Effects of Calcium Channel Blockade on Angiotensin II-Induced Peritubular Ischemia in Rats

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ABSTRACT

Recent studies have indicated that derangement of peritubular capillary (PTC) circulation with consequent tubulointerstitial hypoxia plays a pivotal role in the pathogenesis of renal injury. The present study was performed to determine whether azelnidipine, a new dihydropyridine calcium channel blocker, attenuates angiotensin II (AngII)-induced peritubular ischemia in anesthetized rats. The superficial PTCs were visualized directly using an intravital fluorescence videomicroscope system, and the PTC blood flow was evaluated by analyzing the velocity of fluorescein isothiocyanate-labeled erythrocytes. Intravenous infusion of AngII (50 ng/kg/min, 10 min) significantly increased mean arterial pressure (MAP) and renal vascular resistance (RVR) (by 35 ± 3% and 110 ± 32%, respectively), and decreased total renal blood flow (RBF) and PTC erythrocyte velocity (by −34 ± 4 and −37 ± 1%, respectively). Treatment with azelnidipine (5 μg/kg/min i.v., 10 min) had no effect on basal MAP, RBF, RVR, or PTC erythrocyte velocity. However, azelnidipine markedly attenuated the AngII-induced increases in MAP (7 ± 3%) and RVR (40 ± 4%) and decreases in RBF (−24 ± 1%) and PTC erythrocyte velocity (−22 ± 1%). Similar attenuation in the AngII-induced responses of MAP, RBF, RVR, and PTC erythrocyte velocity were observed in rats treated with a higher dose of azelnidipine (20 μg/kg/min i.v., 10 min), which significantly decreased basal MAP and RVR and increased RBF and PTC erythrocyte velocity. These data suggest that calcium channel blockade attenuates AngII-induced peritubular ischemia, which may be involved in its beneficial effects on renal injury.

Renal dysfunction and injury have been reported to show better correlations with structural damage to the renal tubulointerstitium than with damage to the glomeruli (Risdon et al., 1968; Mackensen-Haen et al., 1992). It has also become apparent that tubulointerstitial hypoxia plays a pivotal role in the progression of renal injury (Fine et al., 2000; Matsuzato et al., 2004; Nangaku, 2004). Tubulointerstitial hypoxia may occur as a consequence of a reduction in peritubular capillary (PTC) blood flow, i.e., the peritubular ischemia and injury (Matsuzato et al., 2004; Nangaku, 2004). Indeed, extensive tubulointerstitial injury has been shown to be associated with the loss of PTCs in human kidneys (Serou et al., 1990) and animal models (Thomas et al., 1999; Ohashi et al., 2000), and the degree of loss is strongly related to the progression of renal disease. Recently, Manotham et al. (2004) demonstrated a reduced PTC blood flow and interstitial hypoxia before the development of structural damage to renal tissues in an early phase of remnant kidney model rats. Although the precise mechanisms responsible for the pathogenesis of peritubular ischemia are currently unclear, a potential contribution of the renin-angiotensin system has been suggested (Nobes et al., 1991; Lombardi et al., 1999; Omoro et al., 2000; Franco et al., 2001; Norman et al., 2003; Welch et al., 2003a,b, 2005; Manotham et al., 2004). Acute infusion of angiotensin II (AngII) resulted in renal cortical vasoconstriction (Nobes et al., 1991) or hypoxia (Norman et al., 2003). Likewise, cortical hypoxia was observed in the kidneys of chronically AngII-infused hypertensive rats (Welch et al., 2005) and in the clipped kidney at an early phase of two-kidney, one-clip Goldblatt hypertensive rats (Welch et al., 2003b). Furthermore, chronic infusion of AngII elicited salt-sensitive hypertension with cortical vasoconstriction (Franco...
et al., 2001) and loss of PTCs (Lombardi et al., 1999). In contrast, renal cortical blood flow and oxygenation were increased by infusion of an angiotensin-converting enzyme inhibitor (Omoró et al., 2000) or AngII-type AT₁ receptor antagonists (Norman et al., 2003; Welch et al., 2003a). Recent studies have also revealed that treatment with an AT₁ receptor antagonist restored PTC blood flow and ameliorated renal interstitial hypoxic injury in remnant kidney model rats (Manotham et al., 2004).

Several lines of evidence have indicated that calcium influx through L-type calcium channel-dependent mechanisms represents an essential component of AngII-induced renal vasoconstriction (Loutzenhiser et al., 1987; Carmines and Navar, 1989; Kageyama et al., 1989; Arendshorst et al., 1989, 1999; Iversen and Arendshorst, 1999). In cultured vascular smooth muscle cells from preglomerular vessels, AngII increased calcium entry through activation of dihydropyridine-sensitive L-type calcium channels leading to voltage-dependent pathways (Iversen and Arendshorst, 1999). Other in vitro studies demonstrated that L-type calcium channel blockers (CCBs) attenuated AngII-induced afferent arteriolar vasoconstriction (Carmines and Navar, 1989; Arendshorst et al., 1999). Treatment of isolated perfused rat kidneys (Loutzenhiser et al., 1987) or kidneys of anesthetized dogs (Kageyama et al., 1989) with dihydropyridine CCBs attenuated AngII-induced reductions in renal blood flow (RBF) or glomerular filtration rate. Collectively, these data suggest that dihydropyridine CCBs have protective effects against AngII-dependent vasoconstriction and ischemic changes in renal tissues, including peritubular interstitial areas. However, to the best of our knowledge, there is no direct evidence that AngII actually induces peritubular ischemia. Furthermore, the effects of calcium channel blockade on AngII-induced responses in PTC blood flow have not yet been examined.

In the present study, we first examined the effects of AngII on PTC blood flow in anesthetized rats. To visualize renal superficial PTCs directly in vivo, a fluorescence videomicroscope system was developed, and changes in PTC blood flow were evaluated by analyzing the velocity of fluorescein isothiocyanate (FITC)-labeled erythrocytes (Oyanagi-Tanaka et al., 2001; Li et al., 2004). Using this system, we further determined whether the responses of PTC blood flow to AngII are influenced by treatment with azelnidipine, a new dihydropyridine CCB that is highly lipid-soluble and distributed in cardiovascular tissues (Koike et al., 2002).

Materials and Methods

Animal Preparation

All experimental procedures were performed according to the guidelines for the care and use of animals established by Kagawa University Medical School. The surgical preparation of the animals and basic experimental techniques were identical to those described previously (Nishiyama et al., 2002; Rahman et al., 2002; Fujisawa et al., 2004). Male Sprague-Dawley rats (Clea Japan Inc., Tokyo, Japan) weighing 300 to 350 g were anesthetized with sodium pentobarbital (50 mg/kg body weight i.p.). A polyethylene tube (PE-50) was inserted into the right femoral vein for infusion of drugs and autologous red blood cells, respectively. The left kidney was exposed via a flank incision, isolated from the surrounding tissues, and immobilized in a kidney cup on a specially constructed stage for stable and respiratory movement-free fixation of organs. An ultrasonic flowmeter probe was attached to the left renal artery for measuring total RBF (VF-1; Crystal Biotech, Northborough, MA). Renal vascular resistance (RVR) was estimated as MAP/RBF (mm Hg per milliliter per minute per gram). The rats were allowed to equilibrate for 60 min before initiating the experimental protocols.

Intravenous Observation of the PTC

The superficial PTCs were visualized directly using an in vivo fluorescence microscope system (Eclipse E600; Nikon, Tokyo, Japan) equipped with a 100-W mercury lamp attached to an illuminator with filter blocks for epi-illumination, and images were recorded by a charge-coupled device video camera system (KCC-247; Kocom, Seoul, South Korea). Images on the camera were converted into color video signals every 33 ms (Bitcast browser version 3.0) and recorded on a computer by a video-capturing tool. With 10× salt water immersion objectives (Fluor; Nikon), the technique allowed a magnification of approximately ×400 on the computer monitor.

To measure erythrocyte velocity, a batch of FITC-labeled erythrocytes was injected i.v. as described previously (Oyanagi-Tanaka et al., 2001; Li et al., 2004). In brief, erythrocytes obtained from an experimental rat were separated by centrifugation, washed three times in saline, and incubated with a phosphate-buffered saline solution containing 1 mg/ml FITC at 25°C for 3 h. The labeled erythrocytes were washed with saline containing 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO), and the final hematocrit was adjusted to 50% using isotonic saline. Using captured computer-recorded images, specific line segment was set along the capillary bed, and the erythrocyte velocity was calculated by frame-by-frame analysis (Oyanagi-Tanaka et al., 2001; Li et al., 2004). Data for the erythrocyte velocities at five to seven different points were averaged.

Experimental Protocols

Effects of AngII on Renal Hemodynamics and PTC Blood Flow (Group 1). After a stabilization period of 60 min after completion of the surgery, the experimental protocol was started by recording the basal MAP, HR, RBF, and PTC erythrocyte velocity. Next, AngII (Sigma Chemical Co.) was i.v. infused for 10 min at a rate of 50 ng/kg/min, and MAP, HR, RBF, and PTC erythrocyte velocity were monitored continuously (n = 5). After cessation of the AngII infusion, a period of 40 min was allowed for stabilization of the systemic and renal parameters. Thereafter, AngII infusion was repeated, and MAP, HR, RBF, and PTC erythrocyte velocities were measured, as described above. AngII was dissolved in isotonic saline, and its dose was determined on the basis of results from previous studies on rats (Cervenka and Navar, 1999; Rahman et al., 2002).

Effects of Azelnidipine on Renal Responses to AngII (Groups 2 and 3). In group 2 rats (n = 6), the responses of systemic and renal parameters to AngII were evaluated before and after treatment with azelnidipine (5 μg/kg/min, 10 min). The experimental protocol was started by recording the basal MAP, HR, RBF, and PTC erythrocyte velocity. Next, AngII was i.v. infused for 10 min at a rate of 50 ng/kg/min and MAP, HR, RBF, and PTC erythrocyte velocity were monitored, as described in the experimental protocol for group 1. After cessation of the AngII infusion, a period of 30 min was allowed for stabilization of the systemic and renal parameters. Thereafter, azelnidipine was infused i.v. at a rate of 5 μg/kg/min for 10 min. After cessation of the azelnidipine infusion, AngII infusion (50 ng/kg/min, 10 min) was repeated, and MAP, HR, RBF, and PTC erythrocyte velocity were measured. In preliminary experiments, we observed that azelnidipine at 5 μg/kg/min for 10 min did not significantly change MAP, HR, RBF, or PTC erythrocyte velocity during a 30-min observation period (n = 3, data not shown).

In the other seven rats, the effects of a higher dose of azelnidipine
(20 μg/kg/min, 10 min) on the AngII-induced systemic and renal changes were evaluated (group 3). The experimental protocol for this experiment was identical to that for group 2. In brief, AngII was i.v. infused for 10 min at a rate of 50 μg/kg/min after the control period. After a stabilization period (30 min), azelnidipine was infused i.v. at a dose of 20 μg/kg/min for 10 min. This dose of azelnidipine was determined on the basis of results from previous studies on rats (Oizumi et al., 1989; Koike et al., 2002). After cessation of the azelnidipine infusion, AngII infusion was repeated, and MAP, HR, RBF, and PTC erythrocyte velocity were measured.

Azelnidipine was dissolved in dimethylformamide and then diluted in isotonic saline (final concentration of dimethylformamide, less than 0.1%), as described previously (Oizumi et al., 1989). Preliminary experiments showed that infusion of dimethylformamide did not alter MAP, HR, RBF, or PTC erythrocyte velocity (n = 3, data not shown).

ATP Contents in Renal Tissues

In a separate group of animals, ATP contents in renal tissue were measured using the high-performance liquid chromatography technique to investigate the degree of ischemia, as has been described by Ally and Park (1992). In brief, the left kidney was removed and snap-frozen in liquid nitrogen after 60 min after anesthesia with sodium pentobarbital (50 mg/kg body weight i.p.) in control rats (n = 11). The frozen kidney was weighed and homogenized with 0.4 mol/l HClO4 containing 0.5 mM EDTA. After 10 min on ice, the acid extract was centrifuged at 10,000g for 2 min. Next, the supernatant was neutralized with 0.5 mol/l K2CO3. The neutralized extract was then centrifuged to precipitate insoluble KClO4, and the supernatant was used for high-performance liquid chromatography separation within 1 h. In 12 other rats, AngII was i.v. infused for 10 min at a rate of 50 μg/kg/min after a stabilization period of 60 min. Then, the kidney was removed and processed to ATP measurements, as described for the controls. In eight rats, the effects of a higher dose of azelnidipine on the AngII-induced changes in kidney ATP contents were determined.

Statistical Analysis

The values are presented as means ± S.E. Statistical comparisons of the differences were performed using one- or two-way analysis of variance for repeated measures combined with the Newman-Keuls post hoc test. P < 0.05 was considered statistically significant.

### TABLE 1

Responses to AngII infusion (50 μg/kg/min, 10 min) before and after treatment with azelnidipine

<table>
<thead>
<tr>
<th></th>
<th>Control 1</th>
<th>AngII-1</th>
<th>Control 2</th>
<th>Azelnidipine (5 μg/kg/min)</th>
<th>AngII-2</th>
<th>Recovery (10 min)</th>
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<tbody>
<tr>
<td><strong>Group 1 (n = 6)</strong></td>
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<tr>
<td>MAP (mm Hg)</td>
<td>116 ± 4</td>
<td>156 ± 3*</td>
<td>125 ± 3</td>
<td>122 ± 3</td>
<td>130 ± 4***</td>
<td>118 ± 3</td>
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<tr>
<td>RBF (ml/min/g)</td>
<td>6.9 ± 0.9</td>
<td>4.5 ± 0.7*</td>
<td>6.5 ± 0.9</td>
<td>6.6 ± 1.0</td>
<td>5.0 ± 0.8***</td>
<td>6.5 ± 0.8</td>
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<tr>
<td>RVR (mm Hg/ml/min/g)</td>
<td>19 ± 3</td>
<td>36 ± 4*</td>
<td>21 ± 3</td>
<td>21 ± 3</td>
<td>29 ± 4***</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>PTC erythrocyte velocity (μm/s)</td>
<td>1243 ± 18</td>
<td>789 ± 19*</td>
<td>1281 ± 23</td>
<td>1268 ± 21</td>
<td>987 ± 6***</td>
<td>1230 ± 34</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>341 ± 6</td>
<td>328 ± 5*</td>
<td>344 ± 7</td>
<td>347 ± 7</td>
<td>332 ± 5***</td>
<td>342 ± 9</td>
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<table>
<thead>
<tr>
<th></th>
<th>Control-1</th>
<th>AngII-1</th>
<th>Control-2</th>
<th>Azelnidipine (20 μg/kg/min)</th>
<th>AngII-2</th>
<th>Recovery (10 min)</th>
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<tr>
<td><strong>Group 2 (n = 7)</strong></td>
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<tr>
<td>MAP (mm Hg)</td>
<td>113 ± 2</td>
<td>144 ± 8*</td>
<td>113 ± 4</td>
<td>92 ± 4***</td>
<td>100 ± 5***</td>
<td>86 ± 5</td>
</tr>
<tr>
<td>RBF (ml/min/g)</td>
<td>7.1 ± 0.5</td>
<td>4.1 ± 0.4*</td>
<td>7.0 ± 0.5</td>
<td>7.4 ± 0.4***</td>
<td>5.8 ± 0.3***</td>
<td>6.3 ± 0.4</td>
</tr>
<tr>
<td>RVR (mm Hg/ml/min/g)</td>
<td>17 ± 2</td>
<td>38 ± 5*</td>
<td>17 ± 2</td>
<td>13 ± 1***</td>
<td>19 ± 2***</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>PTC erythrocyte velocity (μm/s)</td>
<td>1210 ± 38</td>
<td>720 ± 32*</td>
<td>1166 ± 35</td>
<td>1257 ± 42**</td>
<td>1039 ± 56***</td>
<td>1224 ± 37</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>361 ± 7</td>
<td>349 ± 5*</td>
<td>365 ± 6</td>
<td>378 ± 6**</td>
<td>367 ± 5***</td>
<td>371 ± 10</td>
</tr>
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</table>

* P < 0.05 vs. control 1.
*** P < 0.05 vs. control 2.
*** P < 0.05 vs. azelnidipine.
tively, whereas AngII-induced decreases in RBF and PTC erythrocyte velocity were 22 ± 3% and 40 ± 1%, respectively (Table 1; Figs. 2–4). The response of HR to AngII infusion was not significantly changed by azelnidipine treatment (Table 1).

In group 3 rats, a higher dose of azelnidipine administration (20 μg/kg/min, 10 min) significantly decreased basal MAP and RVR and increased HR, RBF, and PTC erythrocyte velocity (Table 1). Similar to the results observed for the group 2 rats treated with a subpressor dose of azelnidipine, the higher dose of azelnidipine markedly attenuated the responses of these renal parameters to a second infusion of AngII (P < 0.05 for each), as shown in Figs. 2 to 4 and Table 1. Before the treatment with azelnidipine, AngII increased MAP and RVR by 27 ± 2% and 131 ± 22%, respectively, and decreased RBF and PTC erythrocyte velocity by −39 ± 4% and −40 ± 1%, respectively. After the treatment with azelnidipine, AngII-induced increases in MAP and RVR were 9 ± 1% and 43 ± 7%, respectively, whereas AngII-induced decreases in RBF and PTC erythrocyte velocity were −22 ± 3% and −17 ± 3%, respectively (Table 1; Figs. 2 to 4). The response of HR to AngII infusion was not significantly changed by azelnidipine treatment (Table 1).

**Effects of AngII and Azelnidipine on ATP Contents in Renal Tissues.** In control rats, ATP content in whole kidney tissue averaged 0.79 ± 0.04 μmol/g (Fig. 5). Renal tissue ATP content in AngII-infused rats was 0.55 ± 0.03 μmol/g, which was significantly lower than that of control rats (Fig. 5). In azelnidipine (20 μg/kg/min, 10 min)-pretreated AngII-infused rats, renal tissue ATP content was
Calcium influx through L-type calcium channel-dependent mechanisms represents an essential component of AngII-induced renal vasoconstriction (Loutzenhiser et al., 1987; Carmines and Navar, 1989; Kageyama et al., 1989; Arendshorst et al., 1999; Iversen and Arendshorst, 1999). Therefore, we examined the effects of azelnidipine, a dihydropyridine CCB with a high binding affinity for L-type calcium channels (Koike et al., 2002) on AngII-induced peritubular ischemia. The results revealed that treatment with azelnidipine markedly attenuated AngII-induced reductions in PCT blood flow and renal ATP levels, suggesting a protective effect of azelnidipine against AngII-induced peritubular ischemia. Previous studies have shown that treatment with dihydropyridine CCBs improves AngII-dependent renal dysfunction or injury in rats chronically treated with AngII (Huelsemann et al., 1985), two-kidney, one-clip Goldblatt rats (Veniant et al., 1994), and transgenic TGR(mREN2)27 rats (Witte et al., 1999). Thus, it is possible that at least some of the therapeutic effects of CCBs are mediated through inhibition of AngII-induced peritubular ischemia. However, other multiple mechanisms may also be involved in the beneficial effects of CCBs on renal injury (Yamasaki et al., 2000; Gashti and Bakris, 2004; Segura et al., 2005). In addition, the present study did not address the specific action sites of AngII and azelnidipine in renal vessels. Further studies are needed to clarify these issues.

We recently demonstrated that tubulointerstitial injury in spontaneously hypertensive rats was associated with increases in the intrarenal AngII levels (Kobori et al., 2005). Furthermore, treatment with an AngII AT1 receptor antagonist, olmesartan, prevented these increases in the intrarenal AngII level and tubulointerstitial injury. On the other hand, although combination therapies with three different vasodilators (hydralazine, reserpine, and hydrochlorothiazide) prevented the development of hypertension, they had no effect on the intrarenal AngII levels or tubulointerstitial injury (Kobori et al., 2005). These observations suggest that the renoprotective effects of AngII blockade are mediated through blood pressure-independent mechanisms. Azelnidipine has a potent vasodilatory property (Oizumi et al., 1989; Koike et al., 2002), which may be responsible for its inhibitory effects on the AngII-induced responses of PTC blood flow. However, we observed that a lower dose of azelnidipine (5 μg/kg/min) did not affect basal renal hemodynamics and PTC blood flow but significantly attenuated AngII-induced responses of the renal parameters. Thus, these data suggest that these effects of azelnidipine cannot be simply explained by its effect on basal renal vascular tone.

In conclusion, the results of the present study demonstrate that administration of AngII actually results in a reduction in PTC blood flow in vivo. The results further show that L-type calcium channel blockade with azelnidipine markedly attenuates AngII-induced peritubular ischemia. These data suggest that the renoprotective effects of dihydropyridine CCBs are mediated through inhibition of AngII-induced peritubular ischemia, at least in part. Future studies will be performed in pathological models to ascertain any therapeutic effects of chronic treatment with azelnidipine on AngII-dependent renal injury.

Discussion

AngII has been reported to play a pivotal role in the pathogenesis of renal derangements and injury through multiple mechanisms including effects on renal circulation (Navar et al., 1996, 1999; Jackson et al., 1999). Many in vivo animal studies have been performed to investigate the role of AngII in the regulation of renal hemodynamics mostly using clearance techniques, dye dilution methods, Doppler effects, micropuncture techniques, etc. However, these methods do not provide any data regarding the effect of AngII on the PTC microcirculation. In the present study, we developed an in vivo fluorescence microscope system to visualize the renal superficial PTCs in anesthetized rats and investigated the responses of PTC blood flow to AngII administration by analyzing the velocity of FITC-labeled erythrocytes (Oyanagi-Tanaka et al., 2001; Li et al., 2004). This intravital videomicroscope system combined with analysis of the FITC-labeled erythrocyte velocity allow observation of the PTC microcirculation with minimal invasion. Using these methods, the current study presents direct evidence, for the first time, that AngII administration actually decreases PTC blood flow in vivo. We also observed that during acute infusion of AngII, reductions in renal tissue ATP levels were associated with decreases in PTC blood flow. Although AngII-induced reductions in PTC blood flow may largely be a secondary consequence of vasoconstriction of the respective renal vessels, i.e., afferent and efferent arterioles (Navar et al., 1996), the present data support the concept proposed in previous studies (Nobes et al., 1991; Lombardi et al., 1999; Omoro et al., 2000; Franco et al., 2001; Norman et al., 2003; Welch et al., 2003a,b, 2005; Manotham et al., 2004) that PTCs are targets for injuries induced by AngII.

0.78 ± 0.03 μmol/g. This value was not different from that of control rats but was significantly higher than that of AngII-infused rats (Fig. 5), indicating that AngII-induced reduction in renal tissue ATP levels is attenuated by pretreatment with azelnidipine (20 μg/kg/min i.v. for 10 min). *, P < 0.05 versus control rats.
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References


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