12-Hydroxyeicosatetraenoic acid participates in angiotensin II afferent arteriolar vasoconstriction by activating L-type calcium channels

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Abstract **The lipoxygenase (LO) metabolite, 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE], constricts renal vessels, contributes to the vascular response to angiotensin, and has been implicated in cardiovascular and renal diseases. The current studies were performed to determine if renal microvascular 12(S)-HETE production is stimulated by angiotensin and the contribution of L-type calcium channels to the vasoconstriction elicited by 12(S)-HETE. Angiotensin increased renal microvascular 12(S)-HETE production by 64%, whereas cyclooxygenase metabolite production was not altered. Renal microvessels also expressed platelet-type 12-LO and leukocyte-type 12-LO. In the juxtamedullary preparation, afferent arteriolar diameter averaged 21 ± 1 μm and 12(S)-HETE caused a graded decrease in vessel caliber. The afferent arteriolar response to 12(S)-HETE was abolished during L-type calcium channel inhibition. Renal microvascular smooth muscle cells were studied using fluorescence microscopy. Renal myocyte $[Ca^{2+}]_i$ averaged 93 ± 5 nmol/l. The 12(S)-HETE (5 μmol/l) increased myocyte $[Ca^{2+}]_i$ to a peak value of 340 ± 55 nmol/l. The peak $[Ca^{2+}]_i$ response following exposure to 12(S)-HETE was greatly attenuated in the absence of extracellular Ca^{2+} or calcium channel blockade. These results demonstrate that renal microvascular 12(S)-HETE production is increased in response to angiotensin, and activation of L-type calcium channels is an important mechanism responsible for the afferent arteriolar vasoconstriction elicited by 12(S)-HETE.**—Yiu, S. S., X. Zhao, E. W. Inscho, and J. D. Imig. **12-Hydroxyeicosatetraenoic acid participates in angiotensin II afferent arteriolar vasoconstriction by activating L-type calcium channels.** ***J. Lipid Res.*** **2003.** 44: **2391–2399.**

Supplementary key words lipoxygenase • cytosolic calcium • microcirculation • kidney

The arachidonate lipoxygenase (LO) product, 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), has been implicated as a key contributor to the pathogenesis of atherosclerosis, hypertension, and diabetic nephropathy (1–4). 12-LO expression has been detected in renal vascular and glomerular cells, and these cells have the capacity to produce 12(S)-HETE (5–8). In addition to its involvement in glomerular inflammation and vascular smooth muscle cell growth, 12(S)-HETE is a renal vasoconstrictor (5, 8–10). 12(S)-HETE decreases renal blood flow and glomerular filtration independent of cyclooxygenase (COX) activity (9). Additionally, the action of angiotensin II on the renal vasculature involves participation of the LO pathway. A previous study by our laboratory demonstrated that the afferent arteriolar constriction to angiotensin II is attenuated in the presence of 12-LO inhibition (11). In contrast, norepinephrine-mediated renal microvascular vasoconstriction was unaffected by 12-LO inhibition (11). One aim of the current study was to determine renal microvascular production of COX metabolites and 12(S)-HETE in response to angiotensin II and norepinephrine.

Although the importance of 12(S)-HETE in renal and cardiovascular disease is now established, the mechanism by which 12(S)-HETE constricts the preglomerular vasculature remains to be identified. It is known that 12(S)-HETE causes depolarization of the vascular smooth muscle cell membrane and may act through activation of protein kinase C to constrict blood vessels (9). Depolarization of vascular smooth muscle should activate L-type calcium channels. Therefore, a second aim of the present study was to determine the contribution of L-type calcium channel activation to the 12(S)-HETE mediated renal vasoconstriction.

METHODS

Renal microvessel 12(S)-HETE and COX metabolite production

Male Sprague-Dawley rats were anesthetized with sodium pentobarbital (40 mg/kg body weight ip), and the abdominal cavity was

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exposed to permit cannulation of the abdominal aorta via the superior mesenteric artery. Ligatures were placed around the abdominal aorta at sites proximal and distal to the left and right renal arteries, respectively. The kidneys were cleared of blood by perfusion of the isolated aortic segment with an ice-cold, low-calcium physiological salt solution (PSS; pH 7.35) of the following composition (in mmol/l): 125 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 10.0 glucose, 20.0 HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 0.1 $CaCl_2$, and 6% BSA. The kidneys were resected and decapsulated, and the renal medullary tissue was removed. Cortical tissue was pressed through a sieve (180 μm mesh), and the sieve retentate was washed repeatedly with ice-cold low-calcium PSS. Renal microvessels were incubated (37°C) with gentle agitation for 1–1.5 h in a Tyrode's solution containing 0.2 mg/ml each of dithiothreitol, collagenase, trypsin inhibitor, and albumin. Microvessels were transferred to a 100 μm nylon mesh and rinsed. Purified microvessels were examined under a stereomicroscope, and short segments were collected for subsequent use. Purity of the collected microvascular sample was assessed by alkaline phosphatase activity measurement as previously described (12).

Isolated renal microvessels (50–100 mg) were incubated for 20 min at 37°C in 2 ml of 1.8 mmol/l calcium PSS gassed with 95% O_2 and 5% CO_2 and agitated at 60 cycles/min. Angiotensin II (100 nmol/l), norepinephrine (1 μmol/l), or vehicle were added to the PSS at the start of the incubation period. The lipids were immediately extracted in ethyl acetate, evaporated to dryness under liquid nitrogen, and stored at −80°C. The amount of prostaglandin E_2 (PGE_2), PGI_2 (measured as 6-keto-$PGF_{1\alpha}$), thromboxane (TXB_2), and 12(S)-HETE released into the medium was measured by enzyme immunoassay (EIA, PerSeptive Diagnostics). Cross reactivity was determined for 12(R)-HETE and 15(S)-HETE and averaged 3.1% and 0.5%, respectively, for the 12(S)-HETE assay.

Renal microvessel LO mRNA and protein expression

Total RNA was prepared from isolated renal microvessels or kidney cortex using an ultra-pure TRIzol reagent according to the procedure described by the manufacturer (Gibco-BRL, Grand Island, NY). Random hexanucleotide primers were used for reverse transcription (RT) of 2 μg RNA. Oligonucleotide primers were designed from the published cDNA sequences of platelet-type (P-12-LO) and leukocyte-type (L-12-LO) 12-LO, type 2 15-LO (15-LO2), and GAPDH. GAPDH was used as an internal standard. The sequences of the P-12-LO primers are sense 5′-GGA CGA TGT GAC GAT GGA-3′ and antisense 5′-CGG CTA GGC TTG AGG TAT-3′. The sequences of the L-12-LO primers are sense 5′-TGG GGC AAC TGG AAG G-3′ and antisense 5′-AGA GCG CTT CAG CAC CAT-3′. The sequences of the 15-LO2 primers are sense 5′-TGT CTG ATG CTG CGA ATA-3′ and antisense 5′-CAC TGG AGC CAC CAT TTC-3′ (13). The sequences of the GAPDH primers are sense 5′-AAT GCA TCC TGC ACC ACC AA-3′ and antisense 5′-GTA GCC ATA TTC ATT GTC ATA-3′. The expected sizes of the amplified P-12-LO, L-12-LO, 15-LO2, and GAPDH polymerase chain reaction (PCR) products are 261, 312, 627, and 515 base pairs, respectively. RT-PCR was performed as previously described (12). After amplification, 15 μl of each PCR reaction mixture were electrophoresed through a 1.5% agarose gel with ethidium bromide (0.5 μg/ml). The gel was scanned with UV illumination using Digital Imaging and Analysis (Alpha Innotech Corporation).

We confirmed the presence of L-12-LO protein expression in renal microvessels with a commercially available antibody. Renal microvessels and kidney cortex were harvested and processed as previously described (12). Samples were separated by electrophoresis on a 10% stacking Tris-glycine gel, and proteins were transferred electrophoretically to a nitrocellulose membrane. The primary antibody used was rabbit anti-murine L-12-LO polyclonal antibody (1:1,000; Cayman Chemical Co.). The blots were then washed in a PBS-0.1% Tween 20 solution and incubated with the secondary antibody (anti-rabbit 1:100,000), conjugated to horseradish peroxidase for 1 h at room temperature, and washed. Detection was accomplished using enhanced chemiluminescence Western blotting (Amersham Corp.), and blots were exposed to X-ray film (Hyperfilm-ECL, Amersham Corp.).

Afferent arteriolar response to 12(S)-HETE

Experiments were performed on male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) weighing an average of 350–450 g. Tulane University and Medical College of Georgia Animal Care and Use Committees approved the experimental procedures. The rats were anesthetized with sodium pentobarbital (40 mg/kg body weight ip), the right carotid artery was cannulated, and a midline abdominal incision was made. The right renal artery of the kidney was cannulated via the superior mesenteric artery, and the kidney was immediately perfused with a Tyrode's solution containing 6% albumin and a mixture of L-amino acids (11).

Blood was collected through the carotid artery cannula into a heparinized syringe (2,000 U). Erythrocytes were separated from plasma and leukocytes by centrifugation and were reconstituted in Tyrode's solution containing 6% albumin to yield a hematocrit of 20%. The reconstituted red blood cell containing solution was filtered and stirred continuously in a closed reservoir that was pressurized by a 95% O_2-5% CO_2 tank. The kidney was removed and maintained in an organ chamber at room temperature throughout the isolation and dissection procedure. The juxtamedullary microvasculature was isolated for study as previously described (11). The Tyrode's solution was replaced by reconstituted blood, and renal artery perfusion pressure was set to 100 mmHg. The organ chamber was warmed and the tissue surface was continuously superfused with a Tyrode's solution containing 1% albumin at 37°C. Following a 20 min equilibration period, an afferent arteriole was chosen for study, and baseline diameter was measured using video-microscopy techniques as previously described (11). Afferent arteriolar inside diameters were measured at 15 s intervals using a digital image-shearing monitor. The image-shearing device is accurate to within 0.2% of the screen width or 0.2 μm and measurement reproducibility is within 0.5 μm.

Following the equilibration period, baseline diameter measurements of the afferent arteriole were made. The arteriole was subsequently exposed to increasing concentrations of 12(S)-HETE, 12(R)-HETE, and 15(S)-HETE (0.01–5 μmol/l), and diameter changes were monitored for 3 min at each concentration. The afferent arteriolar diameter 12(S)-HETE response in the presence of diltiazem was determined to assess the involvement of L-type calcium channels. The kidney was exposed to 10 μmol/l diltiazem for a minimum of 20 min, and the afferent arteriole diameter response to 12(S)-HETE (5 μmol/l) was assessed. Steady-state diameter was attained by the end of the second minute, and the average diameter of the third minute of each treatment period was utilized for statistical analysis.

Signaling mechanisms involved in the renal microvascular smooth muscle cell calcium response to 12(S)-HETE

Male Sprague-Dawley rats were anesthetized with sodium pentobarbital (40 mg/kg body weight ip), and the kidney tissue was collected as described for the renal microvessel production studies. The cortical tissue was pressed through a sieve (180 μm mesh), and the sieve retentate was washed repeatedly with ice-cold low-calcium PSS and enzymatically digested to obtain renal microvascular smooth muscle cells as previously described (14). Dispersed renal microvascular smooth muscle cells were gently suspended in 1.0 ml DMEM supplemented with 20% fetal calf se-

rum, 100 U/ml penicillin, and 200 μg/ml streptomycin. Renal microvascular cell suspensions were stored on ice until use.

Experiments were performed using a standard microscope-based fluorescence spectrophotometry system. The excitation wavelengths were set at 340 nm and 380 nm, and the emitted light was collected at 510 ± 10 nm. Measurements of fluorescence intensity were collected at five data points per second, and the data were collected and analyzed with the aid of Photon Technology International software. Calibration of the fluorescence data was accomplished as previously described (14).

Measurement of $[Ca^{2+}]_i$ in single microvascular smooth muscle cells was performed as previously described (14). Suspensions of freshly isolated renal microvascular cells loaded with the calcium sensitive fluorescent probe, fura 2 acetoxymethyl ester (4.0 μmol/l). An aliquot of cell suspension was transferred to the perfusion chamber and mounted to the stage of a Nikon Diaphot inverted microscope. The cells were continuously superfused (1.3 ml/min) with a 1.8 mmol/l calcium PSS solution of the following composition (in mmol/l): 125 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 10.0 glucose, 20.0 HEPES, 1.8 $CaCl_2$, and 0.1 g/l BSA. For each experiment, a single microvascular cell was isolated in the optical field by positioning the adjustable sampling window directly over the cell of interest. All fluorescence measurements were obtained with background subtraction, and a new coverslip of cells was used for each experiment.

The effects of 12(S)-HETE on $[Ca^{2+}]_i$ were determined by exposing single cells to PSS containing 12(S)-HETE concentrations of 1–5 μmol/l. 12(S)-HETE-mediated responses at each concentration were evaluated by determining the average magnitude of the peak and steady-state $[Ca^{2+}]_i$ achieved. Peak responses were defined as the maximum agonist-induced $[Ca^{2+}]_i$ attained during the 200 s of agonist administration. Steady-state responses were obtained by calculating the average $[Ca^{2+}]_i$ over the last 50 s of agonist administration.

Studies were performed to determine the role of extracellular calcium on the increase in $[Ca^{2+}]_i$ induced by 12(S)-HETE. The contribution of calcium influx to the response was determined by exposing single cells to 12(S)-HETE (5 μmol/l) while being bathed in nominally calcium-free PSS. Previous studies have shown that $[Ca^{2+}]_i$ remains unchanged when preglomerular smooth muscle cells are subjected to strong depolarizing conditions while being bathed in nominally calcium-free conditions. These responses were compared with responses obtained from similar cells challenged in normal-calcium PSS. The role of L-type calcium channels in 12(S)-HETE-mediated calcium responses was assessed with 10 μmol/l diltiazem. We have previously reported that diltiazem prevented the depolarization-induced increase in preglomerular smooth muscle $[Ca^{2+}]_i$ in response to 90 mmol/l KCl (14). Addition of vehicle to the superfusate had no effect on the $[Ca^{2+}]_i$ response elicited by 12(S)-HETE (n = 10 cells from three dispersions).

Statistics

Data are presented as mean ± SEM. The significance of differences in renal microvessel COX metabolite and 12(S)-HETE production was evaluated using an unpaired *t*-test. Differences in mean afferent arteriolar diameters between groups were evaluated with a two-way ANOVA for repeated measures followed by Duncan's multiple range test. Differences within and between groups of renal microvascular smooth muscle cell $[Ca^{2+}]_i$ values were analyzed by

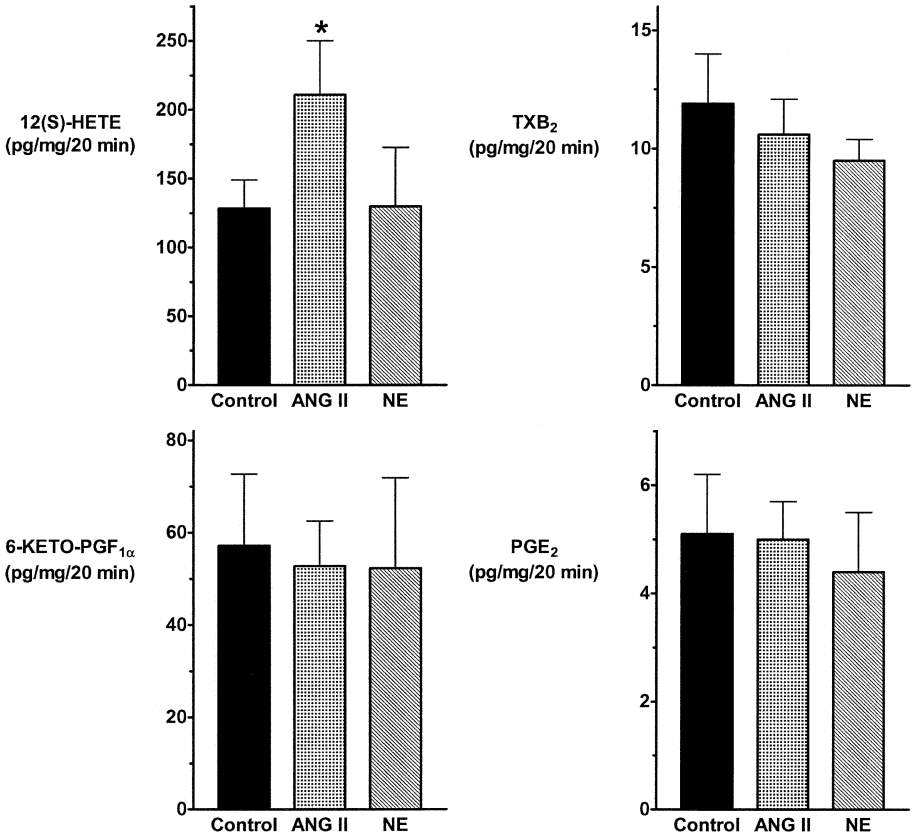

Fig. 1. The effect of angiotensin (ANG II) and norepinephrine (NE) on renal microvascular 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] and COX metabolites production. * Significant difference from control renal microvessel production.

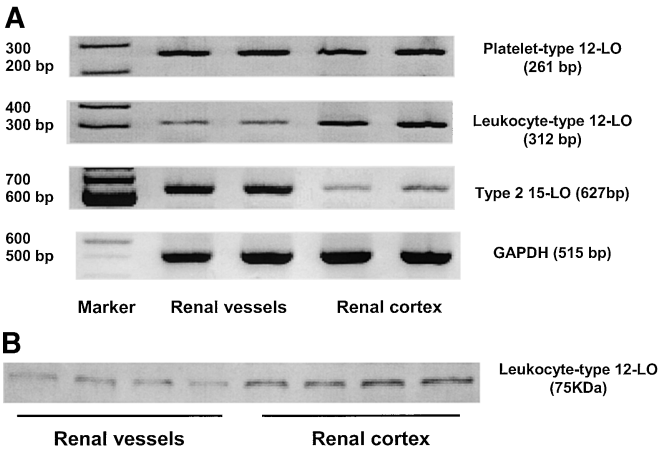

Fig. 2. Renal cortical and microvessel lipoxygenase (LO) mRNA and protein expression. A: RT-PCR analysis of platelet and leukocyte 12-LO, as well as type 2 15-LO mRNA in renal cortex and microvessels. The blots show the expected 261 bp, 312 bp, and 627 bp bands for P-12-LO, L-12-LO, and 15-LO2, respectively. B: Western blot analysis of L-12-LO in renal cortex and microvessels. The blot shows the expected 75 kDa band for L-12-LO in four renal cortical and microvessel samples.

one-way ANOVA followed by Newman-Keuls multiple-range test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Renal microvessel COX and 12(S)-HETE production in response to angiotensin II

We have previously reported that the 12-LO inhibitor, baicalein, attenuated the afferent arteriolar constriction to angiotensin II but did not alter the vasoconstrictor response to norepinephrine (11). In the current study, we provide additional biochemical evidence for the involvement of 12(S)-HETE in the renal microvascular response to angiotensin II. As depicted in **Fig. 1**, angiotensin II significantly increased renal microvessel 12(S)-HETE generation by 64%. In contrast, 12(S)-HETE production by renal microvessels was unaltered when incubated with norepinephrine. Production of the COX metabolites TXB_2, 6-keto-$PGF_{1\alpha}$, or PGE_2 was not changed when renal microvessels were incubated with angiotensin II or norepinephrine. These results provide support for the postulate that 12(S)-HETE is an important mediator of angiotensin II actions on the renal microvasculature. Another set of experiments was conducted to begin identifying the cell signaling pathways influenced by 12(S)-HETE.

Renal microvascular expression of 12-LO and 15-LO

The identity of the LO localized to the renal microvasculature is not known. We demonstrate the presence of at least three LO enzymes in renal microvessels. As depicted in **Fig. 2A**, platelet and leukocyte 12-LO, as well as type 2 15-LO mRNA expression, was detected in the renal cortex and microvessels. Next, we determined the presence of L-12-LO with a commercially available antibody to confirm that mRNA expression translated to protein expression. L-12-LO protein expression was detected in four renal cortical and renal microvascular samples (Fig. 2B). Thus, the renal microvasculature is capable of metabolizing arachidonic acid to 12-HETE and 15-HETE.

Afferent arteriolar diameter response to 12(S)-HETE

Figure 3 depicts the effect of 12(S)-HETE, 12(R)-HETE, and 15(S)-HETE on the diameter of the afferent arteriole. Afferent arteriolar diameter decreased following superfusion with 12(S)-HETE, 12(R)-HETE, and 15(S)-HETE and reached a steady-state diameter by the end of the second minute. 12(R)-HETE and 15(S)-HETE had weak vasoconstrictor activity and decreased afferent arteriolar diameter by 8.1 ± 0.8%. and 7.7 ± 0.8%, respectively, at the highest concentration studied (5 μmol/l). In contrast, 12(S)-HETE (5 μmol/l) produced a robust constriction and reduced the diameter of the afferent arteriole by 20.9 ± 1.4%. Next, we examined the contribution of L-type calcium channels to the 12(S)-HETE-induced preglomerular vas-

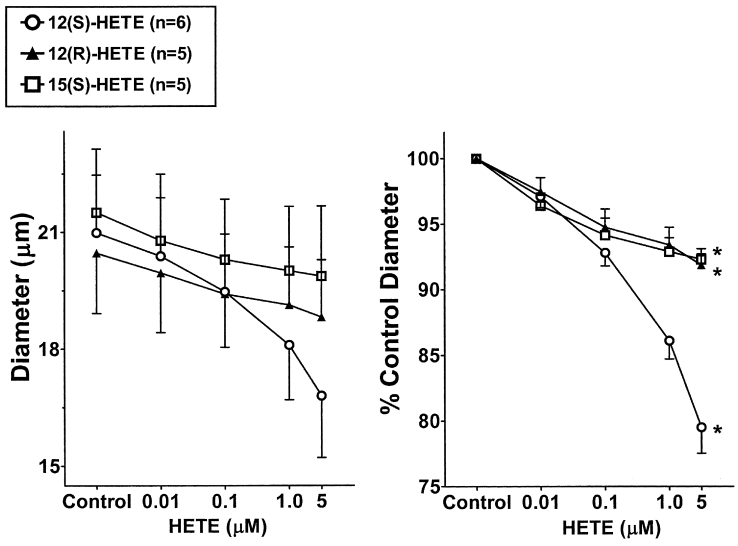

Fig. 3. Cumulative concentration response curve for the effects of 12(S)-HETE on afferent arteriolar diameter (left panel) and percent control diameter (right panel). * Significant difference from control diameter measured at 100 mmHg.

cular constriction. During calcium channel blockade with diltiazem, the afferent arteriolar vasoconstrictor response to 12(S)-HETE was significantly ($P < 0.05$) attenuated. Afferent arteriolar diameter averaged 22.2 ± 1.8 μm and decreased by 1 ± 1% in response to 12(S)-HETE (**Fig. 4**).

Renal microvascular smooth muscle cell calcium response to 12(S)-HETE

A total of 125 single renal microvascular smooth muscle cells prepared from 22 tissue dispersions were examined in the present study. The baseline $[Ca^{2+}]_i$ in cells treated with 12(S)-HETE averaged 93 ± 5 nmol/l (n = 125), and no significant difference was evident in resting $[Ca^{2+}]_i$ between any of the treatment groups.

The first series of experiments determined the effects of 12(S)-HETE on $[Ca^{2+}]_i$ in freshly isolated rat renal microvascular smooth muscle cells. **Figure 5** presents representative traces of the increase in $[Ca^{2+}]_i$ evoked by 1 μmol/l, 3 μmol/l, and 5 μmol/l 12(S)-HETE administered for 200 s. 12(S)-HETE caused a rapid increase in $[Ca^{2+}]_i$ that reached a peak followed by a gradual recovery to a steady state. The peak increase in $[Ca^{2+}]_i$ varied from 30 s to 80 s after 12(S)-HETE administration. In addition, the change in $[Ca^{2+}]_i$ was reversible, and removal of 12(S)-HETE from the bathing solution resulted in a return of $[Ca^{2+}]_i$ to values similar to that of baseline. The average maximum $[Ca^{2+}]_i$ response to each 12(S)-HETE dose is depicted in the Fig. 5 inset. 12(S)-HETE concentrations of 1 μmol/l, 3 μmol/l, and 5 μmol/l increased $[Ca^{2+}]_i$ by 7 ± 1 nmol/l, 217 ± 98 nmol/l, and 321 ± 71 nmol/l, respectively. Sustained $[Ca^{2+}]_i$ elevations observed with 12(S)-HETE concentrations of 1 μmol/l, 3 μmol/l, and 5 μmol/l averaged 3 ± 1 nmol/l, 2 ± 7 nmol/l, and 21 ± 4 nmol/l, respectively, and was significantly greater than baseline at the 5 μmol/l 12(S)-HETE concentration.

We evaluated the role of Ca^{2+} influx in the 12(S)-HETE increase in $[Ca^{2+}]_i$ in single renal microvascular smooth muscle cells. Renal microvascular smooth muscle cells were exposed to a solution containing 5 μmol/l 12(S)-HETE while they were being bathed in Ca^{2+}-free solution. As shown in representative traces, the 12(S)-HETE-mediated increase in $[Ca^{2+}]_i$ was markedly attenuated in cells bathed in Ca^{2+}-free medium (**Figs. 6**, 8). Resting $[Ca^{2+}]_i$ averaged 98 ± 19 nmol/l in the absence of extracellular Ca^{2+} and increased to 179 ± 69 nmol/l after the administration of 12(S)-HETE. The steady-state response to 5 μmol/l 12(S)-HETE was also noticeably reduced when cells were bathed in Ca^{2+}-free solution and averaged 9 ± 7 nmol/l. Next, we determined the effect of calcium channel blockade with diltiazem on the 12(S)-HETE-mediated increase in $[Ca^{2+}]_i$. As depicted in **Figs. 7** and **8**, pretreatment of cells with diltiazem had no detectable effect on $[Ca^{2+}]_i$; however, it greatly reduced the overall response to 12(S)-HETE. Baseline $[Ca^{2+}]_i$ averaged 90 ± 5 nmol/l in a 1.8 mmol/l calcium PSS solution and 88 ± 5 nmol/l after the addition of diltiazem to the bathing medium. Subsequent exposure to 5 μmol/l 12(S)-HETE increased $[Ca^{2+}]_i$ to a peak of 129 ± 16 nmol/l before returning to a steady state $[Ca^{2+}]_i$ of 99 ± 5 nmol/l. The steady-state $[Ca^{2+}]_i$ was not significantly different from the baseline $[Ca^{2+}]_i$ in the presence of diltiazem.

DISCUSSION

The importance of the 12-LO metabolite, 12(S)-HETE, as a significant component in cardiovascular and kidney pathologies has been well established (1–5, 15). Increased cultured vascular smooth muscle and endothelial cell 12(S)-HETE production is elevated by high glucose (2, 16). The 12-LO pathway or 12(S)-HETE levels are elevated in a diabetic pig model, ischemic preconditioned rat hearts, the spontaneously hypertensive rat (SHR), and patients with essential hypertension (1–3, 17). The increased 12(S)-HETE levels in hypertension and diabetes appear to play a key role in mediating the smooth muscle cell hypertrophy, and atherosclerotic and vascular constrictor actions of angiotensin II (3, 8, 18–21). In the kidney, 12(S)-HETE decreases renal blood flow, constricts renal vessels, and is involved in the afferent arteriolar response to angiotensin II (5, 9, 11). Although the renal vascular actions of 12(S)-HETE have been long recognized, the intercellular signaling mechanisms responsible for 12(S)-HETE activity remain poorly defined. The current study provides further

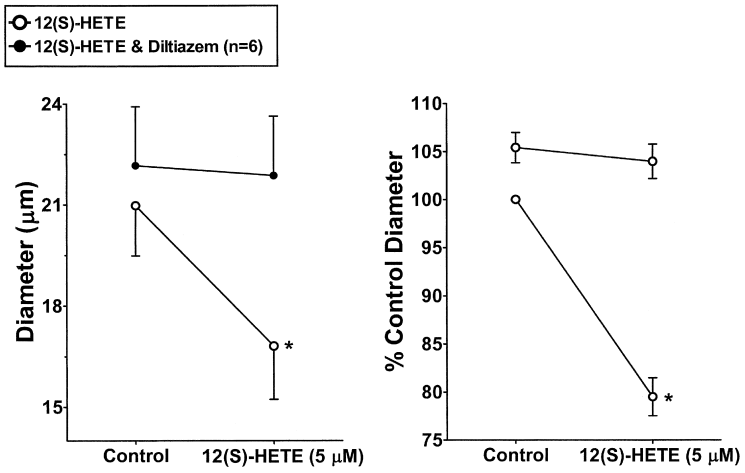

Fig. 4. Effect of diltiazem on the vasoconstrictor response to 12(S)-HETE. The afferent arteriolar diameter (left panel) and percent control diameter (right panel) response to 12(S)-HETE under control conditions and after addition of diltiazem. * Significant difference from control diameter measured at 100 mmHg.

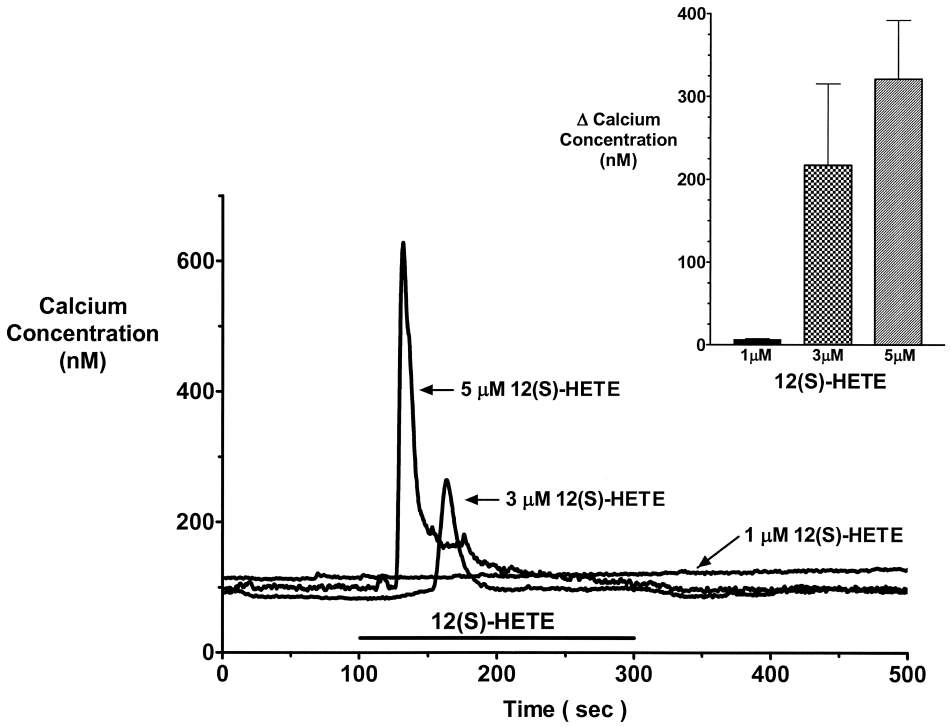

Fig. 5. Response of $[Ca^{2+}]_i$ to 12(S)-HETE under control conditions with 1.8 mmol/l extracellular calcium. Three representative calcium response traces from single microvascular smooth muscle cells are presented. 12(S)-HETE was administered as indicated by the solid horizontal bar. Graph inset depicts the mean peak elevations in $[Ca^{2+}]_i$ in response to 200 s of 12(S)-HETE administration.

evidence that 12(S)-HETE participates in the renal microvascular response to angiotensin II and establishes the contribution of L-type calcium channel activation to the 12(S)-HETE-mediated afferent arteriolar constriction.

First, we evaluated renal microvessel COX metabolite and 12(S)-HETE production in response to angiotensin II or norepinephrine. The main finding of this set of experiments was that angiotensin II but not norepinephrine increased renal microvascular 12(S)-HETE generation. In addition, we determined the LO enzymes that could be responsible for renal microvascular 12(S)-HETE production. Renal microvessels contained platelet- and leukocyte-type 12-LO, as well as type 2 15-LO. The finding of L-12-LO mRNA and protein expression in renal microvessels is consistent with previous findings in the porcine aorta and coronary arteries (2, 22). 12(S)-HETE generation could also possibly be due to the presence of P-12-LO, which has been shown to be an endothelial arachidonate 12-LO (23). Interestingly, we detected mRNA expression of 15-LO2 in renal cortical and microvascular tissue. This finding is in contrast to the first description of this enzyme that was unable to detect 15-LO2 in the human kidney (24). 15-LO2 converts arachidonic acid to 15(S)-HETE but is a poor metabolizer of linoleic acid (24). This suggested that, in addition to 12(S)-HETE, other arachidonate LO metabolites could contribute to angiotensin II-mediated renal vasoconstriction.

We compared the effects of 12(S)-HETE, 12(R)-HETE,

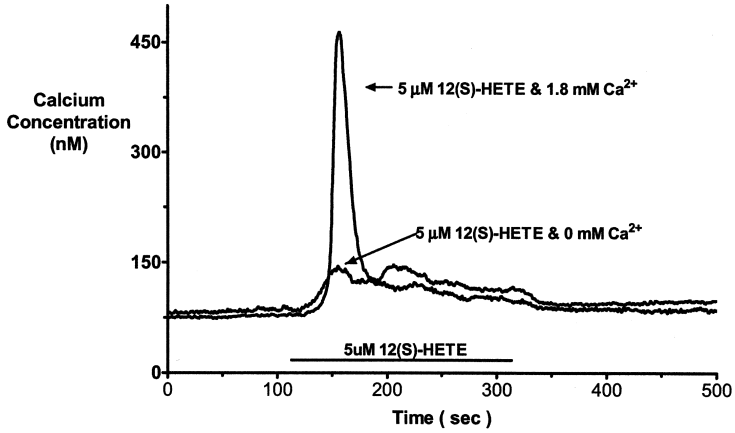

Fig. 6. Effect of Ca^{2+}-free bathing solution on the microvascular smooth muscle cell response to 12(S)-HETE. Representative calcium response traces from single microvascular smooth muscle cells are presented. Ca^{2+}-free solution and 12(S)-HETE were administered as indicated by the solid horizontal bar.

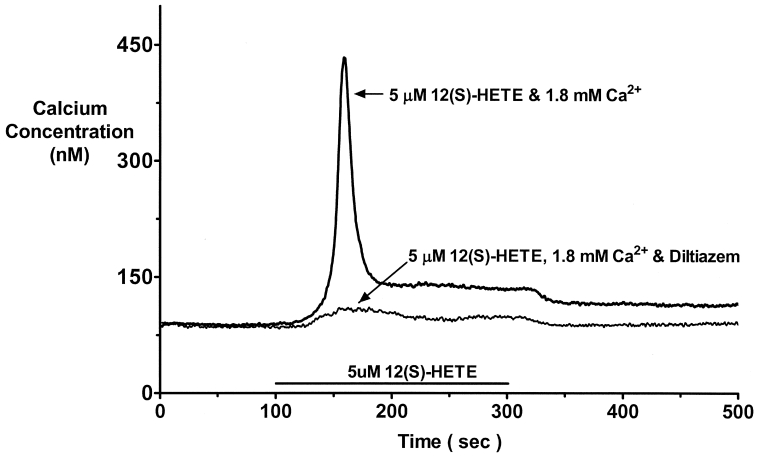

Fig. 7. Effect of ditiazem on the microvascular smooth muscle cell response to 12(S)-HETE. Representative calcium response traces from single microvascular smooth muscle cells are presented. Diltiazem and 12(S)-HETE were administered as indicated by the solid horizontal bar.

and 15(S)-HETE on afferent arteriolar diameter in the next set of experiments. 12(S)-HETE had prominent renal vasoconstrictor activity, whereas 12(R)-HETE and 15(S)-HETE were weak vasoconstrictors. This activity profile of arachidonate LO metabolites is in agreement with a number of previous studies that employed vascular smooth muscle and endothelial cells (4, 18, 21, 23). In contrast, 12(R)-HETE is a more active constrictor of small canine renal arteries (9). The reason for the differences may depend on the species studied, route of administration, subsequent metabolism of the parent compound, or size of the vessel studied. Nevertheless, the vasoconstrictor actions of 12(S)-HETE do appear to require a slightly higher dose when compared with its angiogenic properties (4, 18, 23, 25). A dose of 0.1 μM 12(S)-HETE is near maximal for the vascular growth activities, and a dose between 1 μM and 5 μM 12(S)-HETE was required for maximal actions on the afferent arteriolar diameter. Overall, the findings

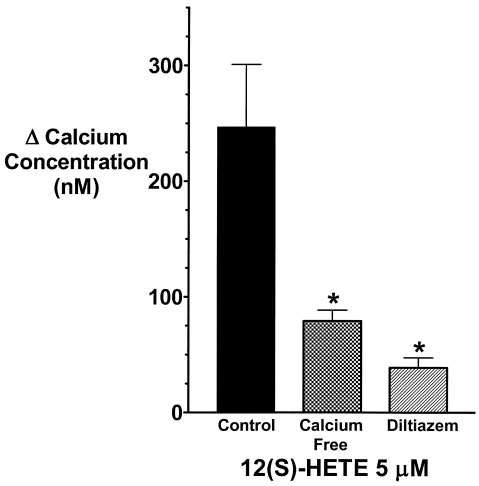

Fig. 8. Effect of Ca^{2+}-free bathing solution and diltiazem on the peak change in $[Ca^{2+}]_i$ exhibited by renal microvascular smooth muscle cell in response to 12(S)-HETE. Representative calcium response traces from single microvascular smooth muscle cells are presented. Ca^{2+}-free solution and 12(S)-HETE were administered as indicated by the solid horizontal bar. * Significant difference from control response to 12(S)-HETE.

of the present study support the concept that 12(S)-HETE is the primary LO metabolite involved in the control of renal vascular resistance.

These findings, combined with the previous observation that inhibition of the 12-LO pathway attenuates the afferent arteriolar response to angiotensin II, provide convincing evidence that 12(S)-HETE is importantly involved in the renal vascular actions of angiotensin II. There are a number of studies that provide corroborating evidence for the view that 12(S)-HETE is a mediator for the vascular intracellular actions of angiotensin II (3, 8, 18–21). The involvement of 12(S)-HETE in the renal vasoconstrictor response to angiotensin II has been demonstrated in several studies (11, 21, 26). Bell-Quilley et al. (26) demonstrated that in the isolated perfused rat kidney, inhibition of the 12-LO pathway attenuated the angiotensin II-mediated decrease in renal blood flow. In contrast, LO inhibition attenuated the renal arcuate artery vasoconstrictor response to norepinephrine and KCl but had no effect on the vascular response to ET-1 (27). Attenuation of the vasoconstrictor response to angiotensin II but not norepinephrine has also been demonstrated for the aorta and large arteries of the skeletal muscle and pulmonary vasculatures (19–21, 28–30). 12(S)-HETE also directly potentiates angiotensin II-mediated contraction of hamster aorta and SHR aorta (3, 31). The concentration of 12(S)-HETE required to potentiate angiotensin-mediated vascular contraction appears to be lower than that required to directly constrict the afferent arteriole (31). Enhanced vascular actions of angiotensin II in the presence of 12(S)-HETE appear to involve increased intracellular calcium signaling in the SHR (3). Even though a link between angiotensin II and 12-(S)-HETE is well established, the mechanism by which 12(S)-HETE contracts the vasculature remains unknown.

Since renal vascular responses to angiotensin II are reported to involve activation of calcium influx pathways (32–34) and 12(S)-HETE depolarizes renal vessels isolated from dogs (9), we postulated that the afferent arteriolar constriction elicited by 12(S)-HETE involves activation of L-type calcium channels. Afferent arteriolar diameter decreased in response to 12(S)-HETE in a dose-

dependent manner. The L-type calcium channel inhibitor, diltiazem, abolished the afferent arteriolar constriction elicited by 12(S)-HETE. Diltiazem was administered at a dose that completely blocks KCl-mediated afferent arteriolar constriction (35). These initial functional studies suggest that L-type calcium channel activation contributes to the 12(S)-HETE-mediated constrictor response. These results are in agreement with studies in renal canine arcuate arteries that demonstrate vascular smooth muscle cell depolarization (9). In contrast, recent studies in the coronary and cerebral vasculatures have provided evidence that 12(S)-HETE is a vasodilator and acts by activating large-conductance Ca^{2+}-activated K^+ channels (17, 36–38). The reason for this apparent discrepancy is not known, but studies performed in renal myocytes demonstrate that 12(S)-HETE increased intracellular calcium levels to a degree consistent with the observed afferent arteriolar constriction.

We performed additional studies to determine the relative contributions of agonist-induced calcium mobilization from intracellular stores and calcium influx to the 12(S)-HETE increases in renal myocyte intracellular calcium. In the studies presented here, the contribution of voltage-dependent L-type calcium channels is demonstrated by the pronounced attenuation of the 12(S)-HETE-induced increase in intracellular calcium by 84%. This finding suggests that calcium influx through L-type calcium channels accounts for a majority of the increase in cell calcium observed during exposure to 12(S)-HETE. We also considered the relative contribution of agonist-induced mobilization from intracellular stores to the 12(S)-HETE-induced increases in intracellular calcium in renal myocytes. The 12(S)-HETE-mediated increase in intracellular calcium was markedly attenuated in cells bathed in nominally calcium-free medium. Interestingly, the response obtained in the absence of extracellular calcium closely resembles the response obtained during blockade of L-type calcium channels. These findings establish that 12(S)-HETE elevates intracellular calcium through two different mechanisms. The renal myocyte response to 12(S)-HETE involves a small intracellular calcium mobilization component, and a large portion involves the influx of extracellular calcium via L-type calcium channels.

Interestingly, the cell signaling pathways that contribute to the 12(S)-HETE-mediated afferent arteriolar constriction are the same pathways utilized by angiotensin II (32–34, 39, 40). The afferent arteriolar response to angiotensin II is largely dependent on influx of calcium through L-type calcium channels, and mobilization of intracellular calcium contributes to a smaller extent (32–34, 39, 40). These parallels between angiotensin II and 12(S)-HETE can be likened to the hypertrophic and matrix protein production actions in vascular smooth muscle cells. Although the angiotensin II cell-signaling pathways for growth and matrix production are different than the pathways responsible for vascular constriction, the fact that 12(S)-HETE appears to mediate the vascular growth and constrictor actions of angiotensin II is worthy of further discussion. A number of studies have provided evidence that 12(S)-HETE activation of the Ras-MAPK pathway to the vascular smooth muscle growth and matrix gene expression mediates the hypertrophic actions of angiotensin II (4, 8). These actions of 12(S)-HETE are consistent with the postulate that angiotensin II activation of the 12-LO pathway plays a key role in atherosclerosis in diabetes and other renal and cardiovascular diseases. 12(S)-HETE is elevated in hypertension and contributes to the pressor actions of angiotensin II (1, 3, 29). Consistent with this possibility, reports have demonstrated that 12(S)-HETE contributes to the angiotensin II-induced vascular constrictor response (19–21, 27–30). In this regard, 12(S)-HETE has been postulated to be a mediator for the intracellular actions of angiotensin II (3, 18); however, the cell-signaling mechanisms by which 12(S)-HETE increased intracellular calcium were not investigated. In the current report we provide evidence that 12(S)-HETE constricts the afferent arteriole by mobilizing small amounts of intracellular calcium and causing a large influx of extracellular calcium through L-type calcium channels.

In summary, we observed an increase in renal microvessel 12(S)-HETE production in response to angiotensin II but not norepinephrine. This finding is in agreement with our previous report that the afferent arteriolar constriction in response to angiotensin II but not norepinephrine involves activation of the 12-LO pathway. We also determined that the 12(S)-HETE-induced afferent arteriolar constrictor response was dependent on activation of L-type calcium channels. Next, we evaluated renal myocyte intracellular calcium regulation in response to 12(S)-HETE. 12(S)-HETE increased intracellular calcium primarily through activation of L-type calcium channels. Taken together, these findings support the postulate that 12(S)-HETE is part of the cell-signaling pathway responsible for the angiotensin II-mediated activation of L-type calcium channels and afferent arteriolar vasoconstriction.

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