Role of cytochrome P-450 4A in oxygen sensing and NO production in rat cremaster resistance arteries

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Kerkhof, Cornel J. M., Erik N. T. P. Bakker, and Pieter Sipkema. Role of cytochrome P-450 4A in oxygen sensing and NO production in rat cremaster resistance arteries. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1546–H1552, 1999.—The role of arachidonic acid metabolism and nitric oxide (NO) in hypoxia-induced changes of vascular tone was investigated in first-order cannulated rat cremaster muscle resistance arteries. Spontaneous tone reduced arterial diameter from 179 ± 2 µm (fully dilated) to 98 ± 3 µm under normoxia (Po2 = 150 mmHg). Hypoxia (Po2 = 5–10 mmHg) had no significant effect on arterial diameter under conditions of spontaneous tone. The effect of hypoxia was not changed after blockade of cyclooxygenase with indomethacin or after blockade of lipoxygenase with nordihydroguaiaretic acid. However, after partial blockade of cytochrome P-450 4A enzymes with 17-octadecynoic acid (17-ODYA), hypoxia increased the diameter by 65 ± 6 µm (P < 0.05). This increase could be inhibited by Nω-nitro-L-arginine (L-NNA) or 20-hydroxyeicosatetraenoic acid (20-HETE). 17-ODYA induced a concentration-dependent dilation under normoxia, which could be blocked by endothelium removal or L-NNA. 17-ODYA did not increase smooth muscle sensitivity to NO. We conclude that, under conditions of spontaneous tone and in the absence of luminal flow, hypoxia (5–10 mmHg) has no effect on the diameter of resistance arteries from the rat cremaster muscle. Inhibition of the cytochrome P-450 4A pathway of arachidonic acid metabolism under normoxia induces NO production by the endothelium. Hypoxia induces an NO-mediated dilation when cytochrome P-450 4A enzymes are partially inhibited.

The contribution of arachidonic acid metabolites in the regulation of vascular diameter in skeletal muscle arteries and resistance arteries during changes in oxygen tension has received much attention. Many cells contain phospholipases that enable them to mobilize arachidonic acid from the sn-2 position of glycerophospholipids (9). Three enzymatic pathways, mediated by cyclooxygenase, lipoxygenase, or cytochrome P-450, then oxygenate free arachidonic acid.

Messina et al. (24) showed that isolated cremaster muscle resistance arteries from the rat are intrinsically sensitive to oxygen and that responses are mediated by cyclooxygenase products from arachidonic acid. During hypoxia, dilator prostaglandin production from the endothelium is stimulated and resistance arteries dilate, whereas an increase in the oxygen tension inhibits prostaglandin production, leading to arterial constriction (25). Also, Fredericks et al. (8) reported that hypoxia induced an endothelium-dependent dilation, which was absent after cyclooxygenase inhibition in isolated skeletal muscle resistance arteries supplying the gracilis muscle. On the other hand, evidence against a role of prostaglandins in mediating oxygen sensitivity in hamster and rat cremaster muscle preparations was presented by Jackson (15) using intravital microscopy. He showed that the cyclooxygenase inhibitors indomethacin and medrofenamate had no effect on the reduction in arterial diameter after an increase in oxygen tension. Furthermore, Pries et al. (31) showed in the rat spinotrapezius muscle, using intravital microscopy, that reductions in arterial diameter following an increase in oxygen tension are not mediated by prostaglandins. Thus, although regional differences may explain some of the contradictory findings, conflicting evidence is reported on the role of cyclooxygenase products in oxygen sensing in cremaster muscle.

In a recent study Harder et al. (13) identified cytochrome P-450 enzymes of the 4A family as a putative microvascular oxygen sensor in the renal and cremaster vascular bed of rats. They showed that the synthesis of 20-hydroxyeicosatetraenoic acid (20-HETE), a potent constrictor and major product of cytochrome P-450 4A enzymes, decreased as the oxygen concentration was lowered, resulting in arteriolar dilation. However, whether the cytochrome P-450 4A enzymes responsible for the oxygen sensitivity are localized in the smooth muscle cells, the parenchymal cells, or the endothelium was not investigated. The cytochrome P-450 pathway is important, because these enzymes are profoundly affected by various infectious and inflammatory stimuli (27). Cytokines, such as interferon-γ, interleukin-1β, and tumor necrosis factor-α, can inactive cytochrome P-450 in a nitric oxide-independent manner (14). Furthermore, endotoxin decreased cytochrome P-450 metabolism via stimulation of NO production (18) and the subsequent binding of NO to the heme moiety of cytochrome P-450 (26). Besides the contribution of arachidonic acid metabolism in vascular responses to alterations in oxygen tension, Pries et al. (31) provided evidence for a role of NO in diameter changes induced...
by alterations in oxygen tension in rat spinotrapezius muscle. They showed that constriction of arterioles in response to elevations in PO2 was inhibited by N\textsuperscript{G}-nitro-L-arginine (L-NNA). Several other studies also showed that NO plays an important role in the vascular sensitivity to hypoxia. However, both an increase (4, 10, 30, 32) and a decrease (28, 38) in NO production have been reported.

Thus several studies indicate a role for metabolites of arachidonic acid and NO in oxygen sensitivity, but their quantitative role and direction of action are equivocal. Furthermore, the interpretation of studies on the role of arachidonic acid metabolites and NO in responses to a variety of stimuli are hampered by the interaction between the NO and arachidonic acid pathways. Recently, we have shown that the production of endothelial cyclooxygenase products from arachidonic acid is increased in the presence of NO (1). Therefore, we studied the three pathways of arachidonic acid metabolism in combination with NO to find out which pathway is involved in the oxygen sensitivity in isolated resistance arteries from the cremaster muscle. This was done by using blockers of cyclooxygenase, lipoxygenase or cytochrome P-450 or using L-NNA. Both the contribution of the oxygen-sensitive arachidonic acid pathway to spontaneous tone and the possible coupling between this pathway and endothelial NO production were studied.

METHODS

Male Wistar rats, weighing 250–300 g, were anesthetized with pentobarbital sodium (Nembutal; 50 mg/kg ip). The left cremaster muscle was isolated according to the procedure described by Messina et al. (24). Briefly, the muscle was exposed by an incision in the skin, cleared of adhering fascia and connective tissue, and isolated from the surrounding tissue. A ventral incision was made over the length of the muscle to remove the testis. The muscle was excised by a transverse incision along its base as close to the origin as possible. The isolated muscle was splayed open and pinned to the silicone bottom of a dissecting dish containing cold (4°C) MOPS-buffered physiological salt solution (PSS) containing (in mM) 145 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 1 MgSO\textsubscript{4}, 1 NaH\textsubscript{2}PO\textsubscript{4}, 5 d-(+)-glucose, 2 pyruvate, 0.02 EDTA, and 3 MOPS. The advantage of using MOPS-buffered PSS is that pH is stable during the preparation period without the need to bubble the solution with gas.

A segment of the first-order resistance artery, 1–2 mm in length, was isolated from the muscle by dissection with microscissors. This segment was placed in a water-jacketed pressure myograph chamber (made in the workshop of the Laboratory of Physiology, Vrije Universiteit Amsterdam) containing a Krebs bicarbonate-buffered PSS containing (in mM) 110 NaCl, 5 KCl, 2.5 CaCl\textsubscript{2}, 1 MgSO\textsubscript{4}, 1 KH\textsubscript{2}PO\textsubscript{4}, 10 d-(+)-glucose, 24 NaHCO\textsubscript{3}, and 0.02 EDTA equilibrated with 21% O\textsubscript{2}-5% CO\textsubscript{2}-74% N\textsubscript{2}, pH 7.35–7.4, at 33°C. The chamber contained an inflow and outflow glass micropipette (tip diameter ~50 μm) and a superfusion inlet and outlet, had a content of 6 ml, and could be closed by a high-quality glass window for observation of the vessel. The proximal end of the resistance artery was mounted on the inflow pipette and the distal end on the outflow pipette using 20-μm nylon sutures. The pressure myograph chamber was placed on the stage of a microscope. The inflow pipette was connected to a tube that was connected to a pressure reservoir; the outflow pipette was temporarily left open to air. The pressure was increased to 25 cmH\textsubscript{2}O by adjusting the height of the PSS in the reservoir, and the vessels were flushed for 1–2 min to clear the lumen of blood. The outflow pipette was then closed, and the fluid level in the pressure reservoir was raised to 88 cmH\textsubscript{2}O (65 mmHg). This pressure has been reported (23) to be in the physiological range for first-order resistance arteries in the cremaster muscle of anesthetized rats. After the vessel was checked for leakage by observing the level of PSS in the pressure reservoir, the temperature was slowly raised to 33°C, which is the physiological temperature for the cremaster resistance arteries. Vessels were studied in the absence of perfusion flow. The resistance arteries were superfused with PSS at a flow rate of 6 ml/min.

In some vessels the endothelium was removed. After the resistance artery was mounted on the inflow pipette, the vessel was perfused with a bolus of air (2 ml). This was followed by a short period (1–2 min) of Krebs-buffered PSS perfusion to flush the debris from the lumen. Finally, the distal end of the resistance artery was mounted on the outflow pipette, and the same procedure was followed as with the resistance arteries with intact endothelium.

Internal diameters of first-order resistance arteries were measured continuously using a Melles Griot standard achromatic microscope objective (×10 power; NA 0.25), a charge-coupled device camera (KP-M1E/K, Hitachi Denshi), a Philips video monitor (LDH 2135/10), and a video micrometer (N560 FvD) built in our electronics department. The final magnification on the screen of the video monitor is ×300, and the accuracy of the diameter measurement was ±1 μm.

Vessels included in this study had to fulfill three criteria. First, resistance arteries that showed signs of leakage were excluded from the study. Second, the resistance arteries had to develop spontaneous tone during the 30-min equilibration period. Segments with no or poor (<30%) diameter reduction were not studied. Finally, the resistance arteries with intact endothelium had to dilate in response to ACh (0.1 μM) as an indication of endothelial integrity. Vessels without endothelium were checked for dilation in response to ACh and were discarded if they were found to dilate.

The effect of decreased oxygen tension was assessed by measuring arterial internal diameters before and after the oxygen tension of the Krebs buffer was decreased from 150 mmHg (21% O\textsubscript{2}-5% CO\textsubscript{2}-74% N\textsubscript{2}) to 5–10 mmHg (5% CO\textsubscript{2}-95% N\textsubscript{2}). The oxygen tension in the bath was measured with a fast-responding Clark-type oxygen electrode (19), which was calibrated before and after the experiment. The effect of blockade of different pathways of arachidonic acid metabolism and NO production was tested in different groups of resistance arteries. To block the cyclooxygenase pathway, we used 10 μM indomethacin. The lipoxygenase pathway was blocked by 3 μM nordihydroguaiaretic acid (NDGA). Cyclochrome P-450 4A \textsuperscript{a}-hydroxylase was blocked by 1 μM 17-octadecynoic acid (17-ODYA), 3 μM miconazole, or 5 μM SKF-525A. NO production was blocked by 100 μM L-NNA. To reverse the effect of 17-ODYA, 20-HETE (3 nM), the major reverse product of cytochrome P-450 4A, was used.

To further study the effect of inhibition of cytochrome P-450 4A on tone and the location of this effect, three concentrations (0.3, 1, and 3 μM) of 17-ODYA were applied in three different series of experiments. In the first series 17-ODYA was applied in the absence of any intervention (effect on spontaneous tone). In the second series 17-ODYA was applied after removal of the endothelium, and in the third series, in the presence of 100 μM L-NNA.
To study whether the sensitivity of the smooth muscle cells to NO was increased in the presence of 17-ODYA, three concentrations of the endothelium-independent NO donor sodium nitroprusside (SNP) were applied in the absence and presence of 1 µM 17-ODYA. In this group endogenous NO production was blocked with 100 µM L-NNA.

Drugs. ACh, 17-ODYA, indomethacin, NDGA, SKF-525A, and 20-HETE were obtained from Sigma (St. Louis, MO). L-NNA was obtained from Bachem (Bubendorf, Switzerland). SNP was purchased from Merck (Darmstadt, Germany). Miconazole was from ICN (Zoetermeer, The Netherlands). ACh, NDGA, SKF-525A, L-NNA, and SNP were dissolved in distilled water. 17-ODYA and 20-HETE were dissolved in ethanol. Miconazole was dissolved in DMSO and indomethacin in 0.2 M Na2CO3. The final concentrations of the carriers ethanol and DMSO did not exceed 0.03 and 0.01% vol/vol, respectively, and had no effect on vascular responses. All the drugs used were added to the superfusion solution and reported as final concentrations.

Statistical analysis. Data are presented as means ± SE. Differences between two means were assessed by Student’s t-test with a Bonferroni correction, if necessary. A probability value of P < 0.05 was considered significant for all tests.

RESULTS

General characteristics. The general characteristics of the resistance arteries are depicted in Fig. 1. The passive intraluminal diameter of isolated resistance arteries with intact endothelium averaged 181 ± 2 µm (n = 32) when pressurized to 65 mmHg. During the equilibration period under normoxia, these resistance arteries developed spontaneous tone, reducing the diameter to 101 ± 3 µm. These vessels dilated to 151 ± 5 µm in response to the endothelium-dependent dilator ACh (0.1 µM). The inner diameter of passive resistance arteries without endothelium averaged 166 ± 7 µm [n = 6, not significantly different (NS) vs. diameter of resistance arteries with an intact endothelium]. The diameter of these resistance arteries was reduced during the equilibration period to 79 ± 9 µm (NS vs. diameter of resistance arteries with endothelium). In vessel segments in which the endothelium was removed, the diameter did not change significantly in response to 0.1 µM ACh (79 ± 9 µm before vs. 77 ± 9 µm after 0.1 µM acetylcholine; n = 6).

Hypoxia experiments. Changing the gas mixture from 21% O2-5% CO2-74% N2 (normoxia) to 95% N2-5% CO2 (hypoxia) decreased the oxygen tension in the pressure myograph chamber from ±150 mmHg to 5–10 mmHg. Hypoxia had no significant effect on arterial diameter under conditions of spontaneous tone (Fig. 2A, n = 10, control response). However, when the cytochrome P-450 4A pathway of arachidonic acid metabolism was blocked with 17-ODYA (1 µM), hypoxia induced a diameter increase of 65 ± 6 µm (Fig. 2A, n = 6, P < 0.05 vs. control response). A typical recording of arterial diameter in response to hypoxia in the absence and presence of 17-ODYA (1 µM) is shown in Fig. 2B. Two mechanistically
distinct inhibitors of cytochrome P-450 4A enzymes, SKF-525A (5 µM) and miconazole (3 µM), had similar effects. Hypoxia induced a dilation in the presence of SKF-525A and miconazole of 41 and 50 µm, respectively (means of 2 experiments for both inhibitors). After the inhibition of cyclooxygenase with 10 µM indomethacin and lipoxygenase with 3 µM NDGA, hypoxia increased the diameter by 8 ± 5 and 14 ± 3 µm, respectively (n = 6 for both inhibitors, NS vs. control response). Thus blockade of these pathways of arachidonic acid metabolism had no significant effect on the response of the resistance arteries to hypoxia compared with the effect of hypoxia under control conditions. Hypoxia-induced dilation in the presence of 17-ODYA could be blocked with 100 µM L-NNA, an inhibitor of NO synthesis (Fig. 2A, n = 6, P < 0.05 vs. hypoxia in the presence of 17-ODYA) or prevented by endothelium removal (n = 2; results not shown). Furthermore, 20-HETE (3 nM), the major product of cytochrome P-450 4A enzyme activity, significantly reduced hypoxia-induced relaxation in the presence of 17-ODYA (Fig. 2A, n = 4, P < 0.05 vs. hypoxia in the presence of 17-ODYA). This dose of 20-HETE was chosen because it induced a constriction of 10 ± 3 µm (n = 4) and reversed the dilation of 8 ± 1 µm (n = 4) induced by 17-ODYA.

Effect of 17-ODYA on arterial diameter. To study the effect of the inhibitor of cytochrome P-450 4A under conditions of spontaneous tone, we measured the change of internal diameters after the application of three cumulative concentrations of 17-ODYA (Fig. 3, n = 6). A concentration-dependent dilation was induced by 17-ODYA, which could be significantly blocked and even reversed by endothelium removal (Fig. 3, n = 6, P < 0.05) or 100 µM L-NNA (Fig. 3, n = 6, P < 0.05). Inhibition of cytochrome P-450 4A enzymes with 10 µM 17-ODYA completely relaxed the vessel (n = 2, results not shown). Thus blockade of cytochrome P-450 4A enzymes resulted in a concentration-dependent increase in arterial diameter that was dependent on an intact endothelium and could be blocked by L-NNA.

Smooth muscle cell sensitivity to NO. Because the effect of 17-ODYA (see Fig. 3) could be explained by an increased sensitivity of the smooth muscle cells of the resistance arteries to NO, we studied the effect of the endothelium-independent NO donor SNP during the blockade of endogenous NO production with 100 µM L-NNA. Figure 4 shows that 1 µM 17-ODYA did not significantly change the sensitivity of the smooth muscle cells to SNP (n = 6).

Synergistic inhibition of cytochrome P-450 4A by hypoxia and 17-ODYA. The effect of 17-ODYA during normoxia or hypoxia is shown in Fig. 5. Hypoxia induced a nonsignificant dilation (Fig. 5, replotted from Fig. 2A). The dilation of the resistance arteries induced by 17-ODYA in combination with hypoxia was larger than the sum of the individual effects of 17-ODYA and hypoxia (Fig. 5, P < 0.05).

**DISCUSSION**

Effect of hypoxia alone and during the inhibition of cytochrome P-450 4A enzymes. We showed that hypoxia had no effect on the internal diameter of isolated cannulated cremaster first-order resistance arteries under conditions of intrinsic tone. However, hypoxia caused a marked dilation in the presence of a submaximal concentration of 17-ODYA (1 mM). Harder et al. (11) showed in isolated cat cerebral microvessels that 1 µM 17-ODYA induced submaximal inhibition of the cytochrome P-450 4A enzymes (75% reduction). In another study Harder et al. (13) used 10 µM 17-ODYA to block the response to changes in oxygen tension.
Fig. 5. Effect of hypoxia and 17-ODYA, singly and in combination, on arterial diameter. Data from 6 arteries are means ± SE of diameter increases induced by hypoxia, 17-ODYA (1 µM), and hypoxia + 17-ODYA (1 µM). *Significantly greater than sum of diameter increases induced by hypoxia alone and 17-ODYA alone (P < 0.05).
absent after removal of endothelium or after inhibition of NO production with l-NNA. This suggests that the 17-ODYA-induced dilation is caused by either an increased NO production from the endothelium or an increased sensitivity of the smooth muscle cells to basally produced NO. This second option is disproved because we showed that in the presence of 1 µM 17-ODYA responses to the endothelium-independent NO donor SNP were not altered (Fig. 4). It is thus concluded that 17-ODYA increased the NO production in the endothelium. This observation may be explained as follows. It has been shown that 20-HETE is one of the major products from cytochrome P-450 monoxygenase enzymes in rat renal microsomes (22), cremaster microsomes (13), and cerebral microvessels (11). 20-HETE has been shown to close calcium-sensitive potassium (KCa) channels in several preparations (11). KCa channels are present in vascular smooth muscle cells (2,3) and in vascular endothelial cells (34). In cultured endothelial cells it has been shown that NO production is controlled by the membrane potential (21) and that KCa channels are important in regulating the membrane potential (34). We therefore suggest that the effect of 17-ODYA on endothelial NO production can be explained by a paracrine effect of 20-HETE on endothelial NO production. Inhibition of 20-HETE formation may result in the subsequent opening of KCa channels in the endothelial cells, a hyperpolarization of the membrane, and an increase in the driving force for calcium entrance. Thus an increase in the intracellular calcium concentration follows, which activates endothelial NO synthase.

Others have also reported a paracrine effect of 20-HETE. Escalante et al. (7) and Schwartzman et al. (35) have shown that 20-HETE is a vasoconstrictor after it is converted by cyclooxygenase in the endothelium. Arguments for 20-HETE as an autocrine controller of NO production. Inhibition of 20-HETE formation may result in the subsequent opening of KCa channels in the endothelial cells, a hyperpolarization of the membrane, and an increase in the driving force for calcium entrance. Thus an increase in the intracellular calcium concentration follows, which activates endothelial NO synthase.

Synergistic action of cytochrome P-450 4A inhibition and hypoxia. The dilation of the resistance arteries induced by 17-ODYA in combination with hypoxia was larger than the sum of the individual effects of 17-ODYA and hypoxia (P < 0.05). This indicates that hypoxia potentiates the response to 17-ODYA. This can be explained as follows. First, both 17-ODYA and hypoxia act at the level of the cytochrome P-450 4A enzyme and, by inhibiting the enzyme, induce NO production. A recently published study by Harder et al. (13) identified this enzyme as a putative oxygen sensor. The authors showed that the activity of cytochrome P-450 4A, measured as production of 20-HETE and epoxyeicosatrienoic acid, is dependent on the oxygen tension. The higher the oxygen tension, the more 20-HETE produced. It was concluded that cytochrome P-450 4A enzymes may participate in the oxygen sensitivity of resistance arteries. An additional contribution is the effect of hypoxia on NO degradation. It has been shown that the half-life of NO is decreased after the elevation of oxygen tension and the production of oxygen-derived free radicals (33). Thus, if the oxygen tension is low, fewer oxygen-derived free radicals are produced and the stability of NO may be increased, resulting in a higher effective concentration.

We conclude that 1) hypoxia (5–10 mmHg) has no direct effect on the diameter of rat cremaster resistance arteries under conditions of spontaneous tone and in the absence of luminal flow; 2) inhibition of cytochrome P-450 4A enzymes under normoxic conditions induces production of NO from the endothelium; and 3) hypoxia induces an NO-mediated vasodilation when cytochrome P-450 4A enzymes are partially inhibited.

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