Transfection of CYP4A1 cDNA increases vascular reactivity in renal interlobar arteries

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SMALL ARTERIAL VESSELS MANUFACTURE 20-HETE, a product of arachidonic acid metabolism by cytochrome P-450 enzymes of the 4A family (CYP4A1) (8, 12, 17, 26). Exogenous 20-HETE was reported to contract or relax vascular smooth muscle, depending on the animal species, type of vessels, and experimental conditions. Cyclooxygenase-dependent and -independent mechanisms have been implicated in 20-HETE-induced vascular contraction (6, 16, 17) and vascular relaxation (2, 4).

Renal preglomerular vessels express CYP4A proteins, produce 20-HETE, and are constricted by exogenous 20-HETE. The constrictor action of exogenous 20-HETE in pressurized canine arcuate arteries, rat renal interlobar arteries, and rat renal afferent arterioles is independent of cyclooxygenase activity and has been attributed to inhibition of the opening of Ca2+-activated K+ channels in vascular smooth muscle cells, leading to cellular depolarization and increased Ca2+ entry (8, 12, 14, 23, 28).

Recent studies support the notion that endogenous 20-HETE subserves vasoconstrictor mechanisms in the rat kidney. For example, inhibitors of 20-HETE synthesis were shown to increase blood flow to the kidney (29), to increase the diameter of preconstricted interlobular arteries denuded of endothelium (11), and to attenuate vasoconstrictor responses elicited by endothelin (10, 22), angiotensin II (5), or increases in transmural pressure (14). 20-HETE produced by the smooth muscle isolated from renal preglomerular vessels was also shown to exert a tonic inhibitory influence on the activity of large-conductance Ca2+-activated K+ channels (28).

Previous studies indicated that the responsiveness of small arterial vessels to constrictor stimuli increased after treatment with a blocker of large-conductance Ca2+-activated K+ channels (13). Hence, it is conceivable that 20-HETE of vascular origin is a stimulatory regulator of vascular reactivity to constrictor agonists. To test this hypothesis, we transfected rat renal interlobar arteries with an expression plasmid containing the cDNA of CYP4A1, the low-Km arachidonic acid ω-hydroxylase, and examined the consequences of enhanced CYP4A1 expression on 20-HETE synthesis and vascular reactivity to phenylephrine. We also studied the effect of exogenous 20-HETE and of agents that interfere with the synthesis or the actions of 20-HETE on vascular reactivity in arterial preparations trans-
fected with plasmid containing CYP4A1 cDNA compared with those transfected with the control plasmid.

MATERIAL AND METHODS

Transfection of renal interlobar arteries. Full-length CYP4A1 cDNA (20) was cloned into the expression vector pcDNA3.1 (+) (Invitrogen). The expression plasmid containing CYP4A1 cDNA (pcDNA3.1–4A1) and the control plasmid (pcDNA3.1) were encapsulated into liposomes (DOTAP Liposomal Transfection Reagent, Boehringer Mannheim, Indianapolis, IN). Sprague-Dawley rats (8 wk old) were anesthetized by pentobarbital sodium, the kidneys were perfused with ice-cold Krebs solution, and renal interlobar arteries were isolated by microdissection under a microscope. The vessels were placed in culture dishes containing DMEM with 10% Nu-serum, 100 µg/ml streptomycin, and 100 µg/ml penicillin plus 17 µg of either pcDNA3.1–4A1 or pcDNA3.1. The vessels were maintained in organ culture for 18 h in a humidified incubator (95% air-5% CO2) at 37°C. At the end of the culture period, vessels were used for measurements of CYP4A protein and 20-HETE synthesis and for vascular reactivity studies.

CYP4A protein and 20-HETE synthesis. Microdissected artery segments were homogenized and proteins were separated by electrophoresis on an 8% SDS-Polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and blotted with goat anti-rat CYP4A1 polyclonal antibody (1:1,000; Genetest; Woburn, MA). Immunoreactive proteins were detected by phosphorimaging methods using Vistra enhanced chemiluminescent substrate (20). 20-HETE production was determined by incubating interlobar arteries with arachidonic acid (30 µM) in 1 ml Tyrode’s buffer containing 1 mM NADPH, 10 µM indomethacin, and 10 µM Nω-nitro-L-arginine methyl ester for 1 h at 37°C. The reaction was terminated by acidification to pH 3.5–4.0 with 10 µl of 2 M formic acid. [20,20-2H2]20-HETE (1 ng) was added as an internal standard. The mixture was then extracted twice with 2 ml ethyl acetate. The final extract was evaporated under nitrogen, resuspended in 30 µl of methanol, and subjected to reverse-phase HPLC as described (18). Fractions coeluting with the 20-HETE standard were collected, evaporated to dryness, and derivatized to the pentfluorobenzyl bromide ester trimethylsilyl ether. 20-HETE was quantified by negative chemical ionization-GC-MS as previously described (18). After extraction, the vessels were collected and suspended in 100–300 µl of 1 N NaOH, in which they were left overnight to dissolve. Any intact vessels remaining were manually ground with a glass rod. Protein concentration was determined by using the Bio-Rad assay.

Measurement of isometric tension in vascular rings. Interlobar arteries (~230 µm, internal diameter) were cut into ring segments (2 mm). The rings were mounted on wires in the chambers of a multivessel myograph (JP Trading, Aarhus, Denmark) filled with Krebs buffer (37°C) and gassed with 95% O2-5% CO2. After 30- to 60-min equilibration, the vessels were set to an internal circumference equivalent to 90% of that which they would have in vitro when relaxed under a transmural pressure of 80 mmHg. Isometric tension (mN/mm vessel length) was monitored continuously before and after experimental interventions. Constrictor response to 60 mM KCl was determined in each vessel. Subsequently, a cumulative concentration-response curve to phenylephrine (10^-9–5 × 10^-5 M) or to KCl (10^-3–10^-1 M) was constructed. In some experiments, the response to phenylephrine and KCl was measured in vessels pretreated with N-methylsulfonyl-12,12-dibromodec-11-enamide (DDMS; 30 µM), 20-hydroxyeicosapentaenoic acid (20-HEDE; 10 µM), and 20-HETE (1 or 10 µM) or with tetraethylammonium (TEA; 1 mM). DMDS is a selective inhibitor of arachidonic acid ω-hydroxylation (25). 20-HEDE was reported to block vascular actions of 20-HETE (1). TEA is a blocker of large-conductance Ca2+-activated K+ channels.

Data analysis. Data are expressed as means ± SE. Concentration-response data derived from each vessel were fitted separately to a logistic function by nonlinear regression. Maximum asymptote of the curve and the concentration of agonist producing EC50 were calculated by using commercially available software (Pyrism 2.01, GraphPAD software, San Diego, CA). Concentration-response data were analyzed by a two-way analysis of variance followed by Duncan’s multiple-range test. Other data were analyzed by Student’s t-test for paired or unpaired observations as appropriate. The null hypothesis was rejected at P < 0.05.

RESULTS

Figure 1 displays data on CYP4A protein expression and 20-HETE synthesis by isolated rat renal interlobar arteries incubated for 18 h with either the expression plasmid containing CYP4A1 cDNA (pcDNA3.1–4A1) or the control expression plasmid (pcDNA3.1). Western blot followed by densitometry analysis demonstrated that the levels of CYP4A-immunoreactive proteins in pcDNA3.1–4A1-treated arteries was increased by 263 ± 25% (mean ± SE, n = 3) compared with arteries incubated with the control plasmid (Fig. 1A). Furthermore, as seen in Fig. 1B, the rate of 20-HETE synthesis in arteries incubated with pcDNA3.1–4A1 was two
times higher than that in arteries incubated with the control plasmid.

Figure 2 contrasts renal interlobar arteries treated with pcDNA3.1–4A1 or with the control plasmid pcDNA3.1 in terms of constrictor responsiveness to phenylephrine and KCl. In both treatment groups, these agonists elicited concentration-dependent increases in isometric tension. The concentration-response curve to phenylephrine in vessels treated with pcDNA3.1–4A1 paralleled that in vessels treated with pcDNA3.1 but was clearly shifted to the left, resulting in a pronounced reduction of the EC50 value without alteration of the maximal response (Fig. 2A). In contrast, the concentration-response curve to KCl was nearly identical in vessels treated with pcDNA3.1–4A1 or with pcDNA3.1 (Fig. 2B). Thus transfection of renal interlobar arteries with CYP4A1 cDNA increased the sensitivity of the vessels to phenylephrine but not to KCl.

Additional experiments in renal interlobar arteries transfected with the control plasmid pcDNA3.1 revealed that the EC50 for phenylephrine (0.33 ± 0.03 μM; n = 6) was decreased (P < 0.05) by treatment with 10 μM 20-HETE (0.08 ± 0.02 μM; n = 6), 1 mM TEA (0.09 ± 0.02 μM; n = 6), or both 20-HETE and TEA combined (0.11 ± 0.03 μM; n = 6) without accompanying changes in maximal response (4.32 ± 0.10 mN/mm for control vessels vs. 4.14 ± 0.09, 4.31 ± 0.09, and 3.78 ± 0.33 mN/mm, respectively, for vessels treated with 20-HETE, TEA, or 20-HETE and TEA combined). On the other hand, the EC50 for phenylephrine in vessels transfected with pcDNA3.1–4A1 (0.10 ± 0.01 μM; n = 6) was not reduced further by exposure to 10 μM 20-HETE (0.11 ± 0.01 μM; n = 6), 1 mM TEA (0.09 ± 0.01 μM; n = 6), or 20-HETE and TEA combined (0.08 ± 0.02 μM; n = 6); neither was the maximal response of these vessels to phenylephrine affected by the treatments (4.14 ± 0.14 mN/mm for control vessels vs. 3.98 ± 0.11, 4.10 ± 0.16 and 3.85 ± 0.26 mN/mm, respectively, for vessels exposed to 20-HETE, TEA, or 20-HETE and TEA combined).

In complimentary experiments, neither 10 μM 20-HETE nor 1 mM TEA affected the EC50 for KCl in renal interlobar arteries (n = 6) transfected with the control plasmid pcDNA3.1 (13.20 ± 1.27 mM in control vessels vs. 13.74 ± 1.29 and 15.59 ± 1.97 mM, respectively, in vessels exposed to 20-HETE and TEA); also, the maximal response to KCl was unaffected by the treatment (3.11 ± 0.22 mN/mm in control vessels vs. 2.88 ± 0.33 and 3.19 ± 0.46 mN/mm, respectively, in vessels exposed to 20-HETE and TEA). Similarly, 10 μM 20-HETE and 1 mM TEA were without effect on the EC50 for KCl in renal interlobar arteries (n = 6) transfected with pcDNA3.1–4A1 (14.19 ± 2.28 mM in control vessels vs. 14.62 ± 2.5 and 13.81 ± 2.19 mM, respectively, in vessels exposed to 20-HETE and TEA); also, the maximal response to KCl was unaltered by the treatments (3.23 ± 0.25 mN/mm in control vessels vs. 2.90 ± 0.31 and 3.17 ± 0.39 mN/mm, respectively, in vessels exposed to 20-HETE and TEA).

As shown in Fig. 3, the increased sensitivity to phenylephrine in renal interlobar arteries transfected with CYP4A1 cDNA was greatly attenuated by agents that inhibit the synthesis or action of 20-HETE. DDMS, a selective inhibitor of CYP4A-catalyzed arachidonic acid ω-hydroxylation (25), caused a rightward shift in the concentration-response curve to phenylephrine in pcDNA3.1–4A1-treated vessels, increasing the EC50 by ~10-fold without affecting the maximal response (Fig. 3A). 20-HEDE, a putative antagonist of 20-HETE (1), also caused a rightward shift in the concentration-response curve to phenylephrine in vessels treated with pcDNA3.1–4A1, increasing the EC50 by about eightfold without affecting the maximal response (Fig. 3A). On the other hand, in interlobar arteries treated with the control plasmid pcDNA3.1, DDMS had no effect, whereas 20-HEDE slightly increased the EC50 (Fig. 3B). Thus DDMS and 20-HEDE are effective in attenuating the sensitivity to phenylephrine in vessels treated with pcDNA3.1–4A1, which express higher levels of CYP4A1 protein and manufacture greater amounts of 20-HETE. In complimentary experiments,
DDMS was without effect on the EC\textsubscript{50} for KCl in renal interlobar arteries transfected with pcDNA3.1 (13.20 ± 1.27 vs. 12.90 ± 1.40 mM; n = 6) or pcDNA3.1–4A1 (14.19 ± 2.28 vs. 14.32 ± 1.93 mM; n = 6); DDMS also was without effect on the maximal response to KCl (3.11 ± 0.22 vs. 3.04 ± 0.26 mN/mm in vessels transfected with pcDNA3.1 and 3.23 ± 0.25 vs. 3.29 ± 0.38 mN/mm in vessels transfected with pcDNA3.1–4A1).

If inhibition of 20-HETE synthesis is responsible for the desensitizing effect of DDMS on phenylephrine-induced contraction of vessels transfected with CYP4A1 cDNA, the inclusion of exogenous 20-HETE into the bathing buffer may be expected to offset the DDMS-induced attenuation of vascular reactivity in such vessels. Figure 4 illustrates the results of experiments examining the effect of 20-HETE on constrictor responsiveness to phenylephrine in CYP4A1-transfected interlobar arteries bathed in buffer containing and not containing DDMS. In vessels in which treatment with DDMS had produced a rightward shift in the concentration-response curve to phenylephrine, exogenous 20-HETE negated the DDMS-induced attenuation of vascular reactivity, partially at 1 \textmu M (Fig. 4A) and completely at 10 \textmu M (Fig. 4B). As noted above, 20-HETE did not affect any aspect of the concentration-response curve to phenylephrine in CYP4A1-transfected vessels not exposed to DDMS (Fig. 4, A and B).

**DISCUSSION**

Successful transfer of genes into cells and tissues by means of viral and nonviral vectors is well documented. In the present study, we used a nonviral approach to achieve overexpression of CYP4A1, an arachidonic acid \(\omega\)-hydroxylase, which catalyzes the synthesis of 20-HETE with an efficiency 10 and 40 times greater than that of CYP4A2 and CYP4A3, respectively (20). Rat renal interlobar arteries cultured

**Fig. 4.** Effect of 20-HETE on constrictor responses to phenylephrine in CYP4A1-transfected renal interlobar arteries pretreated and not pretreated with DDMS. Isolated renal interlobar arteries preincubated with the pcDNA3.1–4A1 for 18 h were mounted on a wire myograph. Changes in isometric tension produced by increasing concentrations of phenylephrine in preparations with and without DDMS (30 \textmu M) pretreatment were measured in the absence and presence of 1 \textmu M 20-HETE (Fig. 4A) and 10 \textmu M 20-HETE (Fig. 4B). Values are means ± SE.

*\(P < 0.05\), from control untreated CYP4A1-transfected arteries.
for 18 h in media supplemented with an expression plasmid containing CYP4A1 cDNA under the control of the CMV promoter displayed greater CYP4A protein levels and a higher rate of 20-HETE synthesis than vessels cultured in media supplemented with an expression plasmid not containing CYP4A1 cDNA. That CYP4A protein expression and 20-HETE synthesis are increased in vessels treated with CYP4A1 cDNA-containing plasmid is indicative of successful transfection leading to enhanced vascular expression of functional CYP4A1.

Renal interlobar arteries transfected with CYP4A1 cDNA differed strikingly from nontransfected vessels in terms of contractile responsiveness to phenylephrine. In the CYP4A1-transfected vessels, the concentration-response curve to phenylephrine was shifted to the left so that the EC50 value was reduced, whereas the maximal response was unchanged. These findings imply that the vascular sensitivity to phenylephrine is increased in CYP4A1-transfected vessels. This increase in sensitivity is not generalized, because the contractile responsiveness to KCl was not affected in vessels treated with CYP4A1 cDNA.

According to our studies, both the CYP4A inhibitor DDMS and the 20-HETE antagonist 20-HEDE were effective in offsetting the enhanced sensitivity to phenylephrine displayed by CYP4A1-transfected renal interlobar arteries. These observations are compelling evidence that the augmented production of 20-HETE brought about by increased expression of CYP4A1 is responsible for sensitizing these vessels to phenylephrine. Such a regulatory action of endogenous 20-HETE is consistent with the finding that exogenous 20-HETE elicits concentration-dependent sensitization to phenylephrine in CYP4A1-transfected vessels pretreated with DDMS to inhibit endogenous 20-HETE synthesis.

Sensitization to phenylephrine by exogenous 20-HETE was not demonstrable in CYP4A1-transfected vessels not pretreated with DDMS. Conceivably, under such circumstances, the vascular production of 20-HETE is already sufficiently high to achieve maximal sensitization to the agonist. In vessels pretreated with an empty plasmid not containing CYP4A1 cDNA, the sensitivity to phenylephrine was not significantly reduced by DDMS and was minimally attenuated by 20-HEDE. One likely interpretation of these observations is that the basal production of 20-HETE under such circumstances is below that required for promoting sensitization to phenylephrine. Collectively, our findings support the concept that 20-HETE of vascular origin is a stimulatory regulator of vascular reactivity to phenylephrine.

A relatively high concentration of 20-HETE, 10 \( \mu \text{mol/l} \), is required to bring up the sensitivity to phenylephrine displayed by control vessels not overexpressing CYP4A1, or by CYP4A1-transfected vessels treated with DDMS, to the level of sensitivity in interlobar arteries overexpressing CYP4A1. This may be indicative of differences in metabolic disposition and accessibility to target sites between exogenous and endogenous 20-HETE. For example, the access of exogenous 20-HETE to potential sites of action, that is, cellular binding sites or receptors (1), protein kinase C (15, 21, 24), and mitogen-activated protein kinase(s) (24, 19), may be limited by further metabolism (9, 16), esterification into cellular lipids (3), or interaction with extracellular structures.

Previous studies have demonstrated that the activity of large-conductance Ca2+-activated K+ channels in renal preglomerular vascular smooth muscle cells is decreased by exogenous 20-HETE and increased by CYP4A inhibitors (28). Similar effects were observed in the cerebral microcirculation (7, 15). On the basis of such findings, it has been proposed that 20-HETE of vascular origin is an inhibitory regulator of the Ca2+-activated K+ channels in vascular smooth muscle. Inhibition of these channels is expected to favor depolarization and contractile responsiveness of vascular smooth muscle. Indeed, in our study, TEA pretreatment increased the sensitivity to phenylephrine in renal interlobar arteries not overexpressing CYP4A1. Thus the sensitizing action of 20-HETE on phenylephrine-induced vascular contraction may be a consequence of the inhibitory action of this eicosanoid on the activity of the large-conductance Ca2+-activated K+ channels. It is conceivable that 20-HETE production in vessels transfected with pcDNA3.1–4A1 is sufficiently increased to maximally suppress the activity of such channels. This would be consistent with our finding that neither exogenous 20-HETE nor TEA, alone or in combination, was effective in sensitizing vessels overexpressing CYP4A1 to phenylephrine.

In summary, this study documents overexpression of CYP4A and increased 20-HETE synthesis in rat renal interlobar arteries maintained in culture for 18 h in media that includes an expression plasmid containing CYP4A1 cDNA. The sensitivity of interlobar arteries treated with CYP4A1 cDNA expression plasmid to phenylephrine greatly exceeded that of vessels treated with a plasmid not containing CYP4A1 cDNA. An inhibitor of CYP4A, DDMS, as well as an antagonist of 20-HETE actions, 20-HEDE, were effective in offsetting the increased sensitivity to phenylephrine brought about by CYP4A overexpression. These observations suggest that increased synthesis of 20-HETE in vessels transfected with CYP4A1 cDNA is responsible for their sensitization to phenylephrine. The study calls attention to the possibility that 20-HETE produced by arterial vessels contributes to vasoconstrictor mechanisms by sensitizing the vasculature to the action of constrictor agonists in situations wherein there is increased vascular CYP expression and/or activity. Relative to this point, a recent study documented a role for 20-HETE in the increased sensitivity of small mesenteric arteries of spontaneously hypertensive rats to phenylephrine and vasopressin (27).

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