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Contribution of cytochrome P450 epoxygenase and hydroxylase pathways to afferent arteriolar autoregulatory responsiveness

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1 Previous studies have demonstrated an important role for the cytochrome P450 (CYT-P450) pathway in afferent arteriole autoregulatory responses but the involvement of specific pathways remains unknown. Experiments were performed to determine the role of CYT-P450 epoxygenase and hydroxylase pathways in pressure mediated preglomerular autoregulatory responses.

2 Afferent arteriolar diameter was measured as renal perfusion pressure was increased from 80–160 mmHg. Afferent arteriolar diameter averaged 19 ± 2 μm at a renal perfusion pressure of 80 mmHg and decreased by $15 \pm 2\%$ when pressure was increased to 160 mmHg.

3 Inhibition of the epoxygenase pathway with 6-(2-proparglyloxyphenyl)hexanoic acid (PPOH), enhanced the microvascular response to increasing renal perfusion pressure. In the presence of 50 μM PPOH, afferent arteriolar diameter decreased by $29 \pm 4\%$ when pressure was increased from 80–160 mmHg.

4 Likewise, the sulphonimide derivative of PPOH, N-methylsulphonyl-6-(2-proparglyloxyphenyl) hexanamide (MS-PPOH, 50 μM), enhanced the afferent arteriolar response to increasing renal perfusion pressure.

5 In contrast, the selective CYT-P450 hydroxylase inhibitor, N-methylsulphonyl-12,12-dibromo-dodec-11-enamide (DDMS) attenuated the vascular response to increasing renal perfusion pressure. In the pressure of 25 μM DDMS, afferent arteriolar diameter decreased by $4 \pm 2\%$ when pressure was increased from 80–160 mmHg.

6 These results suggest that CYT-P450 metabolites of the epoxygenase pathway alter afferent arteriolar responsiveness and thereby modify the ability of the preglomerular vasculature to autoregulate renal blood flow. Additionally, these results provide further support to the concept that a metabolite of the hydroxylase pathway is an integral component of the afferent arteriolar response to elevations in perfusion pressure.

Keywords: Kidney; endothelium-derived hyperpolarizing factor (EDHF); epoxyeicosatrienoic acids (EETs); renal blood flow; microcirculation; autoregulation

Abbreviations: CYT-P450, cytochrome P450; DDMS, N-methylsulphonyl-12,12-dibromododec-11-enamide; EDHF, endothelium-derived hyperpolarizing factor; EETs, epoxyeicosatrienoic acids; HETEs, hydroxyeicosatetraenoic acids; MS-PPOH, N-methylsulphonyl-6-(2-proparglyloxyphenyl) hexanamide; PPOH, 6-(2-proparglyloxyphenyl)hexanoic acid

Introduction

One of the main functions of the kidney is to maintain extracellular fluid volume and electrolyte composition. The preglomerular microvasculature plays an integral role in the maintenance of water and electrolyte balance by adjusting afferent arteriolar resistance in response to fluctuations in perfusion pressure, resulting in autoregulation of renal blood flow and glomerular filtration. Efficient autoregulation of renal blood flow and glomerular filtration is accomplished by the interplay between the tubuloglomerular feedback and myogenic responses of the renal microvasculature (Navar *et al*., 1996).

Recent studies have suggested that cytochrome P450 (CYT-P450) metabolites may participate in renal blood flow autoregulation (Imig *et al*., 1994; Kauser *et al*., 1991; Zou *et al*., 1994a, b). *In vivo* infusion of the CYT-P450 inhibitor, 17-ODYA, into the renal artery attenuated renal blood flow autoregulation (Zou *et al*., 1994a). Additionally, pressure-mediated afferent arteriolar vasoconstriction was attenuated by CYT-P450 inhibitors and this attenuation was associated with impaired autoregulation of glomerular capillary pressure (Imig *et al*., 1994). In the kidney, arachidonic acid metabolites of the CYT-P450 pathway include epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs) which are formed *via* epoxygenase and ω-hydroxylase enzymes; respectively. It has been difficult to evaluate the contribution of specific CYT-P450 pathways involved in renal autoregulatory responses because CYT-P450 inhibitors are not very selective and inhibit renal ω-hydroxylation and epoxidation of arachidonic acid with similar potency (Zou *et al*., 1994c). The recent development of selective CYT-P450 epoxygenase and hydroxylase inhibitors (Wang *et al*., 1998) allows determination of the role of each pathway in the afferent arteriolar autoregulatory response.

The present study determined the contribution of the CYT-P450 ω-hydroxylase and epoxygenase pathways to the afferent arteriolar response to elevations in renal perfusion pressure. Studies were performed using recently developed compounds designed to selectively inhibit the CYT-P450 epoxygenase or ω-hydroxylase pathways. A recent study established the selectivity of these new inhibitors on kidney arachidonic acid metabolism

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by rat renal microsomes. The terminal acetylenic compounds, N-methylsulphonyl-6- (2-proparglyoxyphenyl) hexanamide (MS-PPOH), and 6-(2-proparglyloxyphenyl)hexanoic acid (PPOH), selectively inhibit rat renal microsomal arachidonic acid epoxidation, whereas, the acyclic compound, N-methylsulphonyl-12-12-dibromododec-11-enamide (DDMS) selectively inhibits ω-hydroxylation (Wang *et al*., 1998). Direct assessment of afferent arteriolar diameter to step increases in renal perfusion pressure was performed before and during inhibition of CYT-P450 with these newly developed, selective inhibitors for the epoxygenase or ω-hydroxylase pathway.

Methods

Vascular preparation

Experiments were performed on male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, U.S.A.) weighing an average of 352 ± 6 g. All experiments were approved by the Tulane University Animal Care and Use Committee. Rats were anaesthetized with sodium pentobarbitone (40 mg kg^{-1} body wt i.p.), 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 (Sigma Chemical Co., St. Louis, MO, U.S.A.) and a mixture of L-amino acids (Imig *et al*., 1996b).

Blood was collected through the carotid artery cannula into a heparinized syringe (2000 U). Erythrocytes were separated from plasma and leukocytes by centrifugation, as previously described (Imig *et al*., 1996b). The erythrocytes were resuspended in Tyrode's solution containing 6% albumin to yield a hematocrit of 20%. The reconstituted blood 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 dissection procedure. The juxtamedullary microvasculature was isolated for study as previously described (Imig *et al*., 1996b). After the microdissection procedures were completed, the Tyrode's solution was replaced by reconstituted blood and renal artery perfusion pressure, measured at the tip of the cannula, was set to 80 mmHg. The organ chamber and bathing solution were warmed to 37°C and the tissue surface was continuously superfused with a Tyrode's solution containing 1% albumin. Following a 20 min equilibration period, an afferent arteriole was chosen for study and baseline diameter measured.

Afferent arteriolar diameters were measured using videomicroscopy techniques. The tissue was trans-illuminated on the fixed stage of a Leitz Laborlux microscope equipped with a 75-W xenon lamp and a 40× water immersion objective. Video images of the tissue under study were generated by a Newvicon camera, passed through a time date generator, displayed on a monitor and videotaped for later analysis. Vessel diameter was measured using a calibrated image-shearing monitor, which yielded reproducible measurements within 0.5 μm.

Pressure-diameter studies

After a 20 min equilibration period, the control relationship between diameter and perfusion pressure was determined. Perfusion pressure was varied in steps from 80–120 to 160 mmHg by adjusting the flow of gas in the perfusion reservoir. Measurements of afferent arteriolar diameter were made at 15 s intervals for a 5 min period at a single site at least 50 μm from any branch points. Steady state diameter was attained by the end of the second minute and the average diameter of the third through fifth minute at each perfusion pressure was utilized for statistical analysis. Finally, perfusion pressure was returned to 80 mmHg and a 5 min recovery period ensued. Afferent arteriolar diameter averaged 20.3 ± 0.7 and 19.7 ± 0.7 μm ($n = 29$) respectively; at a renal perfusion pressure of 80 mmHg before and after the step increase in pressure.

After the control period, the effects of two recently synthesized CYT-P450 epoxygenase inhibitors, PPOH and MS-PPOH or the selective ω-hydroxylase inhibitor, DDMS were studied. All compounds were synthesized and their structures confirmed in the laboratory of Dr J.R. Falck as previously described (Wang *et al*., 1998). The CYT-P450 inhibitors were dissolved in 50% ethanol and added to the Tyrode's bathing solution and blood perfusate to yield a final ethanol vehicle concentration of $<0.05\%$ (v v^{-1}). Inhibitors were added to the perfusate and superfusate for 30 min to ensure complete tissue blockade. After the 30 min period, the pressure-diameter relationship of the preglomerular vasculature was redetermined. In time control experiments ($n = 3$), addition of vehicle to the superfusate and perfusate had no effect on the response of the afferent arteriole to elevations in perfusion pressure.

Statistics

Data are presented as mean ± s.e.mean. Statistical comparisons of mean values for the dose-response were made using a one way 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 PPOH on the afferent arteriolar response to elevations in perfusion pressure

A previous study has shown that 5 μM PPOH inhibits renal epoxygenase activity by 40% whereas 50 μM PPOH is 90% effective (Wang *et al*., 1998). Therefore, we evaluated the effects of 5 and 50 μM PPOH on the afferent arteriolar diameter response to increasing renal perfusion pressure. The results of these studies are presented in Figures 1 and 2, respectively. Afferent arteriolar diameter averaged 19.9 ± 1.2 μm ($n = 10$) at a control perfusion pressure of 80 mmHg. Increasing perfusion pressure to 120 and 160 mmHg reduced afferent caliber by 8.4 ± 1.3 and $14.3 \pm 2.2\%$, respectively. PPOH at a concentration of 5 μM did not significantly alter the afferent arteriolar response to increasing perfusion pressure from 80–160 mmHg (Figure 1). In contrast, 50 μM PPOH enhanced the afferent arteriolar vasoconstrictor response to elevations in perfusion pressure (Figure 2). In the presence of 50 μM PPOH, afferent arteriolar diameter decreased by $28.7 \pm 4.3\%$ in response to increasing perfusion pressure from 80–160 mmHg.

Effect of MS-PPOH on the afferent arteriolar response to elevations in perfusion pressure

Wang *et al*. (1998) have previously demonstrated that 5 μM MS-PPOH reduces renal epoxygenase activity by 60% whereas

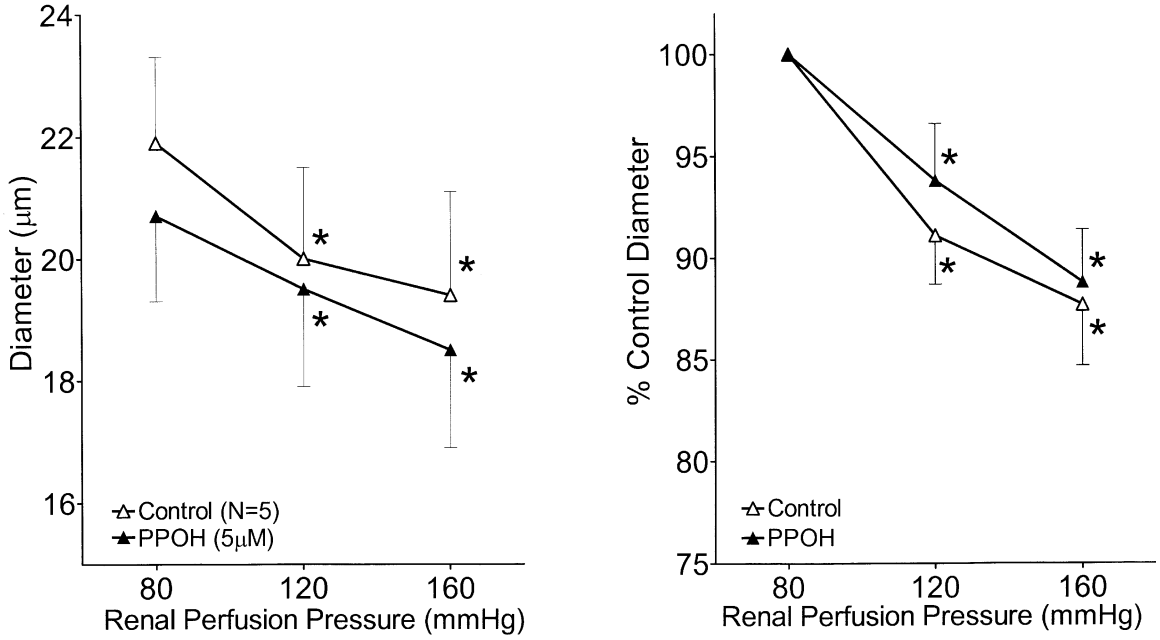

Figure 1 Effect of 5 μM PPOH on the pressure-diameter relationship of afferent arterioles. Data are presented as diameter (left panel) and per cent of control diameter (right panel). *Significant difference from diameter measured at 80 mmHg.

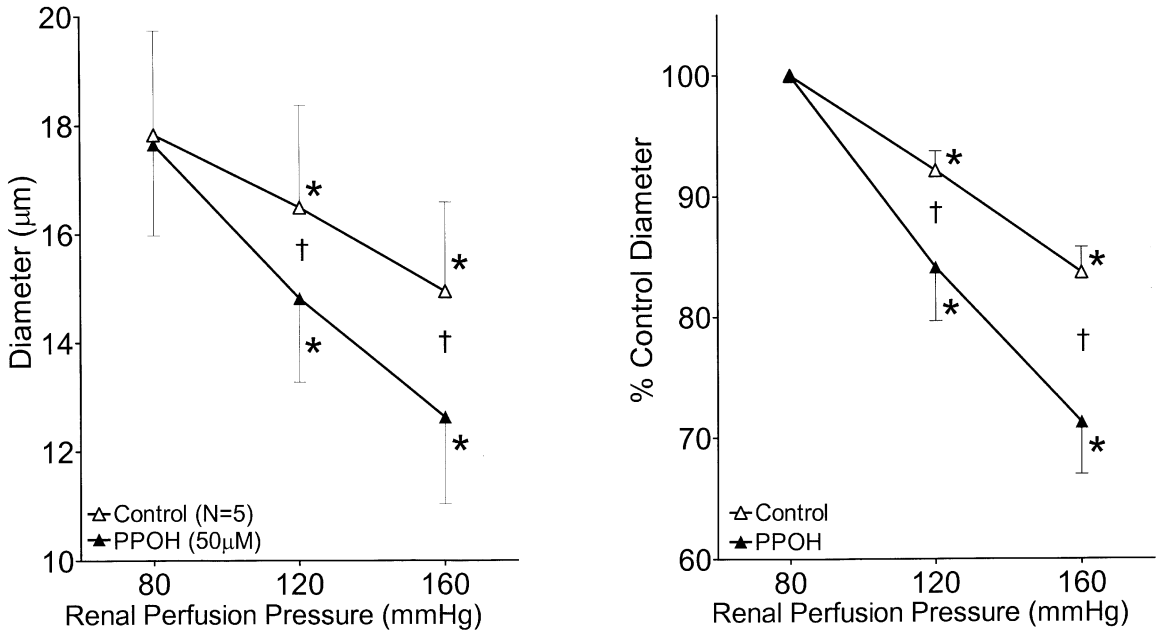

Figure 2 Effect of 50 μM PPOH on the pressure-diameter relationship of afferent arterioles. Data are presented as diameter (left panel) and per cent of control diameter (right panel). *Significant difference from diameter measured at 80 mmHg; †significant difference from corresponding control value.

greater than 80% inhibition is achieved at a concentration of 50 μM MS-PPOH. Accordingly, the effects of MS-PPOH (5 and 50 μM) on the afferent arteriolar response to perfusion pressure were examined. The results of those studies are illustrated in Figures 3 and 4; respectively. The diameter of the afferent arterioles averaged 20.5 ± 1.0 μm ($n = 12$) at a control renal perfusion pressure of 80 mmHg, and this diameter decreased by 6.5 ± 1.9 and 15.9 ± 3.2% in response to elevations in perfusion pressure to 120 and 160 mmHg, respectively. MS-PPOH, at a concentration of 5 μM, significantly decreased afferent arteriolar diameter at all perfusion pressures studied but did not alter the response to increasing perfusion pressure (Figure 3). Like the lower concentration, 50 μM MS-PPOH significantly decreased afferent arteriolar diameter at all perfusion pressures studied (Figure 4). Similar to the results obtained with the other epoxygenase inhibitor, PPOH, the afferent arteriolar vasoconstrictor response to increasing renal perfusion pressure was significantly enhanced after administration of 50 μM MS-PPOH. In the presence of 50 μM MS-PPOH, the afferent arteriolar diameter decreased by 27.4 ± 4.1% in response to elevating renal perfusion pressure from 80–160 mmHg.

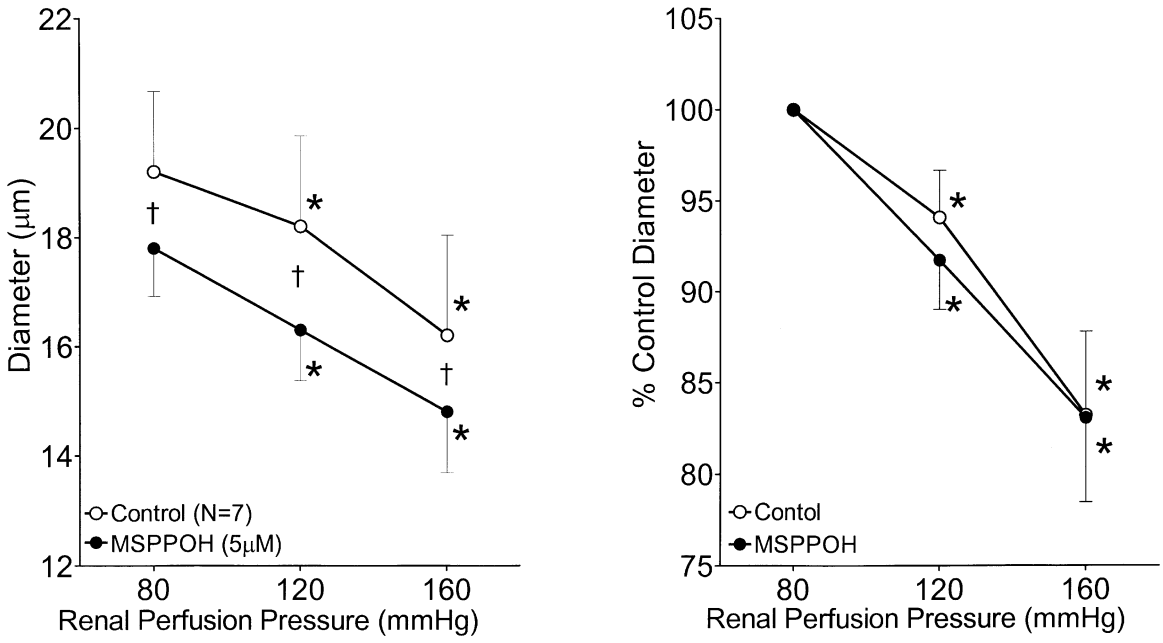

Figure 3 Effect of 5 μM MS-PPOH on the pressure relationship of afferent arterioles. Data are presented as diameter (left panel) and per cent of control diameter (right panel). *Significant difference from diameter measured at 80 mmHg; †significant difference from corresponding control value.

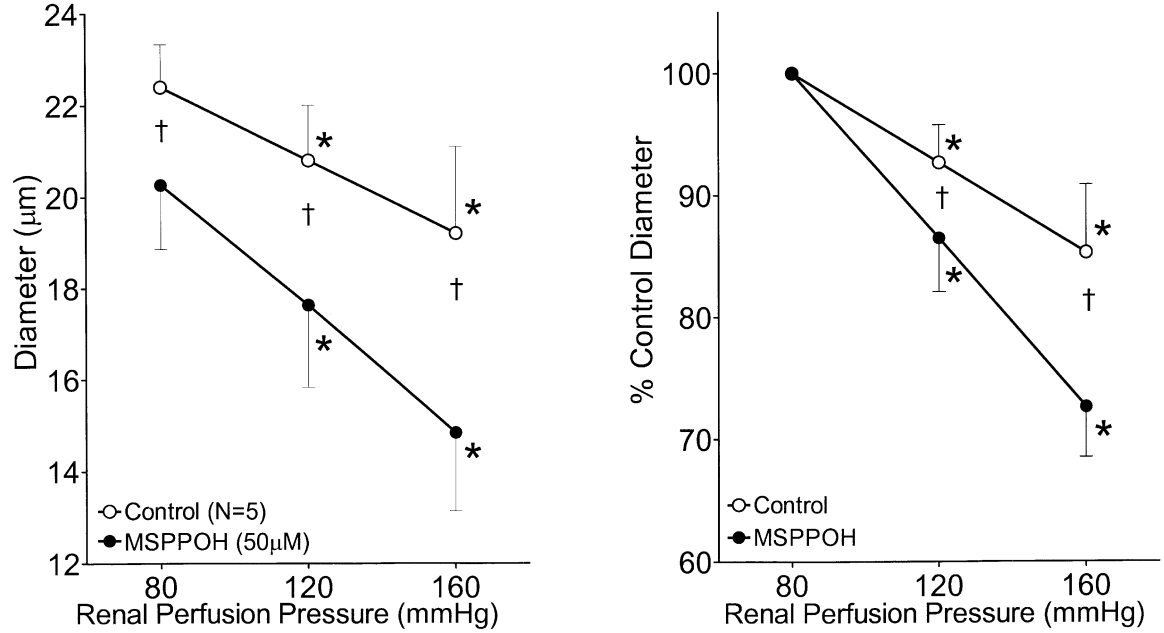

Figure 4 Effect of 50 μM MS-PPOH on the pressure-diameter relationship of the afferent arterioles. Data are presented as diameter (left panel) and per cent of control diameter (right panel). *Significant difference from diameter measured at 80 mmHg; †significant difference from corresponding control value.

Effect of DDMS on the afferent arteriolar response to elevations in perfusion pressure

The effect of the selective ω-hydroxylase inhibitor, DDMS, on the afferent arteriolar pressure-diameter relationship is presented in Figure 5. Under control conditions, the diameter of the afferent arteriole decreased by $12.5 \pm 1.2\%$ as perfusion pressure was increased from 80–160 mmHg. Addition of 25 μM DDMS to the perfusate and superfusate had no effect on basal diameter, but attenuated the vasoconstrictor response to increasing perfusion pressure. After administration of DDMS, the diameter of the afferent arteriole decreased by $4.3 \pm 1.5\%$ in response to the same elevation in renal perfusion pressure.

Discussion

The present study examined the effects of newly developed CYT-P450 inhibitors on the response of the afferent arteriole

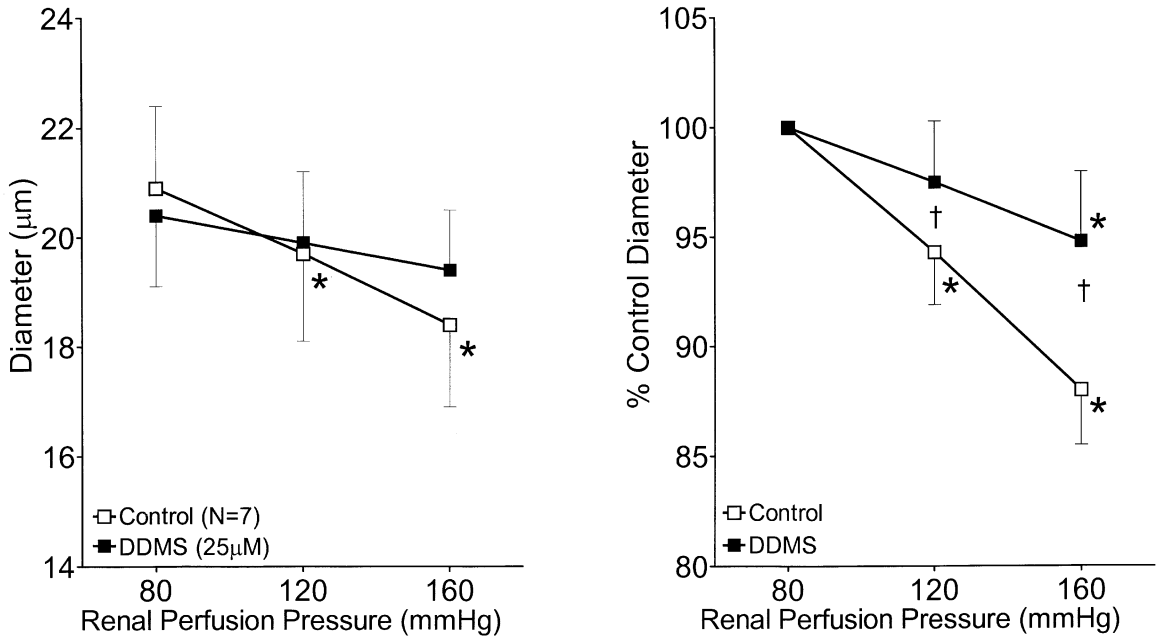

Figure 5 Effect of 25 μM DDMS on the pressure-diameter relationship of afferent arterioles. Data are presented as diameter (left panel) and per cent of control diameter (right panel). *Significant difference from diameter measured at 80 mmHg; †significant difference from corresponding control value.

to changes in perfusion pressure using the *in vitro* perfused rat juxtamedullary nephron microvascular preparation. Administration of the selective epoxygenase inhibitors, PPOH or MS-PPOH, enhanced the vasoconstrictor response, whereas, the selective ω-hydroxylase inhibitor, DDMS, attenuated the decrease in diameter of the afferent arteriole to elevations in renal perfusion pressure. These results suggest that EETs act to limit pressure-mediated vasoconstriction and 20-HETE facilitates the autoregulatory response of the afferent arteriole.

Renal blood flow and glomerular filtration rate are precisely regulated over a wide range of renal perfusion pressures (Navar *et al*., 1996). The extremely efficient autoregulation of renal blood flow results from the complicated interplay between the tubuloglomerular feedback and myogenic responses of the renal microvasculature (Navar *et al*., 1996). Studies in both dog and rat kidneys demonstrate that most of the autoregulatory response of the vasculature, in response to changes in perfusion pressure, is localized to the afferent arteriole (Navar *et al*., 1982; Casellas *et al*., 1985; Imig & Roman 1992; Carmines *et al*., 1992). The mechanisms responsible for adjustments of afferent arteriole caliber are not fully understood. Past studies have suggested that arachidonic acid metabolites of the CYT-P450 pathway may play a major role in renal blood flow autoregulation (Imig *et al*., 1994; Kauser *et al*., 1991; Zou *et al*., 1994a,b). Renal arterial infusions of CYT-P450 inhibitors of arachidonic acid metabolism impair whole kidney blood flow autoregulation (Zou *et al*., 1994a). Additionally, the myogenic response of isolated renal microvessels is abolished by CYT-P450 inhibition (Imig *et al*., 1994). Furthermore, perfusion of the loop of Henle with the ω-hydroxylase and epoxygenase CYT-P450 inhibitor, 17-ODYA, blocks the tubuloglomerular feedback response (Zou *et al*., 1994b). Because the imidazole inhibitors, clotrimazole and miconazole, do not affect autoregulation of renal blood flow at doses which inhibit epoxygenase activity, it has been suggested that a metabolite of the CYT-P450 ω-hydroxylase pathway participates in myogenic and tubuloglomerular feedback responses.

In the present study, administration of the newly developed selective CYT-P450 ω-hydroxylase inhibitor, DDMS, greatly attenuated the afferent arteriolar vasoconstriction to increasing renal perfusion pressure. Even though the CYT-P450 ω-hydroxylase metabolite 20-HETE is an endogenously produced vasoconstrictor of the renal microvasculature, DDMS did not alter basal afferent arteriolar diameter. The current results are similar to a recent study which demonstrated that DDMS does not alter basal diameter of renal arterioles with an intact endothelium but vasodilates these arterioles when the endothelium has been removed (Alonso-Galicia *et al*., 1998). Taken together the results of the current study provide further support to the concept that the ω-hydroxylase metabolite, 20-HETE, is an integral component of afferent arteriolar autoregulatory adjustments.

The effects of 20-HETE on the afferent arteriole are also consistent with a role for this metabolite to maintain constant renal blood flow during changes in renal perfusion pressure. 20-HETE inhibits vascular smooth muscle potassium channels resulting in membrane depolarization and subsequent activation of L-type calcium channels leading to vasoconstriction of the afferent arteriole (Ma *et al*., 1993; Gebremedhin *et al*., 1998; Imig *et al*., 1996b). Interestingly, afferent arteriolar vasoconstriction in response to elevations in renal perfusion pressure involves membrane depolarization and activation of L-type calcium channels (Takenaka *et al*., 1994). Both myogenic and tubuloglomerular feedback responses of the afferent arteriole can be blocked by L-type calcium channel antagonists (Navar *et al*., 1996). Activation of P2 purinoceptors also participate in mediating autoregulatory adjustments of the afferent arteriole (Inscho *et al*., 1996) and results in the release of arachidonic acid from membrane phospholipids in glomerular mesangial cells (Pfeilschifter, 1990). 20-HETE could act as an intracellular signalling molecule for P2 purinoceptors leading to autoregulatory adjustments of afferent arteriolar diameter. Additionally, a study in the isolated mesenteric artery demonstrated that potassium channel blockade reduced vascular responsiveness and

vascular smooth muscle depolarization elicited by pressure elevation (Wesselman *et al*., 1997). Thus, an ω-hydroxylase metabolite may influence the afferent arteriolar autoregulatory response by directly inhibiting vascular smooth muscle cell potassium channels.

Although not an essential component of renal blood flow autoregulatory responses, endothelial vasodilatory factors, such as nitric oxide and prostaglandins, do modify the ability of the preglomerular vasculature to maintain constant blood flow during changes in perfusion pressure (Navar *et al*., 1996). Nitric oxide synthesis inhibition increases afferent arteriolar tone while the ability to autoregulate renal blood flow in response to alterations in perfusion pressure remains well preserved (Imig & Roman, 1992). The contribution of endothelium-derived cyclo-oxygenase metabolites to renal blood flow autoregulation has been studied in a number of species. In cases where renal blood flow has been assessed, cyclo-oxygenase inhibition has failed to alter the autoregulatory response to changes in perfusion pressure (Navar *et al*., 1996). Utilizing a vascular bioassay technique, an unidentified endothelium-derived hyperpolarizing factor (EDHF) has been described which induces vasodilation by activating K^+ channels resulting in membrane hyperpolarization (Mombouli *et al*., 1996). Furthermore, the action of endothelium-derived epoxygenase metabolites to activate K^+ channels resulting in membrane hyperpolarization (Campbell *et al*., 1996) and vasodilate the afferent arteriole (Imig *et al*., 1996a) has led to the putative identification of EETs as EDHF (Campbell *et al*., 1996).

The possible role of epoxygenase metabolites on renal blood flow autoregulatory responses remains unclear. Therefore, experiments were performed to determine the contribution of the CYT-P450 epoxygenase pathway to the afferent arteriolar response to elevations in renal perfusion pressure. Administration of the recently developed selective epoxygenase inhibitors, PPOH or MS-PPOH, enhanced the afferent arteriolar diameter response to elevations in renal perfusion pressure. This enhancement of afferent arteriolar autoregulatory responsiveness appears to be a specific effect of these agents since the vascular response to increasing perfusion pressures was unaltered by inhibitor concentrations which reduced EET activity by less than 50%. The observation that PPOH and MS-PPOH enhance preglomerular autoregulatory responsiveness suggests that the release of vasodilatory epoxygenase metabolites, in response to increases in renal perfusion pressure, attenuates the afferent arteriolar caliber adjustment.

PPOH and MS-PPOH are structurally similar compounds which exert contrasting effects on resting afferent arteriolar diameter. MS-PPOH significantly reduced baseline afferent arteriolar diameter whereas vessel diameter remained unaltered during PPOH treatment. Both agents significantly enhanced autoregulatory behaviour, consistent with their actions to inhibit epoxygenase activity, but the reason for the varying influence on resting diameter remains a mystery.

The results of the current study contrast somewhat with studies using low dose imidazoles to inhibit epoxygenase activity. Although administration of clotrimazole (Sarubbi & Quilley, 1991) or miconazole (Zou *et al*., 1994a) did not significantly alter renal blood flow autoregulation, clotrimazole did decrease renal blood flow at perfusion pressures between 125 and 175 mmHg (Sarubbi & Quilley, 1991). Interestingly, enhancement of renal blood flow autoregulatory responses during long term indomethacin treatment is not related to cyclo-oxygenase metabolite levels and has been proposed to be related to a CYT-P450 vasodilatory pathway (Kramp *et al*., 1995). Thus, the results of the present study demonstrating enhancement of the afferent arteriolar autoregulatory response are consistent with the direct vasodilatory action of EETs and the possibility that EETs are EDHF.

In summary, newly developed selective inhibitors of the CYT-P450 epoxygenase or hydroxylase pathways altered afferent arteriolar autoregulatory responsiveness. The ω-hydroxylase inhibitor, DDMS, attenuated the vasoconstrictor response of afferent arterioles to increases in renal perfusion pressure in the *in vitro* perfused rat juxtamedullary microvascular preparation. These results provide further support to the concept that an ω-hydroxylase metabolite, 20-HETE, participates in afferent arteriolar autoregulatory responses. In addition, the present study demonstrated that the pressure-mediated decrease in afferent arteriolar diameter was enhanced during epoxygenase inhibition with either PPOH or MS-PPOH. These results indicate that CYT-P450 epoxygenase metabolites alter vascular tone in afferent arterioles and thereby modify the autoregulatory efficiency of the preglomerular microvasculature.

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