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

Naoki Kondo, Hideyasu Kiyomoto, Tokunori Yamamoto, Akira Miyatake, Guang-Ping Sun, Matlubur Rahman, Hirofumi Hitomi, Kumiko Moriwaki, Taiga Hara, Shoji Kimura, Youichi Abe, Masakazu Kohno, and Akira Nishiyama

Second Department of Internal Medicine (N.K., H.K., G.-P.S., M.R., H.H., K.M., T.H., M.K.), Life Science Research Center (A.M.), and Department of Pharmacology (M.R., S.K., Y.A., A.N.), Kagawa University Medical School, Kagawa, Japan; and Department of Urology, Nagoya University School of Medicine (T.Y.), Nagoya, Japan

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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; Matsu moto 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 (Matsumoto et al., 2004; Nangaku, 2004). Indeed, extensive tubulointerstitial injury has been shown to be associated with the loss of PTCs in human kidneys (Seron et al., 1990) and animal models (Thomas et al., 1998; 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., 2003b).
et al., 2001) and loss of PTCs (Lombardi et al., 2003). In contrast, renal cortical blood flow and oxygenation were increased by infusion of an angiotensin-converting enzyme inhibitor (Omoro 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., 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 vasconstriction (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 350g were anesthetized with sodium pentobarbital (50 mg/kg body weight i.p.). A polyethylene tube (PE-50) was inserted into the right femoral artery for measuring blood pressure. This arterial catheter was connected to a pressure transducer, and mean arterial pressure (MAP) and heart rate (HR) were continuously recorded via a polygraph system. Two further polyethylene tubes were 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.

**Intravital 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 (Fluo; 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 continuously (n = 5). 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 were examined.
(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 ng/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 delivered 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 HC1O4 containing 0.5 mM EDTA. After 10 min on ice, the acid extract was centrifuged at 10,000×g for 2 min. Next, the supernatant was neutralized with 0.5 mol/l K2CO3. The neutralized extract was centrifuged to precipitate insoluble KClO4, and the supernantant was used for high-performance liquid chromatography separation method.

**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.

**Results**

**Responses of Renal Hemodynamics and PTC Blood Flow to AngII** *(Group 1)*. Intravenous infusion of AngII at a rate of 50 ng/kg/min for 10 min increased MAP by 44 ± 6% (from 102 ± 4 to 147 ± 7 mm Hg) and RVR by 145 ± 15% (from 16 ± 1 to 41 ± 5 mg Hg/ml/min/g) and decreased HR by −3 ± 1% (from 366 ± 5 to 355 ± 4 beats/min) and RBF by −42 ± 2% (from 6.5 ± 0.7 to 3.8 ± 0.5 ml/min/g). The PTC erythrocyte velocity, which reflects the degree of PTC blood flow, decreased by −42 ± 2% (from 1395 ± 31 to 814 ± 29 μm/s).

A second infusion of Ang II resulted in similar changes in these parameters. MAP and RVR increased by 52 ± 5% (from 98 ± 4 to 149 ± 6 mm Hg) and 166 ± 9% (161 ± 4 to 45 ± 4 mm Hg/ml/min/g), respectively, whereas HR, RBF, and PTC erythrocyte velocity decreased HR by −3 ± 1% (from 371 ± 5 to 361 ± 5 beats/min), −45 ± 4% (from 6.2 ± 0.6 to 3.4 ± 0.3 ml/min/g), and −43 ± 2% (1377 ± 17 to 787 ± 18 μm/s), respectively. Thus, time-dependent changes in AngII-induced responses of these renal parameters were not observed, indicating that a recovery period of 40 min after first infusion of AngII is adequate to obtain similar RBF and PTC erythrocyte velocity responses to a second infusion of AngII.

**Responses of Renal Hemodynamics and PTC Blood Flow to AngII after Treatment with Azelnidipine** *(Groups 2 and 3).* The results are summarized as the absolute mean values in the Table 1. Typical responses of the PTC erythrocyte velocity in group 2 rats are shown in Fig. 1. Similar to the results observed for the group 1 rats, Ang II infusion in group 2 rats increased MAP and RVR by 35 ± 3% and 110 ± 32%, respectively, and decreased HR, RBF, and PTC erythrocyte velocity by −4 ± 1%, −34 ± 5%, and −37 ± 1%, respectively, before treatment with azelnidipine (Table 1). In these animals, azelnidipine administration at a rate of 5 μg/kg/min for 10 min did not alter basal MAP, HR, RBF, RVR, or PTC erythrocyte velocity (Table 1). However, azelnidipine treatment markedly attenuated the responses of MAP, RBF, RVR, or PTC erythrocyte velocity to a second infusion of AngII (P < 0.05 for each), as shown in Figs. 2 to 4. After the treatment with azelnidipine, AngII-induced increases in MAP and RVR were 7 ± 3% and 40 ± 4%, respec-

**Table 1**

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

<table>
<thead>
<tr>
<th>Values are means ± S.E.</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>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong> (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<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>
</tr>
<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>6.5 ± 1.0</td>
<td>5.0 ± 0.8***</td>
<td>6.5 ± 0.8</td>
</tr>
<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>28 ± 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>
</tr>
<tr>
<td><strong>Group 2</strong> (n = 7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<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>
</tbody>
</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 \(24 \pm 1\% \) and \(22 \pm 1\% \), 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 \(\mu \)g/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 \((P < 0.05 \text{ for each})\), as shown in Figs. 2 to 4 and Table 1. Before the treatment with azelnidipine, AngII increased MAP and RVR by \(27 \pm 2\% \) and \(131 \pm 22\% \), respectively, and decreased RBF and PTC erythrocyte velocity by \(-39 \pm 4\% \) and \(-40 \pm 1\% \), respectively. After the treatment with azelnidipine, AngII-induced increases in MAP and RVR were \(9 \pm 1\% \) and \(43 \pm 7\% \), respectively, whereas AngII-induced decreases in RBF and PTC erythrocyte velocity were \(-22 \pm 3\% \) and \(-17 \pm 3\% \), 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 \(0.79 \pm 0.04 \ \mu \text{mol/g} \) (Fig. 5). Renal tissue ATP content in AngII-infused rats was \(0.55 \pm 0.03 \ \mu \text{mol/g} \), which was significantly lower than that of control rats (Fig. 5). In azelnidipine (20 \(\mu \)g/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 (Loutzenheriser 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 PCT blood flow. However, we observed that a lower dose of azelnidipine (5 μg/kg/min) did not affect basal renal hemodynamics and PCT 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 PCT 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.
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References


Witte K, Schnecko A, Schmidt T, Voll C, Kranzlin B, and Lemmer B (1999) Cardiovascular, Kagawa University Medical School, 1750-1 Ikenobe, Miki-Cho, Kita-Gun, Kagawa 761-0793, Japan. E-mail: akira@kms.ac.jp

Address correspondence to: Dr. Akira Nishiyama, Department of Pharmacology, Kagawa University Medical School, 1750-1 Ikenobe, Miki-Cho, Kita-Gun, Kagawa 761-0793, Japan. E-mail: akira@kms.ac.jp


Address correspondence to: Dr. Akira Nishiyama, Department of Pharmacology, Kagawa University Medical School, 1750-1 Ikenobe, Miki-Cho, Kita-Gun, Kagawa 761-0793, Japan. E-mail: akira@kms.ac.jp