Transfection of CYP4A1 cDNA decreases diameter and increases responsiveness of gracilis muscle arterioles to constrictor stimuli

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IN THE RAT, 20-hydroxyeicosatetraenoic acid (20-HETE) is a product of arachidonic acid metabolism by cytochrome P450 enzymes of the 4A family (CYP4A) (18). Small arterial vessels express CYP4A isoenzymes and manufacture 20-HETE (10, 15, 19). Participation of this eicosanoid in vasomotor control is subserved vasoconstrictor mechanisms. Recently, we reported that transfection of small renal arterial vessels with an expression plasmid containing the cDNA of CYP4A1, an arachidonic acid ω-hydroxylase that catalyzes the synthesis of 20-HETE with greater efficiency than other CYP4A isofoms (16), increases the vascular expression of CYP4A protein along with the production of 20-HETE (14). In the present study, we used this experimental approach to investigate the vasomotor consequences of increased CYP4A1 expression in rat gracilis muscle arterioles. We compared isolated rat gracilis muscle arterioles transfected with an expression plasmid with and without CYP4A1 cDNA in terms of their vasomotor responses to agents that interfere with the synthesis or actions of 20-HETE.

MATERIALS AND METHODS

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (250–275 g, Charles River, Wilmington, DE) were anesthetized with pentobarbital sodium (60 mg/kg ip). The gracilis anticus muscle was excised and placed on a dish filled with ice-cold Krebs buffer composed of (in mmol/l) 118.5 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 25.0 NaHCO3, and 11.1 dextrose, and first-order gracilis muscle arterioles were immediately dissected for use in various protocols.

Transfection of gracilis muscle arterioles. Full-length CYP4A1 cDNA (16) was cloned into the expression vector pcDNA3.1 (+) (Invitrogen; Carlsbad, CA). 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). First-order gracilis muscle arterioles were placed on culture dishes (35 mm) filled with tissue culture medium (Dulbecco’s modified Eagle’s medium with 10% Nu-serum, and 100 μg/ml streptomycin, and 100 μg/ml penicillin) containing 20 μg/ml of liposome-encapsulated 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, the vessels were used for assessment of CYP4A protein expression and for examination of vasomotor function.

Assessment of CYP4A protein. Samples consisting of six to eight gracilis muscle arterioles, maintained in organ culture as described above, were homogenized, the homogenate was centrifuged, and the 10,000-g supernatant (25 μg of protein) was analyzed for CYP4A protein by immunoblotting with the use of a goat anti-rat CYP4A1 polyclonal antibody (Gentest; Woburn, MA) as previously described (14). Immunoreactive proteins were detected by using the ECL Plus detection system (Amersham; Arlington Heights, IL).

Immunohistochemistry. Gracilis muscle arterioles were fixed in 4% formaldehyde in phosphate-buffered saline (PBS). After being washed with PBS, the vessels were dehydrated in ethanol, embedded in Tissue-Tek OCT (Sakura; Torrance, CA), and cut into 5-μm sections, which were placed onto polylysine-coated slides and incubated for 2 h with blocking solution containing 3% nonimmune goat IgG and 0.25% Triton X-100. The slides were then washed with PBS, incubated with goat anti-rat CYP4A1 polyclonal antibody (1:1,000 dilution; Gentest, Woburn, MA), which cross-reacts with other members of the CYP4A family, followed by further incubation with rabbit anti-goat IgG (1:1,000 dilution) labeled with Alexa Fluor 594 (Molecular Probes; Eugene, OR). Immunostaining was visualized by confocal microscopy.

Evaluation of vasomotor function. Segments (1–2 mm length) of first-order gracilis muscle arterioles, freshly isolated or maintained in organ culture as described above, were mounted between two micropipettes in the chamber (1 ml) of a pressure-myograph (Living Organ Culture in media supplemented with an expression plasmid containing pcDNA3.1–4A1 or the control plasmid pcDNA3.1. The expression of CYP4A protein(s) in vessels treated with pcDNA3.1–4A1 greatly exceeds that in vessels treated with the control plasmid pcDNA3.1. Figure 2 displays sections of gracilis muscle arterioles transfected with pcDNA3.1 (Fig. 2A) or pcDNA3.1–4A1 (Fig. 2B) displayed an immunofluorescent signal indicative of the expression of CYP4A protein. In arterioles transfected with the control

RESULTS

Figure 1 illustrates the results of Western blot analysis of proteins in isolated rat gracilis muscle arterioles maintained in organ culture in media supplemented with an expression plasmid containing pcDNA3.1–4A1 or the control plasmid pcDNA3.1. The expression of CYP4A protein(s) in vessels treated with pcDNA3.1–4A1 greatly exceeds that in vessels treated with the control plasmid pcDNA3.1. Figure 2 shows sections of gracilis muscle arterioles transfected with pcDNA3.1–4A1.
plasmid, the immunofluorescent signal was particularly intense in the intima, whereas in arterioles transfected with pcDNA3.1–4A1, the signal was intense throughout the vessel wall. Little or no immunofluorescence was detected in the control, the sections of arterioles transfected with pcDNA3.1 (Fig. 2C), or pcDNA3.1–4A1 (Fig. 2D) in which incubation with CYP4A antibody was omitted from the immunostaining protocol.

Figure 3 contrasts data on the internal diameter of pressurized (80 mmHg) gracilis muscle arterioles transfected with pcDNA3.1 or pcDNA3.1–4A1 before and during treatment with DDMS (30 μmol/l) or 20-HEDE (1 μmol/l). Before drug treatment, the internal diameter of vessels transfected with pcDNA3.1–4A1 was greatly exceeded (P < 0.05) by that of control vessels transfected with pcDNA3.1. Exposure of these vessels to DDMS or 20-HEDE elicited a dilatory response, which developed maximally over 5 min. DDMS, a CYP4A inhibitor, brought about a slight increase (P < 0.05) of the diameter in arterioles transfected with pcDNA3.1 (6.0 ± 1.4 μm) and a much larger increase (P < 0.05) in arterioles transfected with pcDNA3.1–4A1 (27.7 ± 4.2 μm). Similarly, 20-HEDE, a putative antagonist of 20-HETE, affected marginally the internal diameter of pcDNA3.1-transfected vessels (5.5 ± 3.2 μm) but elicited a robust increase (P < 0.05) of diameter in vessels transfected with pcDNA3.1–4A1 (36.0 ± 3.8 μm). Consequently, much of the difference in the internal diameter of pcDNA3.1- and pcDNA3.1–4A1-transfected vessels was erased after treatment with DDMS or 20-HEDE. A complementary study conducted in pressurized arterioles superfused with calcium-free Krebs buffer revealed that the internal diameter of vessels transfected with pcDNA3.1 (194.5 ± 6.1 μm; n = 4) does not differ significantly from that of vessels transfected with pcDNA3.1–4A1 (191.3 ± 4.9 μm; n = 4). Hence, the difference in the internal diameter of pcDNA3.1- and pcDNA3.1–4A1-transfected arterioles during superfusion with calcium-containing buffer is indicative of differences in the calcium-dependent constrictor tone of the vessels.

Figure 4 compares the effect of stepwise increments of intraluminal pressure on the absolute and the normalized internal diameter of pcDNA3.1- and pcDNA3.1–4A1-transfected gracilis muscle arterioles. Elevations of pressure over the range of 0–40 mmHg did not affect the normalized diameter but induced comparable increases (P < 0.05) of absolute diameter in both groups of vessels. Further increase of intraluminal pressure to a maximum of 100 mmHg effected reduction (P < 0.05) of absolute and normalized diameter in both groups. Importantly, pressure-induced reductions of absolute and normalized diameters in arterioles transfected with pcDNA3.1–4A1 exceeded (P < 0.05) the reductions of diameter obtained at corresponding levels of pressure in arterioles transfected with the control plasmid.

Figure 5 displays data on the pressure-diameter relationship in pcDNA3.1- and pcDNA3.1–4A1-transfected arterioles superfused with buffer containing and not containing DDMS (30

Fig. 2. Immunostaining of CYP4A protein in sections of gracilis muscle arterioles transfected with pcDNA3.1 (A and C) or pcDNA3.1–4A1 (B and D). CYP4A antibody was omitted from the immunostaining protocol in sections shown in C and D. Magnification is ×40 in all panels.
The pressure-induced reduction of internal diameter over the range of 80–100 mmHg was attenuated (P < 0.05) by DDMS, more so in arterioles transfected with pcDNA3.1–4A1 than those with the control plasmid. 20-HEDE also attenuated (P < 0.05) pressure-induced reductions of internal diameter in pcDNA3.1–4A1-transfected vessels but was without effect in vessels transfected with pcDNA3.1. As depicted in Fig. 6, in gracilis muscle arterioles transfected with pcDNA3.1–4A1 and superfused with buffer containing DDMS, the reduction of internal diameter effected by elevations of intraluminal pressure over the range of 60–100 mmHg was intensified (P < 0.05) by the inclusion of 20-HETE (1 μmol/l) in the superfusion buffer. Moreover, at both 80 and 100 mmHg, the internal diameter of arterioles exposed to 10 μmol/l 20-HETE was decreased (P < 0.05) relative to that of arterioles exposed to 1 μmol/l 20-HETE.

Figure 7 displays data on the pressure-diameter relationship in freshly isolated gracilis muscle arterioles superfused with buffer containing and not containing DDMS (30 μmol/l) or 20-HEDE (1 μmol/l). Both DDMS and 20-HEDE attenuated (P < 0.05) the reduction of internal diameter effected by pressure increments over the range of 40–100 mmHg. Pressure-induced vasoconstriction of arterioles exposed to DDMS was intensified (P < 0.05) by inclusion of 20-HETE into the superfusion buffer, which negates expression of the effect prompted by DDMS alone. On the other hand, 20-HETE did not intensify pressure-induced constriction of arterioles exposed to 20-HEDE.

Figure 8 displays data on constrictor responsiveness to phenylephrine in gracilis muscle arterioles transfected with pcDNA3.1 or pcDNA3.1–4A1, pressurized to 40 mmHg, and superfused with Krebs buffer containing and not containing DDMS (30 μmol/l) or DDMS plus 20-HETE (1 μmol/l). During superfusion with Krebs buffer alone, the concentration-response curve to phenylephrine in arterioles transfected with pcDNA3.1–4A1 was shifted to the left of that obtained in pcDNA3.1-transfected vessels. The resulting reduction (P < 0.05) of the phenylephrine EC50 value (from 0.58 ± 0.16 to 0.13 ± 0.01 μmol/l in pcDNA3.1- and pcDNA3.1–4A1-transfected vessels, respectively) is indicative of enhanced sensitivity to the agonist in arterioles transfected with pcDNA3.1–4A1. Addition of DDMS to the superfusion buffer decreased constrictor responsiveness to phenylephrine, more so in vessels transfected with pcDNA3.1–4A1 than with pcDNA3.1. Under such conditions inclusion of 20-HETE in the superfusion buffer intensified (P < 0.05) the constrictor responsiveness to phenylephrine.

**DISCUSSION**

CYP4A1 is a fatty acid ω-hydroxylase that catalyzes the synthesis of 20-HETE from arachidonic acid with greater efficiency than other monooxygenases of the CYP4A family (16). We found that the expression of CYP4A protein is greatly...
increased in gracilis muscle arterioles cultured for 18 h in media supplemented with an expression plasmid containing CYP4A1 cDNA. This observation is in keeping with a previous report that rat renal interlobar arteries treated with pcDNA3.1–4A1 plasmid overexpress CYP4A protein (14).

The major finding of our study is that gracilis muscle arterioles transfected with CYP4A1 cDNA differ functionally from nontransfected vessels. That the internal diameter of pcDNA3.1–4A1-treated arterioles, pressurized to 80 mmHg, is about 70% of the internal diameter of pcDNA3.1-treated arterioles at the same pressure level implies that CYP4A1 expression is conducive to vasoconstriction. That the CYP4A inhibitor DDMS elicits robust dilation of pressurized arterioles transfected with pcDNA3.1–4A1 suggests prominent contribution of a product of fatty acid metabolism by CYP4A1 to the implementation of constrictor tone in these vessels. It is plausible that the product in question is arachidonic acid-derived 20-HETE, because 20-HEDE (1), a putative antagonist of 20-HETE, also was effective in eliciting robust dilation of pressurized gracilis muscle arterioles transfected with pcDNA3.1–4A1. However, CYP4A1 also was reported to catalyze hydroxylation of fatty acids other than arachidonic acid (viz., palmitic, myristic, and oleic acids) (3, 12), raising the possibility that one or more of the resulting products contributes to the constrictor tone displayed by gracilis muscle arterioles overexpressing CYP4A1. Involvement of CYP4A1-derived products in the implementation of constrictor tone in control arterioles transfected with pcDNA3.1 appears to be minimal because little or no dilatory response ensues when such vessels are exposed to DDMS or 20-HEDE. According to our study, CYP4A1 protein expression is diffusely distributed throughout the wall of arterioles treated with pcDNA3.1–4A1, whereas it is somewhat circumscribed to the intima of arterioles treated with pcDNA3.1. It is then conceivable that differences in the intensity of the constrictor tone displayed by these vessels reflect not only differences in the quantity of

Fig. 5. Effect of DDMS (30 μmol/l) and 20-HEDE (1 μmol/l) on the intraluminal pressure-ID relationship in first-order gracilis muscle arterioles transfected with pcDNA3.1–4A1 plasmids. Data are means ± SE. *P < 0.05 relative to corresponding values in control vessels. Numbers in parentheses indicate the number of rats.

Fig. 6. Effect of 20-HETE on the intraluminal pressure-ID relationship in first-order gracilis muscle arterioles transfected with pcDNA3.1–4A1 and pretreated with DDMS (30 μmol/l). Data are means ± SE. *P < 0.05 relative to corresponding control values. Number in parentheses indicate the number of rats.
CYP4A products generated but also in the site of generation, which, in turn, may impact on the number of smooth muscle cells responding to such products.

It is well documented that pressurization of gracilis muscle arterioles promotes development of myogenic constrictor tone (21). 20-HETE of vascular origin is believed to upregulate the expression of myogenic behavior in gracilis muscle arterioles (6) and in small resistance arteries of the mesenteric circulation (20), the kidney (13), and the brain (8). In agreement with this notion, we found that treatment with DDMS or 20-HEDE, which respectively interferes with the synthesis and action of 20-HETE, attenuates the reduction of internal diameter caused by increments of intraluminal pressure in freshly isolated gracilis muscle arterioles. Hence, it is conceivable that the increased vasoconstriction displayed by pressurized gracilis arterioles treated with pcDNA3.1–4A1 is due to magnification of myogenic tone by a CYP4A1-derived product, including 20-HETE. This concept is in keeping with observations that pressure-induced reduction of internal diameter over the pressure range of 40–100 mmHg is significantly enhanced in gracilis muscle arterioles treated with pcDNA3.1–4A1, and that inclusion of DDMS or 20-HEDE into the superfusion buffer brings down the expression of pressure-induced vasoconstriction in such vessels to the level observed in preparations treated with pcDNA3.1. Also supportive of this concept is the finding that exogenous 20-HETE offsets the inhibitory effect of the 20-HETE synthesis inhibitor DDMS on pressure-induced constriction of arterioles overexpressing CYP4A-1.

In agreement with the results of a previous study in rat renal interlobar arteries (14), we found the gracilis muscle arterioles transfected with pcDNA3.1–4A1 are more sensitive to phenylephrine than arterioles transfected with the control plasmid pcDNA3.1. The sensitizing influence of treatment with pcDNA3.1–4A1 is attributable to increased vascular synthesis of a CYP4A1 product, because when the experiments are conducted in the presence of DDMS, pcDNA3.1–4A1-treated

**Fig. 7.** Effect of stepwise increments of intraluminal pressure on the absolute and the normalized ID of freshly isolated first-order gracilis muscle arterioles. Vascular preparation were superfused with control Krebs buffer only, buffer containing DDMS (30 μmol/l) or 20-HEDE (1 μmol/l), or buffer containing 20-HETE (1 μmol/l) along with DDMS (30 μmol/l) or 20-HEDE (1 μmol/l). Data are means ± SE. *P < 0.05 relative to corresponding data in vessels superfused with Krebs buffer only. Number in parentheses indicate the number of rats.

**Fig. 8.** Concentration-dependent constrictor responses to phenylephrine in gracilis muscle arterioles transfected with pcDNA3.1 (A) or pcDNA3.1–4A1 (B) and superfused with control Krebs buffer only, buffer containing DDMS (30 μmol/l), or buffer containing both DDMS (30 μmol/l) and 20-HETE (1 μmol/l). Data are means ± SE. *P < 0.05 relative to corresponding data in vessels superfused with Krebs buffer only. Number in parentheses indicate the number of rats.
arterioles are no longer more sensitive to phenylephrine than arterioles treated with the control plasmid. Hence, enhancement of constrictor responsiveness in gracilis muscle arterioles overexpressing CYP4A1 is not limited to myogenic stimuli but extends to phenylephrine as well.

The present study adds to a large body of evidence that a CYP4A-derived product(s) of vascular origin serves as a facilitatory modulator of microvascular reactivity to diverse constrictor stimuli, including constrictor agonists (22), increased oxygen tension (9–11, 15, 17, 19, 22, 23), and increments of transmural pressure (6, 8, 14). CYP4A-derived 20-HETE is regarded as a prime candidate for playing such a role as production of 20-HETE by small arterial vessels is increased in experimental settings that bring about vasoconstriction, e.g., elevation of transmural pressure (8), augmentation of oxygen tension (11), exposure to angiotensin II (4). Moreover, the known abilities of 20-HETE to inhibit Ca2+-activated K+ channels in vascular smooth muscle (18, 23), to enhance the conductance of Ca2+-activated K+ channels (8, 9, 18), and to activate Rho kinase (17) are all expected to promote amplification of vasoconstrictor responsiveness. It is not known whether 20-HETE shares with CYP4A products arising from hydroxylation of fatty acids other than arachidonic acid the ability to promote vasoconstrictor responsiveness. If it does, such products may be considered along with 20-HETE potential contributors to the functional manifestations of vascular CYP4A product generation.

Studies by other investigators have linked vascular CYP4A product generation to physiological mechanisms of microcirculatory adjustment in the face of elevation of perfusion pressure (5–8) and oxygen tension (11). In addition, augmentation of vasoconstrictor responsiveness in spontaneously hypertensive rats (22), Dahl salt-sensitive hypertensive rats (7), and rats with reduced renal mass hypertension (5) were shown to rely on a mechanism involving CYP4A oxygenases, which is expressed more prominently in hypertensive than in normotensive rats (7, 22).

In summary, this study demonstrates overexpression of CYP4A protein in rat gracilis muscle arterioles maintained in culture for 18 h in media that includes an expression plasmid containing CYP4A1 cDNA. The internal diameter of pressurized arterioles transfected with pcDNA3.1–4A1 was greatly exceeded by that of arterioles transfected with the control plasmid. The constrictor influence of CYP4A overexpression is attributable to facilitation of myogenic vasoconstriction by a CYP4A-derived product.

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GRANTS

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