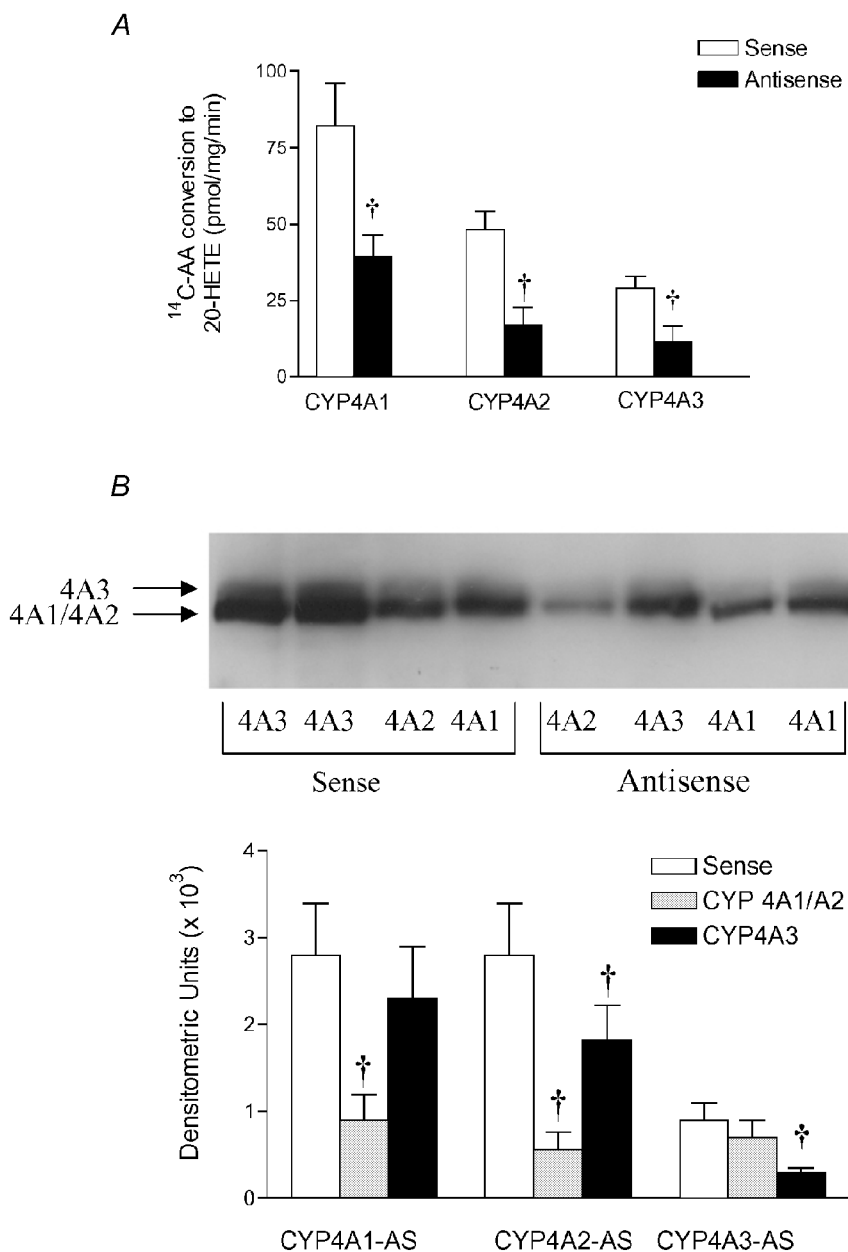

Figure 1. ω-Hydroxylase activity and CYP4A expression in rats treated with sense (S) or antisense (AS) oligonucleotides to CYP4A isoforms

A, ω-hydroxylase activity in whole renal microsomes from rats treated with scrambled (sense) or antisense (antisense) oligonucleotides to CYP4A1, -4A2 and -4A3 . Activity was measured as conversion of [1-^{14}C]arachidonic acid (AA) to 20-HETE. *B*, (upper panel) a typical immunoblot of microsomal samples from rats with scrambled (sense) or antisense (antisense) to CYP4A1 (4A1), -4A2 (4A2) and -4A3 (4A3). The lower panel illustrates a densitometric analysis of the blots obtained from samples from rats treated with antisense oligonucleotides to CYP4A1 (CYP4A1-AS), -4A2 (CYP4A2-AS) and -4A3 (CYP4A3-AS). $\dagger P < 0.05$ *versus* sense.
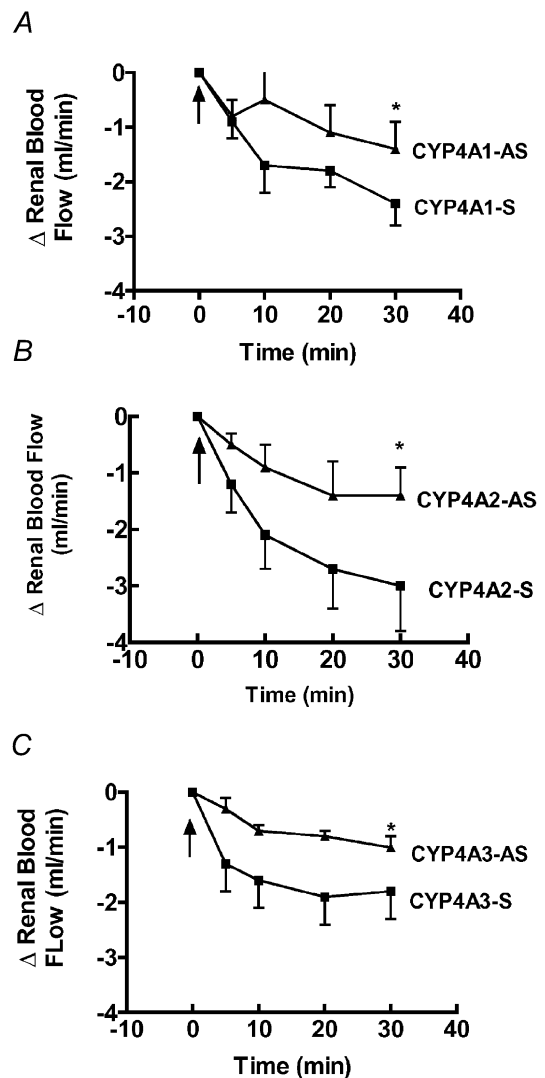
Compared with rats treated with scrambled ODNs, basal RBF or RVR was not significantly different in rats treated with CYP4A3 ASODN. Similarly, there was no difference in RBF or RVR between rats treated with ASODNs or scrambled ODNs to CYP4A1 and -4A2. However, when compared with rats treated with scrambled ODNs, basal MABP was lower in rats treated with ASODNs to CYP4A1 and -4A2. Unlike haemodynamic data, basal renal excretory function was markedly altered in rats treated with ASODNs to all three CYP4A isoforms. For example, in

comparison to rats treated with the respective scrambled ODNs, basal GFR increased by 33 ± 6 or $39 + 5\%$ in rats treated with CYP4A1 or -4A2 ASODN (Table 1). However, there was no difference in basal GFR in rats treated with ASODN to CYP4A3. On the other hand, basal sodium excretion was reduced in rats treated with ASODNs to the three CYP4A isoforms, but the effect was only significant in rats treated with ASODN to CYP4A2 (Table 1). Thus, $U_{Na}V$ was reduced by $18 \pm 8\%$ $(P > 0.05)$, $36 \pm 5\%$ $(P < 0.05)$ and $19 \pm 11\%$ $(P > 0.05)$ in rats treated with ASODNs to CYP4A1, -4A2 and -4A3, respectively, compared with rats treated with the respective scrambled ODNs.
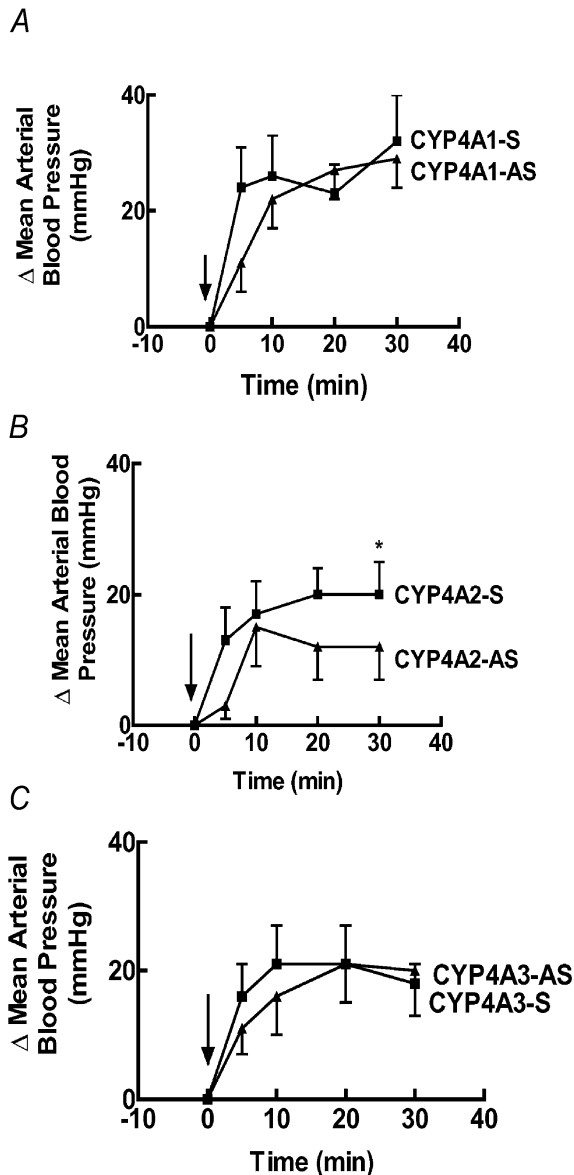
Figure 2. Changes in mean arterial blood pressure in response to L-NAME (25 mg kg^{-1}) in rats treated with scrambled (S) or antisense (AS) oligonucleotides (ODNs)

Responses in male rats to treatment with ODNs to CYP4A1 (CYP4A1-S and CYP4A1-AS) (*A*) and CYP4A2 (CYP4A2-S and CYP4A2-AS) (*B*) and in female rats to treatment with ODNs to CYP4A2 (CYP4A3-S and CYP4A3-AS) (*C*). The arrows show the point of injection of L-NAME. * $P < 0.05$ *versus* scrambled ODN-treated rats.
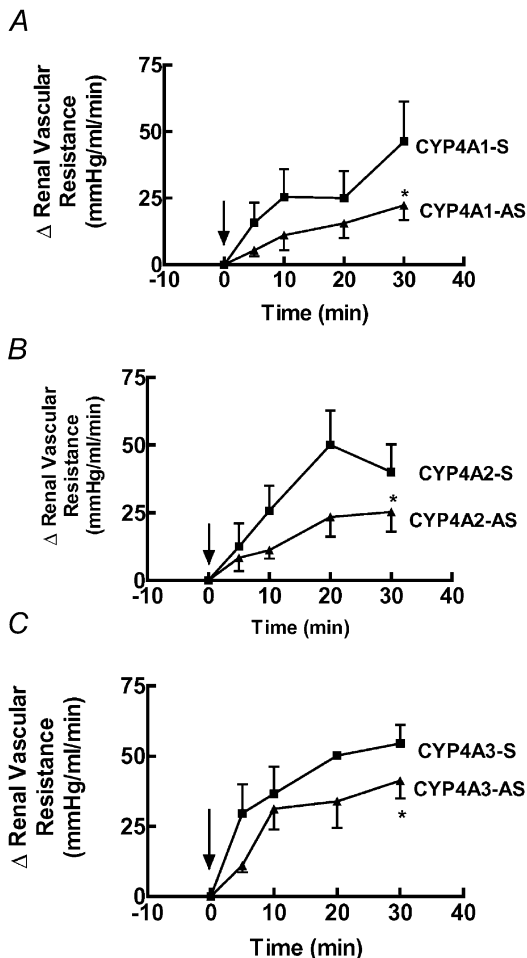

Figure 3. Reductions in renal blood flow evoked by acute administration of L-NAME (25 mg kg^{-1}) in rats treated with scrambled (S) or antisense (AS) oligonucleotides (ODNs)

Responses in male rats to treatment with ODNs to CYP4A1 (CYP4A1-S and CYP4A1-AS)(*A*) and CYP4A2 (CYP4A2-S and CYP4A2-AS) (*B*) and to scrambled or antisense oligonucleotides to CYP4A2 (CYP4A3-S and CYP4A3-AS) in female rats (*C*). * $P < 0.05$ *versus* scrambled ODN-treated rats.

Effect of ASODNs on L-NAME-induced haemodynamics

Injection of L-NAME in rats treated with the scrambled ODNs produced a rapid and pronounced increase in MABP, a reduction in RBF and consequently an increase in RVR. These changes were sustained for at least 30 min (Figs 2, 3 and 4). Accompanying L-NAME-induced changes in systemic and renal haemodynamics was a reduction in GFR that ranged between 42 and 48 %, but a 32–40 % increase in $U_{Na}V$. The increase in $U_{Na}V$ was observed in only 67 % of the test animals as there were reductions in $U_{Na}V$ in 24 % or no change in $U_{Na}V$ in 9 % of the test animals. Data reported were for the increases in $U_{Na}V$ as this was the most frequent effect.
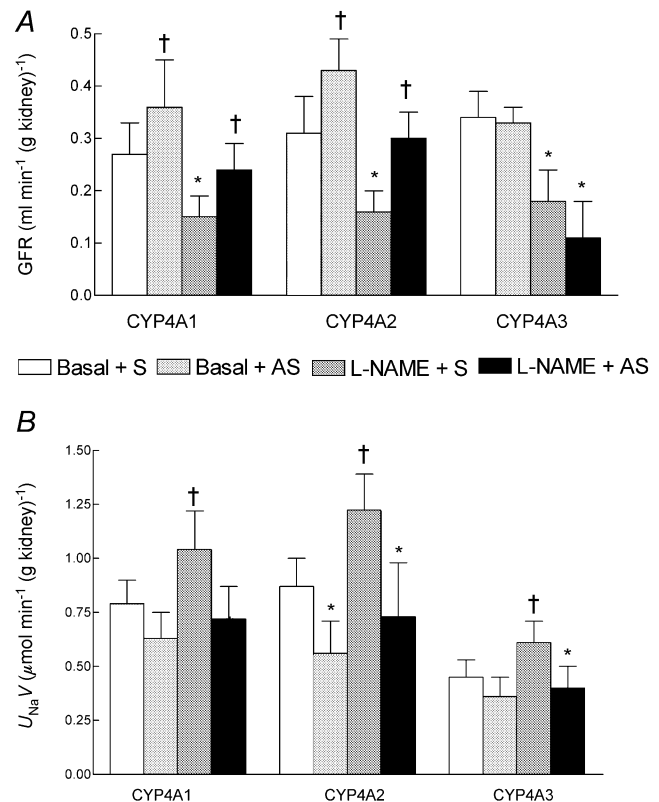
Compared to rats treated with the respective scrambled ODNs, L-NAME-induced increase in MABP was reduced only in rats treated with ASODNs to CYP4A2 (38 ± 9 %, $P < 0.05$) but not -4A1 or -4A3. Figures 2, 3 and 4 show that treatment with CYP4A1 ASODN greatly attenuated the renal haemodynamic and excretory responses, but not the pressor responses to L-NAME. Thus, CYP4A1 ASODN attenuated L-NAME-induced reduction in RBF by 45 ± 12 % ($P < 0.05$) and consequently blunted the increase in RVR by 54 ± 10 % ($P < 0.05$) without affecting L-NAME-induced increase in MABP.

On the other hand, CYP4A2 ASODN markedly attenuated the pressor and renal haemodynamic responses to L-NAME. Thus, CYP4A2 ASODN reduced the increase in MABP induced by L-NAME by 38 ± 9 % ($P < 0.05$, Fig. 2B) and blunted the reduction in RBF by 54 ± 7 % ($P < 0.05$, Fig. 3B) leading to an increase in RVR that was reduced by 49 ± 6 % ($P < 0.05$, Fig. 4B) compared with scrambled ODN-treated rats.

Figure 4. L-NAME-induced increase in renal vascular resistance in rats treated with scrambled (S) or antisense (AS) oligonucleotides (ODNs)

Responses in male rats to treatment with ODNs to CYP4A1 (CYP4A1-S and CYP4A1-AS) (A) and CYP4A2 (CYP4A2-S and CYP4A2-AS) (B) and with scrambled or antisense oligonucleotides to CYP4A2 (CYP4A3-S, CYP4A3-AS) in female rats (C). * $P < 0.05$ *versus* scrambled ODN-treated rats.

Figure 5. Reduction in GFR and natriuresis in rats treated with sense (S) or antisense (AS) oligonucleotides to CYP4A isoforms

Glomerular filtration rate (GFR) (A) and sodium excretion ($U_{Na}V$) (B) in rats treated with scrambled ODNs to CYP4A isoforms (sense) before (basal) or after acute administration of L-NAME (25 mg kg^{-1}) in rats treated with scrambled (S) or antisense (AS) oligonucleotides (ODNs) to CYP4A1 (CYP4A1) and CYP4A2 (CYP4A2) in male rats or of scrambled or antisense oligonucleotides to CYP4A2 in female rats (CYP4A3). † $P < 0.05$ *versus* sense * $P < 0.05$ *versus* basal.

In rats treated with CYP4A3 ASODN there was no difference in the increase in MABP induced by L-NAME (Fig. 2*C*) when compared with rats treated with scrambled ODN to CYP4A3. However, CYP4A3 ASODN attenuated L-NAME-induced reduction in RBF by $42 \pm 7\%$ ($P < 0.05$, Fig. 3*C*) and consequently reduced L-NAME-induced increase in RVR by $34 \pm 5\%$ ($P < 0.05$) (Fig. 4*C*).

Effect of ASODNs on L-NAME-induced glomerular and renal tubular responses

Figure 5 illustrates the effects of treatment with antisense to CYP4A1, -4A2 and -4A3 on changes produced by L-NAME in GFR and $U_{Na}V$. Compared with rats treated with scrambled ODNs, L-NAME-induced reduction in GFR was attenuated in rats treated with ASODNs to CYP4A1 and -4A2 (greater inhibition with CYP4A2 than -4A1) (Fig. 5*A*). On the other hand, treatment with ASODN to CYP4A3 did not inhibit L-NAME-induced reduction in GFR, but instead the L-NAME effect was further reduced. Thus, the reductions by L-NAME in GFR in rats treated with scrambled ODNs to CYP4A1, -4A2 and -4A3 were: 33 ± 4, 30 ± 4 and $66 \pm 7\%$, respectively, compared with reductions of 44 ± 6, 48 ± 7 and $42 \pm 6\%$, respectively, in rats treated with the corresponding ASODNs.

Similarly, treatment with ASODNs to the three CYP4A isoforms attenuated L-NAME-induced increase in $U_{Na}V$ (Fig. 5*B*). Thus, in rats treated with scrambled ODNs to CYP4A1, -4A2, and -4A3, L-NAME increased $U_{Na}V$ by 32 ± 5, 40 ± 6 and $36 \pm 5\%$, respectively. However, in rats treated with the corresponding ASODNs, L-NAME-induced increase in sodium excretion was reduced to 14 ± 4, 30 ± 4 and $11 \pm 3\%$, respectively, giving a potency order of CYP4A3 > CYP4A1 >> CYP4A2.

DISCUSSION

The results of this study demonstrate that after elimination of endogenous NO, the tonic inhibitory effect of NO on CYP AA metabolites, especially 20-HETE, was removed allowing a full expression of its renal functional effect. This thus confirms our earlier study (Oyekan & McGiff, 1998). A pivotal role has been assigned to NO in the complex integrated functions of the kidney on account of its diverse actions and its effect on the many cell types in the kidney. NO exerts a potent influence on renal function through its ability to tonically regulate vasomotor tone and fluid and electrolyte balance (see Ortiz & Garvin, 2002). Acute systemic inhibition of NOS resulted in vasoconstriction and increased blood pressure that resulted 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 (Vallon *et al.* 1995), endothelins (Richard *et al.* 1995) and CYP AA metabolites (see Morgan *et al.* 2001).

We and others have demonstrated that NO inhibits renal ω-hydroxylase activity (Alonso-Galicia *et al.* 1997; Oyekan *et al.* 1999; Wang *et al.* 2002) and that withdrawal of NO leads to increased ω-hydroxylase activity (Wang *et al.* 2002) and expression of CYP4A (Oyekan *et al.* 1999), the protein responsible for 20-HETE synthesis. The effects of 20-HETE on renal vascular resistance, autoregulation of renal blood flow and tubuloglomerular feedback (TGF) (Zou *et al.* 1994*a,b*) are integral to the capacity of the renal vasculature to synthesize 20-HETE and we propose that these can be modulated by NO. In our previous studies, we provided evidence that L-NAME increased 20-HETE production in the whole kidney (Oyekan *et al.* 1999), proximal tubules (Escalante *et al.* 2002) and renal microvessels (Wang *et al.* 2002). Moreover, 20-HETE produced dramatic pertubations in proximal tubular Na^+ transport (Escalante *et al.* 2002), renal haemodynamics, and salt and water excretion (Oyekan & McGiff, 1998) when its production was unregulated in response to inhibition of NO production. The attenuation by inhibition of 20-HETE production of the renal effects of L-NAME (Alonso-Galicia *et al.* 1997; Oyekan & McGiff, 1998) supports our working hypothesis that the control of 20-HETE synthesis by NO is essential to the regulation of renal function. However, two important questions arise. (1) What is the effect of NO on different CYP4A isoforms? (2) Is there a differential expression and/or activity of CYP4A enzymes following inhibition of NO production? In answering these questions, consideration was given to the fact that CYP4A isoforms are differentially expressed in the kidney (Stromstedt *et al.* 1990; Hardwick, 1991; Omata *et al.* 1992*a,b*) and may therefore affect renal function to different degrees. In addition, we considered that the capacity for 20-HETE production is different amongst different CYP4A isoforms, implying that the effect of NO on CYP4A1, -4A2 and -4A3 will have differential effects on 20-HETE-related changes in renal function. Our recent study gave credence to this as we demonstrated that nitrosylation of recombinant CYP4A3 protein and NO-inhibition of its catalytic activity was greater than that of CYP4A1 protein (Wang *et al.* 2002).

Synthesis of 20-HETE has been identified at several intrarenal sites that are pivotal to the regulation of renal function: the proximal tubules, collecting duct, medullary thick ascending limb (Caroll *et al.* 1991; Omata *et al.* 1992*a,b*) and the preglomerular arteriole (Zou *et al.* 1994*a*; Imig *et al.* 1996). Similarly, NOS has also been identified at the same sites (Wu *et al.* 1999; Mattson & Wu, 2000) and NO, like 20-HETE, exerts effects on the vascular smooth muscle and TGF (Wilcox & Welch, 1996) and may influence sodium and/or water transport in the proximal tubule (Gabbai *et al.* 1995; Vallon *et al.* 1995) and collecting ducts (Garcia *et al.* 1996). The colocalization of NOS and CYP4A isoforms at the same renal sites therefore increases the potential for interactions and this may affect renal function. The present study investigating the

contribution of each of the CYP4A isoforms to renal function and the effect of NO on these isoforms as they affect renal function is, to our knowledge, the first study to address this issue.

In the absence of a direct evaluation, we addressed NO–CYP interactions on the premise that the contribution of a particular CYP4A isoform to renal functional response to inhibition of NOS would reflect the effect of NO on that isoform. The relative effect of NO on the different isoforms would in turn be determined by the degree of inhibition observed following selective antagonism of the effect of that particular isoform. In our previous study, antagonism of CYP4A isoforms using their respective ASODNs was used to demonstrate the contribution of 20-HETE of vascular *versus* tubular origin to the regulation of blood pressure (Wang *et al.* 1999). In the present study, the lower basal blood pressure in rats treated with ASODNs to CYP4A1 and -4A2 is in agreement with our earlier observation (Wang *et al.* 1999). In addition, ASODN to CYP4A2 blunted the increase by L-NAME in blood pressure while ASODNs to the three CYP4A isoforms blunted L-NAME-induced increase in renal vascular resistance. By comparing the areas under the curves of MABP, RBF and RVR *versus* time for rats treated with ASODNs to CYP4A isoforms, our data suggest that CYP4A1, -A2 and -A3 contribute to the renal haemo-dynamic effect of L-NAME while only CYP4A2 contributes to its systemic haemodynamic effect, suggesting a significant extrarenal distribution of CYP4A2. By comparing the degree of inhibition by the respective ASODNs of the haemodynamic responses, we deduced that the relative contribution of these isoforms to the effects of NOS inhibition on RBF and RVR is: CYP4A2 = CYP4A3 > CYP4A1; and on blood pressure, the relative contribution is: CYP4A2 >>> CYP4A1 = CYP4A3. Taken together, these data suggest that NO inhibits the 20-HETE generating capability of CYP4A1 and CYP4A2/3 isoforms in the renal vasculature. The observation that ASODN to CYP4A2 produced the greatest inhibition of L-NAME responses is consistent with the fact that CYP4A2 isoform is the major CYP4A isoform in the renal vasculature (Ito *et al.* 1998).

Apart from haemodynamic studies, the present study also evaluated a role for CYP4A isoforms in tubular function. Our data demonstrated that ASODNs to CYP4A1 and -4A2, but not -4A3, increased basal GFR, while only the ASODN to CYP4A2 attenuated basal excretion of sodium suggesting that CYP4A2 makes the greatest contribution to the control of excretory function of the kidney. The lack of effect of CYP4A3 antisense oligonucleotide on basal GFR and $U_{Na}V$ probably reflects that much of the CYP4A3 protein is bound to endogenous NO and this is consistent with our previous data that formation of ferric–nitrosyl complexes was greatest with recombinant CYP4A3 (Wang *et al.* 2002). The decrease in basal $U_{Na}V$ despite an increase in GFR in ASODN-treated

rats is consistent with the known direct tubular effect of 20-HETE that is independent of its vascular effect (see McGiff & Quilley, 1999). Given the fact that NO is produced at the same renal sites and affects Na^+ transport in the same way as 20-HETE, we evaluated NO–CYP4A interactions on excretory function of the kidney. Following NOS inhibition, there was an increase in $U_{Na}V$ in 67 % of the test animals. This observation is contrary to the known effect of NO on Na^+ transport in the nephron (see Ortiz & Garvin, 2002) and at variance with the findings that administration of NOS inhibitors generally led to retention of sodium and water in dogs and rats (see Wu *et al.* 1999, references 10, 14 and 26 therein). However, the present data generally support our previous study and others that demonstrated a natriuresis and diuresis following administration of NOS inhibitors in the anaesthetized rat (Oyekan & McGiff, 1998), conscious rat (Johnson & Freeman, 1992) or in the rat isolated perfused kidney (Ziyyat *et al.* 1996). The natriuresis and diuresis in response to NOS inhibition may result from increased blood pressure (Johnson & Freeman, 1992) as part of a pressure-diuresis mechanism. Given the distribution pattern of CYP4A isoforms in the nephron (Hardwick, 1991; Omata *et al.* 1992*a*,*b*), the inhibition of L-NAME-induced natriuresis by ASODNs to CYP4A1, -A2 and -A3 can be explained on the basis that withdrawal of NO led to increased *ω*-hydroxylase activity (increased 20-HETE) thereby inhibiting Na^+ transport in the proximal tubule, the collecting duct and the mTALH (sites of synthesis of 20-HETE) (see McGiff & Quilley, 1999). This is consistent with the demonstrated inhibitory effects of 20-HETE on Na^+,K^+-ATPase and the $Na^+–K^+–2Cl^-$ cotransporter in the proximal tubule and the mTALH (Escalante *et al.* 1994, 2002). The inhibition of the natriuretic effect of L-NAME by ASODNs to CYP4A1, -4A2 and -4A3 suggests that NO probably reduced the expression and/or activity of all three CYP4A isoforms in the tubules. However, this notion has to be tempered by the lack of absolute specificity of ASODNs in this study, since treatment with ASODN to a particular isoform reduced the expression of the other isoforms. Thus, though the antisense approach was developed as a superior experimental probe to pharmacological inhibitors and is supported by a clear specificity of the CYP4A1 ASODN *in vitro* (Wang *et al.* 1999), this specificity is not absolute *in vivo*. Nonetheless, data from this study demonstrate indirect, albeit inconclusive, evidence that CYP4A1, -4A2 and -4A3 isoforms participate in renal function and contribute to different extents to the changes in renal function as a result of inhibition of NO production in the rat. The evidence suggests that the CYP4A2 isoform makes the greatest contribution to renal and systemic haemodynamics and to tubular excretory function following inhibition of NO production. Therefore, as reported for clofibrate and other hypolipidaemic agents that selectively increase the expression of CYP4A1 and -4A3 (Hardwick, 1991), NO presents another possible addition to the list of agents that differentially regulate the activity and/or expression of

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CYP4A isoforms. Additional studies are clearly required to address the interactions of NOS and CYP4A proteins by evaluating the expression and/or activity of CYP4A isoforms in the preglomerular vessel, glomerulus and specific segments of the nephron as they relate to vasomotor responses and ion transport in these segments.

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