20-HETE–Induced Contraction of Small Coronary Arteries Depends on the Activation of Rho-Kinase

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Abstract—20-HETE is a potent constrictor of small blood vessels and has been suggested to play a crucial role in the generation of myogenic tone and the development of hypertension. In the present study, we investigated the mechanisms by which exogenously applied 20-HETE modulates vascular tone in small porcine coronary arteries. In organ chamber experiments, 20-HETE elicited a concentration-dependent contraction of small porcine coronary artery rings that was partially inhibited by the cyclooxygenase inhibitor diclofenac, the thromboxane and endoperoxide receptor antagonist SQ29548, and the thromboxane A2 synthase inhibitor furegrelate. Removal of endothelium attenuated the response to 20-HETE, whereas preconstriction of endothelium-denuded vessels to 25% of the maximum response with KCl markedly enhanced the response to 20-HETE. This 20-HETE–induced contraction was not associated with a significant increase in the intracellular concentration of Ca2+. 20-HETE–induced contraction was also observed in β-escin–permeabilized arteries precontracted with a submaximal concentration of Ca2+ and was abolished by the Rho-kinase inhibitor Y27632, but was insensitive to the PKC inhibitor RO 31-8220. 20-HETE elicited the phosphorylation of the myosin light chain (MLC20) in coronary artery rings, an effect that was sensitive to Y27632 and mimicked by the thromboxane analog U46619. These data suggest that in small porcine coronary arteries, 20-HETE can induce contraction by 2 mechanisms, one endothelium-dependent involving the cyclooxygenase-dependent generation of vasoconstrictor prostanooids, and the other endothelium-independent. The latter response is associated with the activation of Rho-kinase, phosphorylation of MLC20, and sensitization of the contractile apparatus to Ca2+. (Hypertension. 2003; 41(part 2):●●●●●●.)

Key Words: 20-HETE ■ kinase ■ myosin ■ calcium ■ arteries

As a potent vasoconstrictor, 20-HETE, an ω-hydroxylation product of arachidonic acid catalyzed by cytochrome P450 (CYP) 4A enzymes, is an essential component of the signal transduction cascade activated by several hormonal systems that have central roles in blood pressure regulation (for reviews, see Capdevila and Falck1 and Roman2). Initially, 20-HETE was reported to affect vascular smooth muscle tone indirectly as a result of its metabolism by cyclooxygenase in endothelial cells, inasmuch as indomethacin and the endoperoxide/thromboxane receptor antagonist SQ29548 inhibited endothelium-dependent contraction.3,5 The inhibitory effect of SQ29548, the appearance of labile metabolites with a half-life of ~6 minutes, and the production of 20-hydroxy-PGF2α by SnCl2 reduction indicated that the vasoconstrictor metabolites of 20-HETE are the labile endoperoxides of 20-HETE, 20-hydroxy-PGF2α, and 20-hydroxy-PGF2α.3,5

Although the effects of 20-HETE in large arteries and in the aorta are at least partially cyclooxygenase-dependent, data gathered over the past 5 years have convincingly demonstrated that 20-HETE is generated endogenously within vascular smooth muscle cells from small resistance arteries. 20-HETE is synthesized in response to an increase in [Ca2+]i, elicited by stimuli such as an increase in transmural pressure6–11 and the vasoconstrictor agonists endothelin-112–16 and angiotensin II17–21. Once formed, 20-HETE is thought to increase smooth muscle tone by inhibiting large conductance Ca2+-activated K+ (BK) channels,22,23 further increasing [Ca2+]i via a Rho-kinase–dependent mechanism. The latter response is associated with the activation of Rho-kinase, phosphorylation of MLC20, and sensitization of the contractile apparatus to Ca2+. (Hypertension. 2003; 41(part 2):●●●●●●.)

Vascular Reactivity Studies

Second branches of coronary arteries (internal diameter, 300 to 500 μm) were dissected from the hearts of freshly slaughtered pigs.
cleaned of adventitial adipose and connective tissue, and cut into 4-mm-long segments. In some experiments, the endothelium was removed by intraluminal perfusion with CHAPS (0.5% in Tyrode’s solution) for 30 seconds.

Coronary artery rings were mounted on stainless-steel triangles connected to a force transducer (Hugh Sachs Elektronik-Harvard Apparatus) and a rigid support for measurement of isometric force in organ baths containing Tyrode’s solution of the following composition (in mmol/L): NaCl 132, KCl 4, CaCl₂ 1.6, MgCl₂ 0.98, NaHCO₃ 23.8, NaH₂PO₄ 0.36, glucose 10, and Ca-Titriplex 0.05. The solution was gassed with 20% O₂, 5% CO₂, and 75% N₂ to give a P₀₂ of 2 mmol/L EGTA and contraction elicited with CaCl₂ (10 mmol/L). After a short equilibration period, the buffer was replaced with one of a similar composition containing adjusted to gas described above. After a short equilibration period, the supernatant was removed, and thromboxane B₂ (TXB₂), a stable metabolite of thromboxane A₂ (TXA₂) levels were assayed with a sensitive fluorometric assay as described.30 At the end of each experiment, the vessels were superfused with a buffer containing CaCl₂ (5 mmol/L), ionomycin (0.1 mmol/L). After a further 10 minutes, the supernatant was replaced with Tyrode’s solution (150 mL), and the pellet washed 3 times with water-saturated diethyl ether. Air-dried samples were solubilized in Triton X-100 lysis buffer, and proteins in the supernatant (50 μg) were heated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, separated by SDS-PAGE (12%), and transferred to a nitrocellulose membrane as described.31 Membranes were cut at the 32-kDa marker, and β-actin and myosin light chain 20 (MLC20) were detected by selective monoclonal antibodies (Sigma) and visualized by enhanced chemiluminescence using a commercially available kit (Amersham).

Measurement of [Ca²⁺]i
Contraction and [Ca²⁺]i were measured simultaneously. Contraction was measured as described above, and changes in [Ca²⁺]i were determined by measuring changes in fura-2 fluorescence. For the measurement of [Ca²⁺]i, arterial rings were mounted between 2 tungsten wires (30 μm diameter) connected to a rigid support attached to a micromanipulator and a force transducer, superfused with modified Tyrode’s solution, and placed on the stage of an inverted microscope (Diaphot-TMB; Nikon). Vessels were loaded with fura-2 by incubating with modified Tyrode’s solution containing fura-2/AM (5 μmol/L) and pluronic F₁₂₇ (0.025% w/v) at room temperature for 120 minutes. [Ca²⁺]i was determined fluorometrically by continuous rapid alternating excitation from dual monochromators set at 340 and 380 nm (Deltascan, Photon Technology) as described.30 At the end of each experiment, the vessels were superfused with a buffer containing CaCl₂ (5 mmol/L), ionomycin (1 μmol/L), and KCl (80 mmol/L). After a stable 340:380 fluorescence ratio was achieved, the buffer was changed to a Ca²⁺-free one. The background fluorescence was calculated after the addition of MnCl₂ (10 mmol/L) and subtracted from the original signals. Data are presented as changes in the 340/380 ratio. Using this method, the fluorescence (340/380 nm) was 1.4±0.05 under basal conditions and 3.4±1.3 in response to ionomycin, CaCl₂, and KCl.

Permeabilization of the Small Porcine Coronary Arteries
Arteries were permeabilized using β-escin as described,31 with minor modifications. Briefly, arteries were incubated at room temperature for 45 minutes in PIPES solution (20 mmol/L PIPES, 10 mmol/L creatine phosphate, 5.2 mmol/L Na₃ATP, 5.1 mmol/L MgCl₂, 87 mmol/L KCl, 1 μmol/L leupeptin, 1 μmol/L iomycin, 1 μmol/L calmodulin, and 4 mmol/LEGTA at pH 7.4) supplemented with β-escin (50 μmol/L). Arteries were then mounted in the organ chamber containing PIPES buffer (37°C). Passive tension was adjusted to 1 g as described above. After a short equilibration period, the buffer was replaced with one of a similar composition containing 2 mmol/L EGTA and contraction elicited with CaCl₂ (10 μmol/L). After washing, the arteries were re-equilibrated in the PIPES buffer, and a submaximal concentration of Ca²⁺ (1 μmol/L) was added.

Assay of Thromboxane A₂ Production by Porcine Coronary Endothelial Cells
Porcine coronary endothelial cells were isolated as described39 and seeded onto 48-well culture plates. Six hours after seeding, the cell supernatant was replaced with Tyrode’s solution (150 mL), and the cells were stimulated with solvent (0.1% ethanol), 20-HETE (1 μmol/L), or ionomycin (0.1 μmol/L). After a further 10 minutes, the supernatant was removed, and thromboxane B₂ (TXB₂), a stable metabolite of thromboxane A₂ (TXA₂), levels were assayed with a sensitive fluorometric assay as described.30 At the end of each experiment, the vessels were superfused with a buffer containing CaCl₂ (5 mmol/L), ionomycin (0.1 μmol/L), and vessels exhibiting 80% relaxation were discarded.

Figure 1. Effect of 20-HETE on endothelium-intact rings of small porcine coronary artery. Original recordings (A and B) and statistical summary (C) showing the effect of 20-HETE (0.1 to 1 μmol/L) on the tone of endothelium-intact (E+) rings of small porcine coronary artery. Experiments were performed in the presence of solvent (Solv; 0.1% ethanol), diclofenac (Diclo; 10 μmol/L), furegrelate (Fur; 10 μmol/L), and SQ-29548 (SQ; 10 μmol/L). Results are presented as mean±SEM of data obtained in 8 separate experiments; *P<0.01, **P<0.001 vs solvent.

When the contraction reached a plateau, 20-HETE (1 μmol/L) and GTP (10 μmol/L) were also applied.

Immunoblotting
Endothelium-intact rings of porcine coronary artery were incubated for 30 minutes at 37°C in modified Tyrode’s solution and stimulated as described in Results. Vessels were then frozen in liquid nitrogen, ground to a powder, and suspended in trichloroacetic acid (10% w/v). After 30 minutes at 4°C, the suspension was centrifuged (4°C) at 14 000 g for 30 minutes, the supernatant was discarded, and the pellet washed 3 times with water-saturated diethyl ether. Air-dried samples were solubilized in Triton X-100 lysis buffer, and proteins in the supernatant (50 μg) were heated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, separated by SDS-PAGE (12%), and transferred to a nitrocellulose membrane as described.31 Membranes were cut at the 32-kDa marker, and β-actin and myosin light chain 20 (MLC20) were detected by selective monoclonal antibodies (Sigma) and visualized by enhanced chemiluminescence using a commercially available kit (Amersham).

Statistical Analysis
Contraction is expressed in grams of tension or as a percentage of the maximal contraction (% max) obtained using 80 mmol/L KCl. Data are expressed as mean±SEM, and statistical evaluation was performed with the Student t test for unpaired data or 1-way ANOVA followed by a Bonferroni t test, when appropriate. Values of P<0.05 were considered statistically significant.

Results
Effect of 20-HETE on the Tone of Endothelium-Intact Rings of Small Porcine Coronary Artery
Application of 20-HETE (0.1 to 1 μmol/L) to endothelium-intact rings of small porcine coronary arteries resulted in a concentration-dependent contraction (Figure 1). The kinetics of the response depended on the concentration applied, as the increase in tone observed after the application of lower concentrations of 20-HETE developed slowly, whereas the contraction observed after application of the highest concentration was rapid.

The 20-HETE–induced contraction was significantly attenuated by the cyclooxygenase inhibitor diclofenac (10 mmol/L;
was associated with an increase in [Ca\(^{2+}\)]. Although the subsequent addition of 20-HETE further increased contraction, there was no significant corresponding increase in [Ca\(^{2+}\)], (Figure 3).

Because 20-HETE is reported to elicit contraction by inhibiting BK channels,\(^{22,23}\) depolarizing vascular smooth muscle cells, and opening L-type Ca\(^{2+}\) channels,\(^{24}\) we determined the effect of 20-HETE on the vascular tone of porcine coronary arteries precontracted with KCl in the absence and presence of the BK blocker iberiotoxin and the Ca\(^{2+}\) antagonist, nifedipine. Iberiotoxin (100 nmol/L) did not affect either the basal tone or the 20-HETE-induced contraction of porcine coronary arteries precontracted with KCl (contractions were 45.2±11.9 versus 43.3±8.5% max in the absence and presence of iberiotoxin, respectively; n=4, P=0.77). Significantly higher concentrations of KCl were required to precontract nifedipine-treated coronary artery rings to 25% of the maximal contraction (30 mmol/L versus 80 mmol/L KCl in solvent and nifedipine-treated rings, respectively). The presence of nifedipine, however, did not significantly affect the maximal increase in tone that developed in response to the addition of 20-HETE (contractions were 50.0±6.8 versus 41.5±9.04% max in the absence and presence of nifedipine, respectively; n=6, P=0.46).

**Effect of 20-HETE on the Tone of Permeabilized Arteries**

Because the ability of 20-HETE to increase the tone of small porcine coronary arteries was markedly enhanced by precontraction with KCl, but independent of a further increase in [Ca\(^{2+}\)], we assessed the ability of 20-HETE to increase tone in β-escin–permeabilized arteries. After permeabilization, arteries were contracted using a submaximal concentration of Ca\(^{2+}\) (1 μmol/L), and once a stable contraction level was reached, 20-HETE and GTP were added to the vessel chamber. The addition of GTP (10 μmol/L) alone was without effect (data not shown), whereas the addition of 20-HETE and GTP elicited a rapid contraction (Figure 4). This contractile response was not significantly affected by RO 31-8220.
but was abolished by Y27632 (Figure 4). Identical results were obtained in endothelium-intact and endothelium-denuded arterial rings.

**Effect of 20-HETE on MLC20 Phosphorylation**

Because Y27632 inhibited the contraction to 20-HETE and Ca$^{2+}$ sensitization in several different arteries has been linked to phosphorylation of MLC20, we determined the effects of 20-HETE on the phosphorylation of MLC20. In extracts of unstimulated porcine coronary artery, MLC20 was detected as a single band (Figure 5). Stimulation of coronary arteries with 20-HETE (1 μmol/L) induced a shift in the mobility of this protein, which was not observed in extracts from arteries treated with 20-HETE and Y27632. A similar phenomenon was observed in coronary arteries stimulated with the known Ca$^{2+}$-sensitizing agent U46619 (Figure 5).

**Discussion**

The results of the present investigation indicate that in small porcine coronary arteries, 20-HETE enhances vascular tone by a mechanism involving the activation of Rho-kinase, phosphorylation of MLC20, and the sensitization of the contractile apparatus to Ca$^{2+}$. When the vascular endothelium was intact, the contractile response appeared to be dependent on the metabolism of 20-HETE by cyclooxygenase, whereas the latter enzyme was not involved in the contractile response to 20-HETE in endothelium-denuded arteries.

The metabolism of 20-HETE in endothelial cells by cyclooxygenase has been appreciated for a number of years, and although we found that 20-HETE elicited the generation of TXB2 from freshly isolated endothelial cells, it is most likely that the endothelium-dependent contractile response obtained can be attributed to the labile peroxides, 20-hydroxy-PGG$_2$, and 20-hydroxy-PGH$_2$.3–5

Although 20-HETE is reported to be a potent constrictor of isolated endothelium-denuded renal skeletal muscle and cerebral arteries, contraction of endothelium-denuded small porcine coronary arteries to 20-HETE was only observed when high concentrations were used. The contractile response was, however, markedly potentiated when vessels were precontracted to ∼25% of the maximal KCl-inducible tone, suggesting that 20-HETE may potentiate contraction by sensitizing the contractile proteins to the increase in [Ca$^{2+}$], induced by KCl.

20-HETE is reported to induce vascular smooth muscle contraction by inhibiting BK channels,22–24 thus depolarizing the membrane and increasing Ca$^{2+}$ entry via L-type Ca$^{2+}$ channels.16,24 However, in the present investigation, we found no evidence linking 20-HETE to this mechanism, as the contractile response to 20-HETE was not affected by the BK channel blocker iberiotoxin. 20-HETE had also no direct effect on Ca$^{2+}$ signaling as, although we observed a clear increase in smooth muscle [Ca$^{2+}$], after the stimulation of arteries with KCl, the subsequent application of 20-HETE did not further increase [Ca$^{2+}$]. Moreover, blocking Ca$^{2+}$ entry via L-type Ca$^{2+}$ channels with nifedipine failed to affect the contractile response to 20-HETE. In the small porcine coronary artery, therefore, 20-HETE induces contraction chiefly by increasing vascular smooth muscle sensitivity to Ca$^{2+}$. The best evidence for a 20-HETE–induced Ca$^{2+}$ sensitization came from experiments using β-escin–permeabilized arteries, in which [Ca$^{2+}$], was clamped at a constant value. Because, under the experimental conditions used, contraction was unrelated to a change in [Ca$^{2+}$], the 20-HETE–induced contraction can be attributed to an increase in the sensitivity of the contractile apparatus to Ca$^{2+}$. In general, small porcine coronary arteries respond poorly to Ca$^{2+}$-elevating agonists such as norepinephrine and phenylephrine but are sensitive to the TXA$_3$ analog U46619. Indeed, the plateau phase of the U46619-induced contraction is achieved mainly by enhancing the sensitivity of the contractile apparatus to a relatively
small increase in [Ca\(^{2+}\)], by a mechanism involving the activation of Rho-kinase.\(^{33}\)

As PKC-mediated and Rho-kinase–mediated Ca\(^{2+}\) sensitization mechanisms are operative in small coronary arteries,\(^{33}\) we determined the effects of PKC and Rho-kinase inhibitors on the 20-HETE–induced increase in coronary artery tone. The PKC inhibitor, RO 31-8220, did not significantly affect the 20-HETE–induced contraction of endothelium-denuded arteries, whereas the response was abolished by the Rho-kinase inhibitor Y27632. A similar abolition of the 20-HETE–induced contraction in the presence of Y27632 was also observed in \(\beta\)-escin–permeabilized arteries.

Although the intracellular mechanisms underlying the phenomenon of calcium sensitization have not been fully elucidated, numerous studies have shown that it is accompanied by an increase in MLC\(_{20}\)|. The extent of MLC\(_{20}\) phosphorylation and contraction, was abolished in arteries preincubated with the Rho-kinase inhibitor Y27632 (assessed as a mobility shift in Western blotting experiments) in small porcine coronary arteries. This response, like the potentiation of MLC\(_{20}\) phosphorylation and contraction,\(^{36,37}\) whereas PKC phosphorylates CPI-17, a myosin light chain phosphatase that dephosphorylates and inactivates MLC\(_{20}\) (for reviews, see Somlyo and Somlyo\(^{34,35}\) and Fukata\(^{35}\)). PKC and Rho-kinase can both sensitize vascular smooth muscle cells to Ca\(^{2+}\), although the molecular mechanisms eventually leading to enhanced MLC\(_{20}\) phosphorylation are distinct; Rho-kinase phosphorylates and inhibits myosin light chain phosphatase, leading to an increased MLC\(_{20}\) phosphorylation and contraction,\(^{36,37}\) whereas PKC phosphorylates CPI-17, a myosin light chain phosphatase inhibitor.\(^{38}\) In the present study, we observed that 20-HETE-induced phosphorylation of MLC\(_{20}\) (assessed as a mobility shift in Western blotting experiments) in small porcine coronary arteries. This response, like the potentiation of contraction, was abolished in arteries preincubated with the Rho-kinase inhibitor. A similar effect was also observed in arteries treated with U46619, which also sensitizes small porcine coronary arteries to Ca\(^{2+}\) via the activation of Rho-kinase.\(^{39}\)

Taken together, the results of the present investigation indicate that 20-HETE is able to sensitize small porcine coronary artery smooth muscle cells to Ca\(^{2+}\) by activating Rho-kinase and enhancing the phosphorylation of MLC\(_{20}\).

**Perspectives**

20-HETE is currently characterized as a prohypertensive eicosanoid and has the potential to play a dual role in the regulation of blood pressure by virtue of its ability to induce contraction, as well as to inhibit sodium reabsorption. Several reports have demonstrated that the expression of CYP 4A and the production of 20-HETE are altered in genetic and experimental models of hypertension (for review, see Capdevila and Falck\(^{3}\) and Roman\(^{2}\)). It is therefore tempting to speculate that some of the potentially beneficial effects of Rho-kinase inhibitors in hypertension\(^{26,39}\) may be related to the inhibition of 20-HETE–induced Ca\(^{2+}\) sensitization. Moreover, although a direct role for 20-HETE in coronary artery vasospasm has not been described to-date, the fact that Rho-kinase inhibitors effectively reduce vasospasm in porcine models,\(^{40,41}\) as well as in patients with vasospastic angina,\(^{42}\) may be indicative of a role for this eicosanoid in vasospasm.

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**References**


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