20-HETE agonists and antagonists in the renal circulation

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Alonso-Galicia, Magdalena, John R. Falck, Koman-dla Malla Reddy, and Richard J. Roman. 20-HETE agonists and antagonists in the renal circulation. Am. J. Physiol. 277 (Renal Physiol. 46): F790–F796, 1999.—The present study examined the effects of a series of 20-hydroxyeicosatetraenoic acid (20-HETE) derivatives on the diameter of renal arterioles to determine the structural requirements of the vasoconstrictor response to 20-HETE. The vascular responses to 5-, 8-, 12-, 15-, 19-, 20-, 21-HETEs, arachidonic acid (AA), and saturated, partially saturated, dimethyl, carboxyl, and 19-carbon derivatives of 20-HETE (10-8 to 10-6 M) were assessed in rat renal interlobular arteries (65–125 μm). 20-HETE, 21-HETE, dimethyl-20-HETE, and a partially saturated derivative of 20-HETE, 20-hydroxyeicos-5(Z),14(Z)-dienoic acid, reduced vessel diameter by 19 ± 3, 17 ± 3, 16 ± 2, and 28 ± 2%, respectively. In contrast, 5-, 8-, 12-, 15-, and 19-HETE, AA, saturated, partially saturated, carboxyl, and the 19-carbon derivatives of 20-HETE had no effect on vessel diameter. Pretreatment with 5-, 15-, and 19-HETE, the 19-carbon derivative or 20-hydroxyeicos-6(Z),15(Z)-dienoic acid (1 μM) completely blocked the vasoconstrictor response to 20-HETE in renal arterioles. Pretreatment with AA, carboxyl, saturated 19-carbon, and saturated 20-HETE derivatives (1 μM) partially blocked the response, whereas 8- and 12-HETE (1 μM) had no effect on the vasoconstrictor response to 20-HETE. These findings suggest that 20-HETE agonists and antagonists require a carboxyl or an ionizable group on carbon 1 and a double bond near the 14 or 15 carbon. 20-HETE agonists also require a functional group capable of hydrogen bonding on carbon 20 or 21, whereas antagonists lack this reactive group.

Cytochrome P-450; renal arterioles; vasoconstriction; hydroxyeicosatetraenoic acids

RECENT STUDIES HAVE indicated that arachidonic acid (AA) is primarily metabolized by a P-450 4A-dependent pathway to 20-hydroxyeicosatetraenoic acid (20-HETE) in the kidney and the peripheral vasculature (3, 6, 19). 20-HETE serves as a critical second messenger in the regulation of renal and peripheral vascular tone, as well as renal tubular function. In this regard, 20-HETE is a potent vasoconstrictor that inhibits the opening of Ca2+-activated K+ channels in vascular smooth muscle (VSM) cells (12, 32). It promotes Ca2+ entry by depolarizing VSM cells secondary to blockade of Ca2+-activated K+ channels (17) and by increasing the conductance of L-type Ca2+ channels (5). Inhibitors of the formation of 20-HETE block the myogenic response of renal, cerebral, and peripheral arterioles to elevations in transmural pressure and autoregulation of renal and cerebral blood flow in vivo (7, 11, 33, 34). Selective P-450 4A inhibitors attenuate the vasoconstrictor response to endothelin (21), angiotensin II (2, 22), elevations in tissue Po2 (8, 16), and the mitogenic actions of growth factors in VSM and renal mesangial cells (15, 31). They also delay or prevent the development of hypertension in spontaneously hypertensive rats and other experimental models of hypertension (18, 24, 27). In the kidney, 20-HETE is also produced by renal tubular cells, where it participates in the regulation of sodium transport in the proximal tubule and thick ascending limb of the loop of Henle (20, 22, 23). 20-HETE is also produced by the airways in human and rabbit lungs, where it serves as a bronchodilator (13).

Despite the importance of 20-HETE in the regulation of renal function, vascular tone, and airway resistance, little is known about its mechanism of action. Recent studies have indicated that the mitogenic actions of 20-HETE and its effects on vascular tone and sodium transport are associated with activation of protein kinase C and mitogen-activated protein kinase signal transduction cascades (4, 20, 29). Activation of these pathways are usually triggered by receptor-mediated events; however, presently there is no evidence for a 20-HETE receptor. There have been no studies to determine whether 20-HETE binds to membrane proteins or whether the vasoconstrictor properties of 20-HETE can be mimicked by other analogs. Thus the purpose of the present study was to perform structure activity studies with a series of synthetic 20-HETE analogs to evaluate the structural requirements for its vasoconstrictor actions in interlobular arteries microdissected from the kidney of rats.

METHODS

General. Experiments were performed on 10- to 12-wk-old male Sprague-Dawley rats purchased from Harlan Sprague Dawley Laboratories (Indianapolis, IN). The rats were housed in the animal care facility at the Medical College of Wisconsin, which is approved by the American Association for the Accreditation of Laboratory Animal Care. The animals had free access to food and water. All protocols involving animals received approval by the Animal Care Committee of the Medical College of Wisconsin.

Synthesis of 20-HETE agonists and antagonists. The following compounds: 5(S)-, 8(S)-, 12(S)-, 15(S)-, and 19(S)-HETE, were used to examine the effect of the position of the hydroxy group on the vasoconstrictor response to 20-HETE. All of these analogs are commercially available (Biomol, Plymouth Meeting, PA) and have the same number of carbons (20), double bonds (4), and molecular weight as 20-HETE (Fig. 1). The only difference between these compounds and 20-HETE...
the position of the hydroxyl group along the carbon chain. Additional experiments were also performed with AA (Sigma, St. Louis, MO) and 20-carboxy-AA, which are structurally similar to 20-HETE but lack a hydroxyl group on the 20th carbon, and 20-hydroxyeicosa-6(Z),15(Z)-diene acid [6(Z),15(Z)-20-HETE], which has the carboxy and hydroxy moieties on the 1st and 20th carbon reversed (Fig. 1). The synthesis of 20-HETE and the 20-HETE analogs are described in detail in the United States patent application USSN 60/076,091 (25).

Additional experiments were performed to assess the importance of the double bonds and the length of the carbon chain to the vasoconstrictor response to 20-HETE. In these experiments, the double bonds in the 20-HETE molecule were removed, creating the partially saturated 20-HETE analog 20-hydroxyeicosa-5(Z),14(Z)-diene acid [5(Z),14(Z)-20-HETE] and a saturated 20-HETE derivative, 20-hydroxyeicosanoic acid (20-HE). The 20-HETE molecule was also shortened by one carbon, creating a 19-carbon analog, 19-hydroxyeicosanoic acid (C19 analog), and the double bonds were removed, creating a saturated 19-carbon analog, 19-hydroxyeicosanoic acid (C19 analog). In addition, one carbon was added to the 20-HETE molecule, creating a 21-carbon analog, 21-hydroxyeicosa-5(Z),8(Z),11(Z),14(Z)-tetaenoic acid (21-HETE). Finally, the hydrogen molecules on the 20th carbon were replaced with methyl groups to form 20,20-dimethyl-20-HETE (Fig. 1). The synthesis of these 20-HETE derivatives is also described in detail in the United States patent application USSN 60/076,091 (25).

Structural requirements of the vasoconstrictor response to 20-HETE: isolated vessel studies. Interlobular arteries (65–125 µm inner diameter) were microdissected from the kidneys of rats. The vessels were mounted on glass micropipettes in a perfusion chamber containing physiological saline solution maintained at 37°C and equilibrated with 95% O2-5% CO2 gas mixture. The vessels were secured to the pipettes with 10-0 silk suture and stretched to its in vivo length using an eyepiece micrometer. This was achieved by stretching the vessel until the inner diameter was close to the initial inner diameter measured in situ before microdissection. The inflow pipette was connected to a pressurized reservoir to control intraluminal perfusion pressure, which was monitored using a transducer (Cobe, Lakewood, CO). The outflow cannula was clamped off, and intraluminal pressure was maintained at 90 mmHg. Vessel diameter was measured with a video system composed of a stereomicroscope (Carl Zeiss), a television camera (KP-130AU, Hitachi), a videocassette recorder (AG-7300, Panasonic), a television monitor (CVM-1271, Sony), and a video measuring system (VIA-100, Boeckeler Instrument, Tucson, AZ). The composition of the perfusate and the bath was (in mM) 119 NaCl, 4.7 KCl, 1.17 MgSO4, 1.6 CaCl2, 12 NaHCO3, 1.18 NaH2PO4, 0.03 EDTA, and 10 glucose, pH 7.4. Indomethacin (5 µM), baicalein (0.5 µM), and 17-octadecynoic acid (17-ODYA, 1 µM) were added to the bath to block the endogenous formation and metabolism of eicosanoids via the cyclooxygenase, lipoxigenase, and cytochrome P-450 pathways (1, 17). After the vessels were mounted and the inhibitors were added to the bath, a 30-min equilibration period was allowed before the cumulative dose-response curves were generated.

After the equilibration period, control inner diameter was measured and a cumulative dose-response curve was generated for each of the 20-HETE analogs (10-8 to 10-6 M). After the compounds were added to the bath, 2 to 3 min were allowed before recording the steady-state inner diameter of the vessels. The dose-response curve to each compound was completed within 10 min after measuring the control inner diameter of the vessel.

The inactive analogs of 20-HETE were further tested for antagonist activity. A cumulative dose-response curve to 20-HETE (10-8 to 10-6 M) was generated under control conditions. Then the bath was replaced with fresh physiological saline solution and inhibitors, and a 30-min period was allowed for the vessels to reequilibrate. After the equilibration period, one of the following inactive 20-HETE analogs was added to the bath: 5(S)-HETE (0.5 µM), 8(S)-HETE (1 µM), 12(S)-HETE (0.5 µM), 15(S)-HETE (1 µM), 19(S)-HETE (1 µM), C19 analog (1 µM), AA (1 µM), 20-carboxy-AA (1 µM),


RESULTS

Effect of hydroxy group position on the vasoconstrictor response to 20-HETE. The vasoconstrictor responses to the 20-HETE derivatives are presented in Fig. 2. 20-HETE (10⁻⁸ to 10⁻⁶ M) reduced the diameter of renal interlobular arteries in a concentration-dependent manner to 19 ± 3% of control (−4.3 ± 0.8 to −22.5 ± 1.5 µm, n = 7). The threshold concentration of 20-HETE that reduced vascular diameter was 10 nM (P < 0.05). In contrast, 5(S), 8(S), 12(S), 15(S), and 19(S)-HETE had no significant effect on the diameter of renal interlobular arteries even at a concentration as high as 1 µM (Fig. 2A).

The effects of altering the length of the carbon chain on the vasoconstrictor response to 20-HETE are presented in Fig. 2B. 21-HETE and 20,20-dimethyl-20-HETE were just as potent vasoconstrictors as 20-HETE, 21-HETE reduced vessel diameter in a dose-dependent manner to a maximum of 17 ± 3% of control (−4.8 ± 0.9 to −21.1 ± 2.4 µm, n = 6). Dimethyl-20-HETE reduced vessel diameter to a maximum of 15 ± 2% of control (−4.2 ± 0.8 to −18.4 ± 1.2 µm, n = 6). In contrast, AA, which lacks a hydroxyl group on the 20th carbon, C₁₉ analog, and 20-carboxy-AA had no significant effect on vessel diameter. Similarly, the saturated 20-HETE derivative 20-HE, in which the double bonds were eliminated, had no effect on vessel diameter (Fig. 2B). The partially saturated 20-HETE derivative 5(Z),14(Z)-20-HEDE, in which two of the four double bonds were removed, was as potent a constrictor as 20-HETE. The 5(Z),14(Z)-20-HEDE produced a concentration-dependent fall in vascular diameter to a maximum of 28 ± 2% of control (−6.4 ± 1.2 to −29.1 ± 0.7 µm, n = 3). Interestingly, 6(Z),15(Z)-20-HEDE in which the positions of the hydroxy and carboxy groups on the 1st and 20th carbon are reversed, had no effect on vascular diameter (Fig. 2B).

Determinants of 20-HETE antagonist activity. We also studied whether the inactive analogs of 20-HETE could serve as antagonists of the vasoconstrictor response to 20-HETE. Concentration response curves to 20-HETE were generated in isolated perfused renal interlobular arteries before and after adding 0.5–1 µM of the various inactive 20-HETE derivatives to the bath. Under control conditions, 20-HETE (10⁻⁸ to 10⁻⁶ M) produce a dose-dependent fall in vessel diameter to a maximum of 25.5 ± 4.4% of control (−5.4 ± 0.9 to −20.6 ± 1.4 µm, n = 9). Addition of 5(S)-0.5 µM, n = 5) or 15(S)-HETE (1 µM, n = 5) to the bath completely blocked the vasoconstrictor response to 20-HETE (Fig. 3A). Similarly, addition of 19(S)-HETE (n = 5, 1 µM), the C₁₉ analog (n = 5, 1 µM), or 6(Z),15(Z)-2-HEDE (n = 3, 1 µM) to the bath, eliminated the vasoconstrictor response to 20-HETE (Fig. 3B).

Other experiments examined the effects of placing the hydroxyl group closer to the hydrophobic head of the 20-HETE molecule on the antagonist activity. In this study, 20-HETE (10⁻⁸ to 10⁻⁶ M) produced a fall in vessel diameter to a maximum of 22.6 ± 3.4% of control (−5.5 ± 0.6 to −19.5 ± 1.1 µm, n = 7) under control
conditions. Addition of 8(S)-HETE (1 µM) or 12(S)-HETE (0.5 µM) had no significant effect on the vasoconstrictor response to 20-HETE (Fig. 4A). In the presence of 8(S)-HETE (1 µM), 20-HETE (10⁻⁸ to 10⁻⁶ M) decreased the diameter of these vessels by 26.3 ± 1.3% of control (−2.8 ± 0.7 to −16.9 ± 0.2 µm, n = 3). Similarly, in the presence of 12(S)-HETE (0.5 µM), 20-HETE (10⁻⁸ to 10⁻⁶ M) reduced vascular diameter by 21.7 ± 5.7% of control (−5.5 ± 1.7 to −16.2 ± 2.9 µm, n = 5). Derivatives of 20-HETE lacking a hydroxyl group appeared to serve as true competitive antagonists of the vasoconstrictor response to 20-HETE. For example, AA (1 µM) reduced the vasoconstrictor response to 1 µM 20-HETE as expected by about 50%. However, 20-carboxy-AA had no significant antagonistic properties (Fig. 4B).

We next evaluated the importance of double bonds on 20-HETE-agonist activity. In these experiments, 20-HETE (10⁻⁸ to 10⁻⁶ M) reduced vessel diameter in a concentration-dependent manner to 28.3 ± 2.9% of control (−5.3 ± 0.4 to −19.5 ± 1.3 µm, n = 16). Addition of sC₁₉ analog to the bath, reduced the vasoconstrictor response to 20-HETE by about 50% (Fig. 4B). In the presence of the sC₁₉ analog (1 µM), 20-HETE (10⁻⁸ to 10⁻⁶ M) decreased the diameter of these vessels to a maximum of 11.9 ± 1.8% of control (−1.7 ± 0.8 to −9.8 ± 1.5 µm, n = 5). Similarly, the saturated 20-HETE (20-HE) derivative exhibited competitive antagonist activity. In the presence of 20-HE (1 µM), 20-HETE (10⁻⁸ to 10⁻⁶ M) only reduced vascular diameter to a maximum of 9.2 ± 3.8% of control (−0.9 ± 1.3 to −7.0 ± 2.9 µm, n = 5) (Fig. 4B).

A comparison of the structures of the compounds that have agonist or antagonist activity is presented in Fig. 5. 20-HETE agonists and antagonists both require a carboxyl or an ionizable group on carbon 1. They also require a double bond at a distance equal to 14 or 15 carbons from the 1carboxyl or ionizable group. A 20-HETE agonist requires a hydroxyl or another ionizable or functional group capable of hydrogen bonding at a distance equivalent to 20 or 21 carbons from the ionizable group on the 1st carbon. A 20-HETE antagonist has a similar structure as an agonist but lacks a reactive group on the 20th of 21st carbon.

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**Fig. 3.** A: antagonist activity of various 20-HETE derivatives on vasoconstrictor response to 20-HETE in isolated, pressurized rat renal interlobular arteries. Cumulative concentration response curves to 20-HETE were generated under control conditions and after addition of 5(S)-HETE (0.5 µM, n = 5 vessels) and 15(S)-HETE (1 µM, n = 5 vessels) to bath. B: cumulative concentration response curves to 20-HETE were generated under control conditions and after addition of 19(S)-HETE (1 µM, n = 6 vessels), C₁₉ analog (1 µM, n = 5 vessels), and 6(Z),15(Z)-20-HEDE (1 µM, n = 3 vessels) to bath. Results are expressed as absolute change in vessel diameter. Values are means ± SE. *Significant difference (P < 0.05) from control values.

**Fig. 4.** A: effect of 8(S)- and 12(S)-HETE on vasoconstrictor response to 20-HETE in isolated, pressurized rat renal interlobular arteries. Cumulative concentration response curves to 20-HETE were generated under control conditions and after addition of 12(S)-HETE (0.5 µM, n = 5 vessels) and 8(S)-HETE (1 µM, n = 3 vessels) to bath. B: antagonist activity of various 20-HETE analogs on vasoconstrictor response to 20-HETE. Cumulative concentration response curves to 20-HETE were generated under control conditions and after addition of sC₁₉ analog (1 µM, n = 5 vessels), 20-HE (1 µM, n = 5 vessels), AA (1 µM, n = 3 vessels), and 20-carboxy-AA (1 µM, n = 3 vessels) to bath. Results are expressed as absolute change in vessel diameter. Values are means ± SE. *Significant difference (P < 0.05) from control values.
analogs of 20-HETE were compared with that of the renal responses to saturated and partially saturated fatty acids required for constrictor activity. Therefore, the vascular tone might induce some secondary structure that is lost in the hydrogenation of the double bonds. These results indicate that these double bonds are an important structural requirement for the vasoconstrictor response to 20-HETE.

The most exciting finding of the present study is that analogs of 20-HETE that lack vasoconstrictor activity can serve as antagonists. In this regard, AA, 5(S)-, 15(S)-, 19(S)-HETE, and the C19 analog were all effective antagonists of the vasoconstrictor response to 20-HETE. We also found that there are predictable structural determinants of antagonist activity. Thus it appears that preserving the hydrophobic nature of 20-HETE is critical to retain antagonist activity. For example, molecules such as 12(S)- or 8(S)-HETE, which have a hydroxyl group near the head of the molecule, could not interact with the putative 20-HETE binding site and did not antagonize the vasoconstrictor response to 20-HETE. From these findings, one would predict that 11(S)- and 7(S)-HETE as well as 8,9- and 11,12-epoxyeicosatrienoic acid (8,9-EET and 11,12-EET) and dihydroxyeicosatetraenoic acid (Di-HETEs) would probably be ineffective as 20-HETE antagonists.

We also found that the double bonds of the 20-HETE analogs influence antagonist activity. In this regard, saturated C19 (sC19 analog) and C20 analogs of 20-HETE (20-HE) were not as effective as the parent compounds in antagonizing the vasoconstrictor response to 20-HETE. Similar to our findings with 20-HETE derivatives possessing agonist activity, we found that only one pair of double bonds (between the 5,6- and 14,15-carbons) is required to fully preserve antagonist activity. In this regard, the partially saturated analog 6(Z),15(Z)-20-HEDE completely blocked the vasoconstrictor response to 20-HETE. This compound is particularly interesting since it might be more biologically stable than the other analogs because removal of the double bonds across the 8,9- and 11,12-carbons would block metabolism of this compound by cyclooxygenase and lipooxygenase enzymes. Stability of this compound could also be enhanced by adding an ionizable sulfonamide group to the carboxyl group to block metabolism by β-oxidation and esterification. It is likely that addition of a sulfonamide group would not alter the biological properties of this compound since addition of this group does not alter the effects of 20-HETE, EETs, and mechanism-based inhibitors of the synthesis of 20-HETE (1, 10).

The present findings demonstrating the structural requirements for the vasoconstrictor actions of 20-HETE and the fact that closely related, inactive analogs serve as 20-HETE antagonists provide the first evidence for a 20-HETE receptor. The nature of this receptor or binding site, however, remains to be determined. Classic receptors are generally associated with the extracellular side of the membrane and are members of the seven or more transmembrane domain family of proteins. The effects of 20-HETE on K+

**DISCUSSION**

The present study examined the effects of a series of 20-HETE analogs on the diameter of renal interlobular arteries to determine the structural requirements for the vasoconstrictor response to this compound. Our results indicate that AA, which differs from 20-HETE by the lack of a hydroxyl group on the 20th carbon, does not constrict renal interlobular arteries. Similarly, 5(S)-, 8(S)-, 12(S)-, 15(S)-, and 19(S)-HETE, in which the hydroxyl group is located on other carbons, have no effect on vascular diameter. Moreover, a C19 analog of 20-HETE, which is one carbon shorter, has no effect on vascular tone. In contrast, 21-HETE and dimethyl-20-HETE are just as potent constrictors as 20-HETE. These results indicate that the vasoconstrictor response to 20-HETE is critically dependent on the presence of a hydroxyl group on the 20th or 21st carbon.

We also examined the importance of double bonds to the vasoconstrictor response to 20-HETE analogs in renal arteries. One could speculate that the interactions between the double bonds in the 20-HETE molecule might induce some secondary structure that is required for constrictor activity. Therefore, the vascular responses to saturated and partially saturated analogs of 20-HETE were compared with that of the native compound. We found that 20-HE, the saturated 20-HETE analog, had no effect on vascular tone, whereas the partially saturated analog 5(Z),14(Z)-20-HEDE with double bonds between the 5,6- and 14,15-carbons retained agonist activity. These findings indicate that these double bonds are an important structural requirement for the vasoconstrictor response to 20-HETE.

**Fig. 5.** Comparison of structures of 20-HETE analogs that exhibit agonist or antagonist activity. 20-HETE agonists include 21-hydroxyeicosapentaenoic acid (21-HETE), 20-HETE, 5(Z),14(Z)-20-HEDE, and 20,20-dimethyl-20-HETE. 20-HETE antagonists include 5(S)-, 15(S)-, and 19(S)-hydroxyeicosatetraenoic acid (HETE), C19 analog, and 6(Z),15(Z)-20-HEDE.
channel activity, vascular tone and growth, as well as sodium transport in renal tubules are associated with activation of protein kinase C and mitogen-activated protein kinase signal transduction cascades (4, 20, 29). However, there is also evidence that 20-HETE (30) and epoxyeicosatrienoic acids (14) can inhibit $K^+$ channel activity in detached membrane patches, suggesting that they may act as intracellular lipid activators of various kinases rather than as a hormone or paracrine factor acting on an extracellular receptor.

In summary, the present structure activity studies suggest that 20-HETE agonists and antagonists both require a carboxyl or other ionizable group at one end of the molecule to serve as an anchor point with the putative receptor or binding site. They also require a double bond at a distance equal to 14 or 15 carbons from the putative receptor or binding site. They also require a hydrogen bonding to interact with the receptor at a distance equivalent to 20 or 21 carbons from the ionizable group on the 1st carbon. A 20-HETE antagonist has a similar structure as an agonist, but it lacks a reactive group on the 20th or 21st carbon.

perspectives

Recent studies have indicated that 20-HETE plays an important role as a second messenger in autoregulation of renal blood flow, tubuloglomerular feedback, renal sodium transport, pulmonary function, and the mitogenic and vasoconstrictor responses to numerous vasoactive hormones and growth factors (2, 6–8, 11, 15–18, 21–25, 29–34). The formation of cytochrome P-450 metabolites of AA is altered in genetic and experimental models of hypertension, diabetes, hepato-renal syndrome, and toxemia of pregnancy (1, 20, 23). Given the critical role of this substance in the regulation of renal and pulmonary function, vascular tone, and the control of arterial pressure, it is likely that stable 20-HETE antagonists and analogs may have therapeutic potential in the treatment of some of these diseases. At the very least, these analogs should provide researchers with important, new tools to investigate 20-HETE signaling pathways and the role of this substance in the control of renal and cardiovascular function.

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REFERENCES


