Role of Tyrosine Kinase and PKC in the Vasoconstrictor Response to 20-HETE in Renal Arterioles

Cheng-Wen Sun, John R. Falck, David R. Harder, Richard J. Roman

Abstract—The present study examined the hypothesis that activation of protein kinase C (PKC), components of the mitogen-activated protein (MAP) kinase pathway, or both contributes to the inhibitory effects of 20-hydroxyeicosatetraenoic acid (20-HETE) on K^+-channel activity and its vasoconstrictor response in renal arterioles. 20-HETE (0.1 to 50 \( \mu \)mol/L) dose-dependently produced a 30% increase in PKC activity and a fivefold rise in the expression of active extracellular signal-regulated kinase 1 (ERK1) and ERK2 proteins in renal microvessels. 20-HETE (0.01 to 1 \( \mu \)mol/L) reduced the diameter of isolated perfused renal interlobular arterioles by 33±2%. Blockade of PKC activity with an N-myristoylated PKC pseudosubstrate inhibitor (Myr-PKCi, 100 \( \mu \)mol/L) or calphostin C (0.5 \( \mu \)mol/L) had no significant effect on the vasoconstrictor response to 20-HETE. In contrast, the tyrosine kinase inhibitors genistein (30 \( \mu \)mol/L) and tyrphostin 25 (10 \( \mu \)mol/L) reduced the response to 20-HETE by 76.5±2.1% and 67.5±1.8%, respectively. A specific inhibitor of mitogen-activated extracellular signal-regulated kinase (MEK), PD98059, had no effect on the vasoconstrictor response to 20-HETE. In cell-attached patches on renal vascular smooth muscle cells, 20-HETE reduced the open state probability of a large-conductance K^+ channel (from 0.0026±0.0004 to 0.0006±0.0001). The Myr-PKCi (100 \( \mu \)mol/L) did not alter the inhibitory effects of 20-HETE on this channel. In contrast, the tyrosine kinase inhibitor genistein (30 \( \mu \)mol/L) blocked the inhibitory effects of 20-HETE on the large-conductance K^+ channel. These data suggest that 20-HETE activates the MAP kinase system in renal arterioles and that the activation of a tyrosine kinase, which is proximal to MEK in this cascade, contributes to the inhibitory effects of 20-HETE on K^+-channel activity and its vasoconstrictor effects in the renal arterioles. (Hypertension. 1999;33[part II]:414-418.)

Key Words: kinase ■ muscle, smooth, vascular ■ arachidonic acid ■ renal circulation ■ cytochrome P450 ■ potassium channels

Recent studies have indicated that renal arterioles produce a potent vasoconstrictor, 20-hydroxyeicosatetraenoic acid (20-HETE), and that this compound serves as an intracellular signaling system that plays an important role in the regulation of vascular tone by influencing the open state probability (NPo) of the calcium-activated potassium (K\(_{Ca}\)) channel.1,2 In this regard, inhibitors of the formation of 20-HETE block the myogenic response of renal arterioles in vitro and the autoregulation of renal blood flow in vivo.3,4 Changes in the production of 20-HETE also contribute to the vasoconstrictor response to angiotensin II and the vasodilator response to nitric oxide in the renal circulation.5,6 However, the mechanisms by which 20-HETE inhibits K^+-channel activity and vascular tone, particularly in the renal circulation, remain to be determined.

There is evidence that 20-HETE activates protein kinase C (PKC) and that the inhibitory effects of 20-HETE on K^+-channel activity in cat cerebral vascular smooth muscle (VSM) are dependent on this pathway.7 Others have reported that the inhibitory effect of 20-HETE on Na^+-K^+-ATPase activity in renal proximal tubular cells is also PKC-dependent.8 On the other hand, 20-HETE has been reported to activate the mitogen-activated protein kinase (MAPK) signaling cascade, and there is increasing evidence that stimulation of the production of 20-HETE contributes to the growth-promoting actions of several mitogens (eg, epidermal growth factor [EGF], norepinephrine).9,10 There are also data suggesting that several growth factors and components of the MAPK signaling cascade can mimic the effects of 20-HETE on K^+ channels and promote vasoconstriction.11–13 Therefore, the present study examined the hypothesis that activation of PKC, components of the MAPK signaling pathway, or both, contributes to the inhibitory effects of 20-HETE on K^+ channel activity and its vasoconstrictor effects in the renal microcirculation.

Methods

Experiments were performed on 10- to 13-week-old male Sprague-Dawley rats purchased from Harlan Sprague Dawley Laboratories.
Effects of 20-HETE on PKC and MAPK Activity in Renal Arterioles

Preglomerular renal arterioles were isolated from the kidneys of rats with the Evans blue sieving procedure. Intact renal microvessels were resuspended in physiological salt solution and incubated with vehicle or 20-HETE (0.1 to 50 μmol/L) with or without PKC or tyrosine kinase inhibitors for 20 minutes at 37°C. The vessels were then pelleted by centrifugation and homogenized in a lysis buffer containing 50 mmol/L Tris-HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L NaF, 1 mmol/L phenethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin. The homogenate was centrifuged at 5000g for 5 minutes and 9000g for 15 minutes, and the supernatant was used in all assays.

PKC activity was measured with a Spinkzyme assay kit (Pierce) with the N-acetylated portion of myelin protein basic protein (X-PLSRTLVAAKK; where X=Lissamine Rhodamine B) as a substrate. Vessel homogenates (20 μg protein) were incubated with this substrate for 30 minutes at 30°C in a reaction containing (in mmol/L) the following: 2 ATP, 10 MgCl₂, 0.1 CaCl₂, 20 Tris (hydroxymethyl)aminomethane, 0.2 phosphatidyl L-serine, and 0.002% Triton X-100, pH 7.4. The reactions were loaded onto a spin column that binds phosphorylated substrate and washed with a buffer containing 25 mmol/L Tris-HCl and 0.15 mol/L NaCl, pH 7.2. The phosphorylated substrate was eluted with the use of 0.1 mol/L NH₄HCO₃ and 0.02% sodium azide, pH 8. The fluorescence of the samples was measured with a microtiter plate fluorescence spectrophotometer at wavelengths of 573 nm (excitation) and 589 nm (emission).

The effects of 20-HETE on the MAPK system were assessed by determining the levels of active extracellular signal-regulated kinase 1 (ERK1) and ERK2 proteins with an antibody that recognizes the phosphorylated forms of these proteins (Promega). Twenty micrograms of vessel homogenate was separated on a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were blocked overnight in TBS containing 10% nonfat dry milk and incubated with a 1:20 000 dilution of an anti–dual phosphorylated MAPK antibody (Promega) for 2 hours. The membranes were then incubated with a horseradish peroxidase–conjugated secondary antibody (1:10 000 dilution; Santa Cruz) and developed with a chemiluminescent substrate (Supersignal; Pierce). The membranes were then incubated with the N-acetylated portion of myelin basic protein (X-PLSRTLVAAKK) and the N-acetylated portion of myelin basic protein with the N-acetylated portion of myelin basic protein (X-PLSRTLVAAKK).

Isolated Vessel Experiments

The role of PKC and the MAPK pathway to the vasoconstrictor effects of 20-HETE was studied with the use of isolated renal interlobular arterioles (ID, <100 μm). The vessels were microdissected from the kidneys of rats, mounted on glass micropipettes, and placed in physiological salt solution equilibrated with 95% O₂/5% CO₂ and maintained at 37°C. Intraluminal pressure was maintained at 90 mm Hg. Indomethacin (5 μmol/L), baicalein (0.5 μmol/L), and 17-ODYA (20 μmol/L) were added to the bath to block the endogenous metabolism of arachidonic acid via the cyclooxygenase, lipooxygenase, and cytochrome P-450 pathways.

After a 30-minute equilibration period, cumulative concentration-response curves for 20-HETE (10⁻⁶ to 10⁻⁷ mol/L) were constructed before and after blockade of PKC activity with N-myristoylated PKC pseudosubstrate inhibitor (Myr-PKCi) (100 μmol/L) or calphostin C (0.5 μmol/L); the tyrosine kinase pathway with genistein (30 μmol/L) or tyrphostin 25 (10 μmol/L); or mitogen-activated extracellular signal-regulated kinase (MEK) with PD98059 (10 μmol/L). Additional experiments were performed with the inactive tyrosine kinase inhibitor analogues daidzein (30 μmol/L) and tyrphostin 1 (10 μmol/L) to control for nonspecific effects.

Statistical Analysis

Values are expressed as mean±SEM. The significance of the differences in mean values within and between groups was examined using ANOVA for repeated measures followed by a Duncan’s multiple range test. P<0.05 with a 2-tailed test was considered to be significant.

Results

Effects of 20-HETE on PKC and the MAPK System

The results of these experiments are presented in Figure 1. At concentrations of 0.1 to 10 μmol/L 20-HETE had no significant effect on PKC activity in renal microvessels. At a higher concentration (50 μmol/L), 20-HETE increased PKC activity...
by approximately 30%. Arachidonic acid had a similar effect, and it was just as potent a stimulator of PKC as 20-HETE.

The results of immunoblot experiments to determine the effects of 20-HETE on the expression of the active forms of MAPK (P44/ERK1 and P42/ERK2) are presented in Figure 2. Two immunoreactive bands were detected at 42 and 44 kDa in homogenates of renal microvessels. These bands correspond to the active forms of ERK1 and ERK2. 20-HETE increased the expression of these proteins in a concentration-dependent manner. The threshold concentration needed to increase active ERK levels was in the nanomolar range, which corresponds to the concentrations needed to constrict renal microvessels. At higher concentrations (5 μmol/L), 20-HETE produced a fivefold increase in the levels of active ERK1 and ERK2, and it was equally as effective as EGF in stimulating this pathway. The ability of 20-HETE to stimulate phosphorylation of ERK1 and ERK2 proteins in renal microvessels was blocked by the tyrosine kinase inhibitors tyrphostin 25 and genistein (30 μmol/L); TYR, tyrphostin 25 (10 μmol/L); PD, PD98059 (10 μmol/L); CAL, calphostin C (10 μmol/L); PKCi, Myr-PKCi (100 μmol/L); and CON, control.

Isolated Vessel Studies
The results of these experiments are presented in Figure 3. 20-HETE (10 to 1000 nmol/L) reduced the diameter of renal interlobular arteries in a concentration-dependent manner. Blockade of PKC with Myr-PKCi (100 μmol/L) and calphostin C (0.5 μmol/L) had no significant effect on the vasoconstrictor response to 20-HETE. In contrast, blockade of tyrosine kinase activity with genistein (30 μmol/L), tyrphostin 25 (10 μmol/L), and the MEK inhibitor PD98059 (10 μmol/L) on the vasoconstrictor response to 20-HETE in rat renal interlobular arteries. Values are mean±SEM. Numbers in parentheses indicate the number of vessels studied. *P<0.05 indicates a significant difference from control.

Patch-Clamp Experiments
We also examined the effects of 20-HETE on K⁺-channel activity (Figure 4, top). Three types of K⁺ channels with large (8.0±0.3 pA), intermediate (6.5±0.1 pA), and small (2.8±0.1 pA) conductances were recorded from cell-attached patches on renal VSM cells under control conditions. In 6 cells, 20-HETE (100 nmol/L) reduced the NPo of the large-conductance K⁺ channel that we had previously identified in renal VSM cells as the KCa channel on the basis of its conductance and sensitivity to inhibitors.² Mean open time
fell from 2.2±0.2 to 0.7±0.1 ms, and the number of channel openings fell from 103±23 to 21±11 events per 2 minutes. The unitary current of this channel was not significantly altered by 20-HETE. The effects of blockade of the PKC and tyrosine kinase pathways on the inhibitory effects of 20-HETE on this channel are presented in Figure 4, bottom. Blockade of PKC activity with Myr-PKCi or of tyrosine kinase activity with genistein increased baseline NPo of the large-conductance K⁺ channel in renal arteriolar VSM cells. NPo was unchanged by 20-HETE. The effects of blockade of the PKC and tyrosine kinase pathways both exerted a tonic inhibitory influence on the activity of the Kₐᵥ channel in renal VSM cells. However, the PKC inhibitor had no significant effect on the inhibitory actions of 20-HETE on this channel, NPo still fell by 63.6±3.8% in cells pretreated with the PKC inhibitor Myr-PKCi (n=6 cells). In contrast, genistein markedly attenuated the inhibitory effect of 20-HETE on this channel, and NPo fell only 5.5±4.1% after 20-HETE.

Discussion
The present study examined the role of PKC and the MAPK system in mediating the inhibitory effects of 20-HETE on K⁺-channel activity and its vasoconstrictor effects in the renal microcirculation. The results indicate that 20-HETE and arachidonic acid increase PKC activity but that the concentration required exceeds that needed to maximally constrict renal arterioles. Moreover, inhibition of PKC activity with Myr-PKCi had no significant effect on the ability of 20-HETE to reduce K⁺-channel activity in renal arteriolar VSM cells or on the renal vasoconstrictor response to 20-HETE. The failure of this inhibitor to alter the vasoconstrictor response to 20-HETE was not due to an inability to block PKC activity. Indeed, the Myr-PKCi reduced baseline PKC activity in renal microvessels by 68% and completely blocked 20-HETE–induced activation of PKC. It also blocked the vasoconstrictor response to phorbol esters in renal arteries. Similar results were observed when the chemically dissimilar PKC inhibitor calphostin C was used; it also had no effect on the vasoconstrictor response to 20-HETE in renal arterioles. These findings indicate that activation of PKC does not mediate the vasoconstrictor response to 20-HETE in the renal microcirculation of the rat.

Overall, the results of the present study were unexpected in that we have previously reported that the inhibitory effects of 20-HETE on K⁺-channel activity and the vasoconstrictor response to 20-HETE in cat cerebral arteries are mediated by PKC. Also, it is fairly well established that activation of PKC mimics the inhibitory effects of 20-HETE on K⁺-channel activity and increases myogenic tone and vascular responsiveness in most vascular beds. The reason for the difference in the PKC dependence of the vasoconstrictor response to 20-HETE in renal versus cerebral arteries remains to be determined. However, it is possible that there may be differences in the expression of PKC isoforms, the types of K⁺ channels in the renal vasculature of the rat versus the cerebral vasculature of the cat, or both that influence the vascular response to 20-HETE.

The present study also examined the effects of 20-HETE on the MAPK signaling cascade and the contribution of this system to its vasoconstrictor response. We found that 20-HETE activates the MAPK pathway in renal arterioles as effectively as the classic stimulus EGF. Moreover, the concentration of 20-HETE needed to activate this pathway corresponds well to those needed to inhibit K⁺-channel activity and to constrict renal vessels in vitro. In other experiments, we found that 2 chemically and mechanistically different inhibitors of tyrosine kinase blocked the activation of the MAPK system and the effects of 20-HETE on K⁺-channel activity and vascular tone. In contrast, a specific inhibitor of MEK, at a concentration that completely blocked the phosphorylation of ERK1 and ERK2 in renal microvessels, had no effect on the vasoconstrictor response to 20-HETE. These findings suggest that 20-HETE stimulates the MAPK pathway and that activation of a tyrosine kinase contributes to the inhibitory effects of 20-HETE on K⁺-channel activity and vascular tone. However, the tyrosine kinases involved are proximal to MEK in this cascade, because the PD98059 had no effect on the vasoconstrictor response to 20-HETE.

Our finding that 20-HETE activates the MAPK system in renal arterioles is consistent with recent results indicating that...
20-HETE activates this pathway and is a potent mitogen in rat aortic VSM cells.\textsuperscript{10} It is also consistent with a number of reports indicating that 20-HETE mediates the growth-promoting effects of other mitogens such as EGF and norepinephrine in a variety of cell types.\textsuperscript{3,10}

Activation of the MAPK system activates a number of tyrosine kinases that are important in mediating the effects of mitogens on cell growth and oncogenesis.\textsuperscript{15} Increasingly, there is evidence that these kinases also influence $K^+$-channel activity and vascular tone.\textsuperscript{16} This view is supported by the observations that growth factors such as platelet-derived growth factor and EGF constrict VSM.\textsuperscript{11,12} and that this constrictive response can be blocked by tyrosine kinase inhibitors.\textsuperscript{17}

Others have reported that the vasoconstrictor responses to angiotensin II, serotonin, and norepinephrine can also be attenuated by tyrosine kinase inhibitors.\textsuperscript{18–20} The view that there is emerging is that tyrosine kinases activated secondary to stimulation of the MAPK signal transduction cascade play an important role in the regulation of $K^+$-channel activity and vascular tone. Thus, it is feasible that the vasoconstrictor effects of 20-HETE in renal arterioles could also be linked to activation of this pathway.

The mechanism by which 20-HETE activates the MAPK system and the tyrosine kinases involved in phosphorlyating $K^+$ channels and altering vascular tone remains to be determined. This pathway is generally thought to be activated by agonists binding to membrane-bound receptors. However, there is no compelling evidence for the existence of a 20-HETE receptor. One possibility is that 20-HETE may directly bind to and activate ras or raf proteins in the MAPK pathway, because these proteins are known to be activated by arachidonic acid and other lipids that inhibit $K^+$-channel activity.\textsuperscript{21–23} Activation of small G proteins then increases tyrosine kinase activity.

In summary, 20-HETE is a potent activator of the MAPK signal transduction pathway, and activation of a tyrosine kinase proximal to MEK contributes to the inhibitory effects of 20-HETE on $K^+$-channel activity and its vasoconstrictor response in renal arterioles.

References


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