Afferent Arteriolar Vasodilation to the Sulfonimide Analog of 11,12-Epoxycosatrienoic Acid Involves Protein Kinase A


Abstract—The current study determined the contribution of protein kinase-A (PKA) and protein kinase-G (PKG) to the vasodilation elicited by the N-methylsulfonimide analog of 11,12-epoxycosatrienoic acid (11,12-EET). Experiments were performed, in vitro, using the juxtamedullary nephron preparation combined with videomicroscopy. The response of afferent arterioles to the sulfonimide analog of 11,12-EET, was determined before and after inhibition of PKA, PKG, or guanylyl cyclase. Afferent arterioles, preconstricted with 0.5 μmol/L norepinephrine, averaged 18±1 μm (n=25) at a renal perfusion pressure of 100 mm Hg. Superfusion with 0.01 to 100 nmol/L of the 11,12-EET analog caused a graded increase in diameter of the afferent arteriole. Vessel diameter increased by 11±1% and 15±1%, respectively, in response to 10 and 100 nmol/L of the 11,12-EET analog. The afferent arteriolar response to 10 and 100 nmol/L of the 11,12-EET analog was significantly attenuated during inhibition of PKA with 10 μmol/L H-89 (n=7) or 5 μmol/L myristilated PKI (n=6), such that afferent arteriolar diameter increased by only 5±2% and 2±1%, respectively, in response to 100 nmol/L of the 11,12-EET analog. In contrast, the afferent arteriolar vasodilatory response to the 11,12-EET analog was unaffected by PKG or guanylyl cyclase inhibition. In the presence of 200 μmol/L histone H2B (n=5) or 10 μmol/L ODQ (n=7), the afferent arteriolar diameter increased by 16±3% and 12±2%, respectively, in response to 100 nmol/L of the 11,12-EET analog. These results demonstrate that activation of PKA is an important mechanism responsible for the afferent arteriolar vasodilation elicited by the sulfonimide analog of 11,12-EET.

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Key Words: metabolites, cytochrome P450 ■ kidney ■ endothelium-derived hyperpolarizing factor ■ arterioles ■ cyclic adenosine monophosphate ■ cyclic guanosine monophosphate

Epoxycosatrienoic acids (EET) produced by the kidney affect both renal blood flow and tubular transport function. In this regard, renal enzymatic epoxygenase activity increases in some strains of rats on a high-salt diet and has been shown to be altered during the development of hypertension. Thus, renal epoxygenase metabolites have been implicated in the maintenance of water and electrolyte balance.

More recently, EETs have been demonstrated to mediate agonist-induced endothelium-dependent vasodilation and have been considered an endothelium-derived hyperpolarizing factor (EDHF). In the renal circulation, EDHF appears to mediate a large portion of the vasodilatory response to bradykinin and an endothelial-derived cytochrome P450 metabolite of arachidonic acid has been implicated as an EDHF. We previously demonstrated that 11,12-EET and 14,15-EET vasodilate interlobular arteries and afferent arterioles. Additionally, the major epoxide produced by the rat kidney, 11,12(R, S)-EET, vasodilated the interlobular arteries and afferent arterioles whereas, 11,12(S, R)-EET did not affect microvascular tone. Vasodilation to 11,12-EET resulted from a direct action of the epoxide on pregglomerular vascular smooth muscle and 11,12-EET has also been shown to dilate renal arteries and activate Ca2+-activated K+ channels (Kca). These actions of 11,12-EET on renal vessels and Kca channels are consistent with the possibility that 11,12-EET is an EDHF.

Vasodilation and activation of vascular smooth muscle K+ channels can occur via stimulation of cAMP-dependent and cGMP-dependent protein kinases. Therefore, the present study determined the contribution of cAMP- and cGMP-dependent protein kinases in mediating the 11,12-EET effect on afferent arteriolar diameter. Experiments were performed with the newly synthesized N-methylsulfonimide analog of 11,12-EET which is designed to resist esterification and β-oxidation while retaining full biological activity. Preglomerular vascular responses to the 11,12-EET analog and the involvement of protein kinases were determined utilizing the in vitro perfused juxtamedullary nephron preparation.

Methods

Vascular Preparation

Experiments were performed on male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) weighing an average of 408...
Synthesis of the N-Methylsulfonylamine Analog of 11,12-EET

(±)-11,12-EET was prepared as previously described. The (±)-11,12-EET (18 mg, 0.05 mmol) and N-hydroxysuccinimide (7.1 mg, 0.06 mmol) were added to a dry reaction vessel and dissolved in dry tetrahydrofuran (1 mL) and cooled to 0°C. To this solution, 1.0 mL of a mixture of N,N-diisopropylethylamine (47.6 mg, 0.5 mmol), and 4-dimethylaminopyridine (6.1 mg, 0.05 mmol) were added, and the mixture was stirred. After 12 hours at room temperature, the mixture was filtered and the residue purified via preparative TLC using EtOAc/hexane (2:1). The residue was then recrystallized from diethyl ether/hexane to give a colorless solid.

Afferent Arteriolar Vasodilation to the 11,12-EET Analog

After preconstriction with norepinephrine, the afferent arteriolar response to the cell permeable cAMP analog was determined. The ability of H-89 or myristolated PKI to inhibit the afferent arteriolar vasodilatory response to the 11,12-EET analog was determined. The PKA inhibitor H-89 or myristylated PKI was administered to the perfusate and superfusate for 20 minutes to ensure complete blockade. The afferent arteriolar dose response to the 11,12-EET analog was then repeated in the presence of protein kinase A (PKA) inhibition with H-89 (10 μmol/L; Biomol) or myristoylated PKI (14–22 amide (5 μmol/L; Biomol). The response of the afferent arterioles to the 11,12-EET analog was also determined before and after inhibition of protein kinase G (PKG) with histone H2B (29–35) (200 μmol/L; Biomol). The response of the afferent arterioles to the 11,12-EET analog was also determined before and after inhibition of protein kinase G (PKG) with histone H2B (29–35) (200 μmol/L; Biomol). The response of the afferent arterioles to the 11,12-EET analog was also determined before and after inhibition of protein kinase G (PKG) with histone H2B (29–35) (200 μmol/L; Biomol). The response of the afferent arterioles to the 11,12-EET analog was also determined before and after inhibition of protein kinase G (PKG) with histone H2B (29–35) (200 μmol/L; Biomol). The response of the afferent arterioles to the 11,12-EET analog was also determined before and after inhibition of protein kinase G (PKG) with histone H2B (29–35) (200 μmol/L; Biomol). No differences in repeat afferent arteriolar responses to the N-methylsulfonylamine analog of 11,12-EET (n=4) were observed in time control experiments.

Additional experiments were performed to determine the effectiveness of the protein kinase inhibitors for blocking protein kinase activity. The ability of H-89 or myristoylated PKI to inhibit the afferent arteriolar vasodilatory response to the cell permeable cAMP analog, 8-bromo-cAMP was determined. After the afferent arteriolar response to 8-bromo-cAMP (1 nmol/L to 100 μmol/L; Biomol) was determined, the PKA inhibitor H-89 or myristoylated PKI was administered to the perfusate and superfusate for 20 minutes to ensure complete blockade. The afferent arteriolar dose response to the 11,12-EET analog was then repeated in the presence of protein kinase A (PKA) inhibition with H-89 (10 μmol/L; Biomol) or myristoylated PKI (14–22 amide (5 μmol/L; Biomol). The response of the afferent arterioles to the 11,12-EET analog was also determined before and after inhibition of protein kinase G (PKG) with histone H2B (29–35) (200 μmol/L; Biomol). No differences in repeat afferent arteriolar responses to the N-methylsulfonylamine analog of 11,12-EET (n=4) were observed in time control experiments.

Statistics

Data are presented as mean±SEM. Significance of differences in mean values for the dose-response effect was evaluated by analysis of variance for repeated measures followed by Duncan’s multiple range test. A value of *P<0.05 was considered statistically significant.

Results

Effect of PKA Inhibitors on the Afferent Arteriolar Response to the 11,12-EET Analog

Initial studies were performed comparing the vascular activity of 11,12-EET and the N-methylsulfonylamine analog of 11,12-EET on afferent arteriolar diameter. Both 11,12-EET and the sulfonylamine analog of 11,12-EET elicited similar vasodilatory responses. Afferent arteriolar diameter averaged 18±2 μm (n=3) and increased by 11±2% and 15±3% in response to 10 and 100 nmol/L of the 11,12-EET analog. Likewise, afferent arteriolar diameter increased by 12±3% and 17±4% in response to 10 and 100 nmol/L 11,12-EET.

Figure 1 depicts the effect of the PKA inhibitor, H-89, on the afferent arteriolar vasodilatory response to the 11,12-EET analog. Afferent arteriolar diameter increased after superfusion with the 11,12-EET analog and reached a steady state within 2 minutes. In the presence of the PKA inhibitor H-89,
the afferent arteriolar vasodilation in response to the sulfonimide analog of 11,12-EET was significantly attenuated. The effect of PKA inhibition on the steady state responses to the 11,12-EET analog is presented in Figure 2. The vasodilatory response to 100 nmol/L of the 11,12-EET analog averaged 13 ± 2% and was greatly attenuated in the presence of H-89 and averaged 5 ± 2%. Likewise, the increase in the afferent arteriolar diameter to 100 nmol/L of the 11,12-EET analog averaged 15 ± 4% and was significantly attenuated by the PKA inhibitor, myristolated PKI. In the presence of myristolated PKI, 100 nmol/L of the 11,12-EET analog increased the afferent arteriolar diameter by only 2 ± 1%.

To control for the possibility that PKA inhibition might nonspecifically interfere with microvascular vasodilatory responses, we confirmed the ability of these arterioles to vasodilate in response to acetylcholine. Acetylcholine (1 μmol/L), administered at the end of the experiment, increased afferent arteriolar diameter by 17 ± 2% (n = 2) and 21 ± 5% (n = 2) in the presence of H-89 and myristolated PKI, respectively.

**Effect of PKG and Guanylyl Cyclase Inhibition on the Afferent Arteriolar Response to the 11,12-EET Analog**

Figure 3 presents the effect of the PKG inhibitor (left panel) and the guanylyl cyclase inhibitor, ODQ (right panel), on the afferent arteriolar response to N-methylsulfonimide analog of 11,12-EET. Afferent arteriolar diameter averaged 19 ± 1 μm (n = 12) and increased by 15 ± 1% in response to superfusion of 100 nmol/L of the 11,12-EET analog. The PKG inhibitor, histone H2B, did not significantly alter the vasodilation and afferent arteriolar diameter increased by 15 ± 3% in response to 100 nmol/L of the 11,12-EET analog during PKG inhibition. The afferent arteriolar response to the sulfonimide analog of 11,12-EET was not altered by the guanylyl cyclase inhibitor ODQ. In the presence of ODQ, afferent arteriolar diameter increased by 12 ± 2% in response to 100 nmol/L of the 11,12-EET analog.

**Effect of Protein Kinase Inhibitors on the Afferent Arteriolar Vasodilation Response to 8-Bromo Analogs**

The effect of the PKA inhibitors, H-89 and myristolated PKI, on the afferent arteriolar vasodilation to 8-bromo-cAMP is depicted in the top panel of Figure 4. 8-Bromo-cAMP dose-dependently increased afferent arteriolar diameter. The afferent arteriolar vasodilatory response to 8-bromo-cAMP was significantly attenuated in the presence of PKA inhibition. 8-bromo-cAMP (100 μmol/L) increased afferent arteriolar diameter by 11 ± 3% before the addition of PKA inhibitors and decreased diameter by 2 ± 2% and 1 ± 1% in the presence of H-89 and myristolated PKI, respectively.

Experiments were also performed to determine the ability of histone H2B to inhibit the afferent arteriolar vasodilation to 8-bromo-cGMP. Afferent arteriolar diameter increased dose dependently in response to 8-bromo-cGMP (Figure 4, bottom panel). The afferent arteriolar diameter response to 10 and 100 μmol/L 8-bromo-cGMP was 9 ± 2% and 10 ± 3%, respectively, under control conditions. In contrast, in the presence of histone H2B, these responses were attenuated to 2 ± 2% and 1 ± 1%, respectively.
which is designed to resist esterification and juxtamedullary nephron preparation. The 11,12-EET analog, EET on the afferent arteriole using the in vitro blood-perfused the newly synthesized analog of 11,12-EET. We directly examined the response of arteriole, whereas inhibition of PKG or guanylyl attenuated the 11,12-EET analog-mediated vasodilation of the afferent arteriole, whereas inhibition of PKG or guanylyl cyclase did not alter the response. These data suggest that the increase in afferent arteriolar diameter elicited by the 11,12-EET analog involves activation of the cAMP-dependent protein kinase pathway.

**Discussion**

The present study determined the contribution of cAMP- and cGMP-dependent protein kinase pathways to the afferent arteriolar vasodilation elicited by the N-methylsulfonimide analog of 11,12-EET. We directly examined the response of the newly synthesized N-methylsulfonimide analog of 11,12-EET on the afferent arteriole using the in vitro blood-perfused juxtamedullary nephron preparation. The 11,12-EET analog, which is designed to resist esterification and β-oxidation, had very similar biological properties compared with 11,12-EET. In cultured renal epithelial cells, the sulfonimide analogs of 11,12-EET and 14,15-EET have the same mitogenic activity and utilize the same cellular signaling pathways as their endogenous counterparts. Likewise, the addition of the N-sulfonimide to cp450 inhibitors did not change the potency or selectivity of their inhibitory activity. In the present study, the afferent arteriolar diameter increased dose-dependently in response to superfusion of the 11,12-EET analog and this response paralleled the vasodilation observed with the native 11,12-EET. Inhibition of PKA significantly attenuated the 11,12-EET analog-mediated vasodilation of the afferent arteriole, whereas inhibition of PKG or guanylyl cyclase did not alter the response. These data suggest that the increase in afferent arteriolar diameter elicited by the 11,12-EET analog involves activation of the cAMP-dependent protein kinase pathway.

EETs are synthesized by endothelial cells, activate vascular smooth muscle K⁺ channels, hyperpolarize vascular smooth cells, and vasodilate blood vessels. Thus, EETs have been considered an EDHF. The major epoxygenase metabolites of arachidonic acid produced in the kidney are 11,12-EET and 14,15-EET. Renal microvessels and microsomes prepared from these vessels metabolize arachidonic acid to 11,12-EET and 14,15-EET and their corresponding diols. Recently, it has been suggested that 11,12-EET is the EDHF that mediates a large proportion of the bradykinin-induced vasodilation in coronary and renal arteries. 11,12-EET elicits endothelium- and cyclooxygenase-independent vasodilation of the pregglomerular vasculature, whereas the epoxide hydrolase metabolite 11,12-dihydroxyeicosatrienoic acid did not significantly affect afferent arteriolar tone. The vasodilation of larger caliber renal arteries by 11,12-EET was significantly attenuated by K⁺ channel inhibitors. Likewise, 11,12-EET increased the outward K⁺ current of the large conductance Kᵥ channel active in renal arterial vascular smooth muscle cells. In these experiments, the large conductance Kᵥ channel activity was unaltered in renal arteriolar smooth muscle cells exposed to 11,12-EET on the cytoplasmic or external face of excised membrane patches. This suggests that cytoplasmic signal transduction factors are required for activation of K⁺ channels by 11,12-EET. The results of the present study demonstrate that vasodilation of afferent arterioles by the sulfonimide analog of 11,12-EET requires activation of the cAMP-dependent protein kinase pathway and suggest that PKA participates as a cellular signaling factor for 11,12-EET to activate K⁺ channels.

The ability of 11,12-EET to relax vascular smooth muscle and control organ blood flow is not limited to the renal vasculature. However, the ability of EETs to vasodilate does appear to depend on the vasculature from which the vessels are isolated. EETs do not affect cell membrane potential or cause relaxation of internal carotid arteries isolated from guinea pig, whereas coronary and cerebral arteries generate epoxygenase metabolites and, like the renal vasculature, 11,12-EET causes vasodilation. The vasodilatory action of 11,12-EET on the cat cerebral vasculature is associated with activation of vascular smooth muscle cell Kᵥ channels. In contrast, the 11,12-EET-induced vasodilation of the newborn pig cerebral circulation requires an intact prostanoi system and prostacyclin receptor activation.

In freshly isolated coronary artery smooth muscle cells, 11,12-EET causes membrane hyperpolarization and appears to be selective for the Kᵥ channel since the delayed rectifier K⁺ (K₁) channels were unaffected by 11,12-EET. Interestingly, the prostaglandin I₂ analog iloprost increases the activity of the Kᵥᵩ channels without affecting the Kᵥ channels in the coronary vasculature. Activation of the coronary artery Kᵥ channels by 11,12-EET appears to be due to activation of a GTP binding protein, G, and depends on cytoplasmic signaling factors.

Although Gₛ can activate adenyl cyclase and cause PKA-dependent phosphorylation of the Kᵥ channel, bovine coronary artery cAMP levels were not significantly affected by incubation with 11,12-EET. This finding is not consistent with the observation of the present study that the
11,12-EET analog-mediated vasodilation of the afferent arteriole is greatly attenuated by 2 chemically distinct inhibitors of PKA. One possible explanation for this discrepancy could be that the sensitivity of the cAMP assay was not sufficient to allow for detection of physiologically significant increases in activity. 11,12-EET activation of the cAMP-dependent pathway has been observed in heart cells. 37 Ventricular myocytes incubated with 11,12-EET did significantly elevate intracellular cAMP levels and in this cell type 11,12-EET may act to modulate L-type Ca2+ channel current. 37 Another possibility is that the vascular tissue cAMP measurements have been done on larger caliber vessels that appear to be less sensitive to the vasorelaxant effects of EETs 13 and therefore may not utilize the cAMP pathway to the same extent as smaller caliber vessels. Further studies are required to clearly delineate the cellular-signaling steps responsible for the afferent arteriolar vasodilation in response to 11,12-EET.

Recent studies have shown that PKG can be the mediator of not only cGMP-dependent but also cAMP-dependent vasodilators. 38 The present results demonstrate that inhibition of PKG or guanylyl cyclase did not affect the afferent arteriolar vasodilation to the sulfonimide analog of 11,12-EET. This finding is supported by a number of previous studies which have demonstrated that the cGMP-dependent protein kinase pathway is not involved in the vasodilation produced by endothelial epoxyenase metabolites. In the rat kidney, arachidonic acid is metabolized by the endothelium to a cP450 vasodilatory product and this vasorelaxation was not influenced by guanylyl cyclase inhibition. 39 Likewise, epoxyenase metabolites, when incubated with bovine coronary arteries, significantly decreased tissue cGMP content. 13 In addition, brefeldin A inhibited the EDHF-mediated vasorelaxation and production of EDHF elicited by bradykinin in porcine coronary arteries but did not affect the accumulation of cGMP. 40 Thus, the results of the current study and those of previous studies do not support a role for the cGMP-dependent protein kinase pathway in the vasodilatory response to 11,12-EET.

In summary, the N-methylsulfonimide analog of 11,12-EET vasodilated afferent arterioles of juxtamedullary nephrons. The afferent arteriolar vasodilation elicited by the 11,12-EET analog was significantly attenuated by inhibitors of PKA but was unaltered by inhibition of PKG or guanylyl cyclase. These results demonstrate that activation of PKA is an important intracellular-signaling mechanism responsible for the afferent arteriolar vasodilation elicited by the sulfonimide analog of 11,12-EET.

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