Possible Contributions of Reactive Oxygen Species and Mitogen-Activated Protein Kinase to Renal Injury in Aldosterone/Salt-Induced Hypertensive Rats

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Abstract—Studies were performed to test the hypothesis that reactive oxygen species (ROS) and mitogen-activated protein kinase (MAPK) contribute to the pathogenesis of aldosterone/salt-induced renal injury. Rats were given 1% NaCl to drink and were treated with one of the following combinations for 6 weeks: vehicle (0.5% ethanol, SC, n=6); aldosterone (0.75 µg/H, SC, n=8); aldosterone plus a selective mineralocorticoid receptor antagonist; eplerenone (0.125% in chow, n=8); aldosterone plus an antioxidant; and tempol (3 mmol/L in drinking solution, n=8). The activities of MAPKs, including extracellular signal-regulated kinases (ERK)1/2, c-Jun-NH2-terminal kinases (JNK), p38MAPK, and big-MAPK-1 (BMK1) in renal cortical tissues were measured by Western blot analysis. Aldosterone-infused rats showed higher systolic blood pressure (165±5 mm Hg) and urinary excretion of protein (106±24 mg/d) than vehicle-infused rats (118±3 mm Hg and 10±3 mg/d). Renal cortical mRNA expression of p22phox, Nox-4, and gp91phox, measured by real-time polymerase chain reaction, was increased in aldosterone-infused rats by 2.3, 4.3, and 3.0-fold, respectively. Thiobarbituric acid-reactive substances (TBARS) content in renal cortex was also higher in aldosterone (0.23±0.02) than vehicle-infused rats (0.09±0.01 nmol/mg protein). ERK1/2, JNK, and BMK1 activities were significantly elevated in aldosterone-infused rats by 3.3, 2.3, and 3.0-fold, respectively, whereas p38MAPK activity was not changed. Concurrent administration of eplerenone or tempol to aldosterone-infused rats prevented the development of hypertension (127±2 and 125±5 mm Hg), and the elevations of urinary excretion of protein (10±2 and 9±2 mg/day) or TBARS contents (0.08±0.01 and 0.11±0.01 nmol/mg protein). Furthermore, eplerenone and tempol treatments normalized the activities of ERK1/2, JNK, and BMK1. These data suggest that ROS and MAPK play a role in the progression of renal injury induced by chronic elevations in aldosterone. (Hypertension. 2004;24:841-848.)

Key Words: mineralocorticoids • kidney • rats

In recent years, attention has focused on the role of aldosterone in the pathophysiology of hypertension and cardiovascular disease. It has been shown that patients with primary aldosteronism, in which angiotensin II (Ang II) levels are low, have a higher incidence of cardiovascular complications than do patients with essential hypertension.1

The Randomized Aldactone Evaluation Study (RALES) demonstrated that adding a nonspecific mineralocorticoid receptor (MR) antagonist, spironolactone, to standard therapy, including angiotensin-converting enzyme inhibitors, loop diuretics, and digoxin, significantly reduced morbidity and mortality in patients with moderate to severe heart failure.2

More recently, several clinical and experimental animal data also support a contribution of aldosterone to the progression of renal injury.3–10 Quan et al1 showed that adrenalectomy ameliorated nephropathy despite large doses of replacement glucocorticoid. Severe glomerular injury with renal vascular fibrinosis and inflammation were observed in uninephrectomized rats treated with aldosterone/salt.4–5 Likewise, exogenous infusion of aldosterone reversed the reno-protective effects of Ang II blockade in remnant kidney hypertensive rats6 and stroke-prone spontaneously hypertensive rats.7 Administration of spironolactone did not alter blood pressure but markedly ameliorated renal injury in stroke-prone spontaneously hypertensive rats.8 Chrysostomou and Becker9 reported that adding spironolactone to angiotensin-converting enzyme inhibitors did not have hemodynamic effects but markedly reduced urinary excretion rate of protein (Uprotei V) in patients with chronic renal failure. Further studies by White et al10 showed that a selective MR antagonist, eplerenone, has a similar blood pressure lowering effect compared with a calcium antagonist, amlodipine, but reduced the urinary

Received November 10, 2003; first decision December 5, 2003; revision accepted January 13, 2004.

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Hypertension is available at http://www.hypertensionaha.org

DOI: 10.1161/01.HYP.0000118519.66430.22

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albumin/creatinine ratio to a larger extent than did amlodipine. Thus, these data suggest that aldosterone has deleterious effects on the kidney that cannot be simply explained by blood pressure changes.

Reactive oxygen species (ROS) have been implicated as important mediators of the progression of renal injury in different animal models of hypertension. Exaggerated ROS production in damaged renal tissue has been observed in spontaneously hypertensive rats, deoxycorticosterone acetate (DOCA)-salt hypertensive rats, cyclosporine A-induced hypertensive rats, and Dahl salt-sensitive hypertensive rats. Furthermore, treatment with a cell membrane-permeable radical scavenger, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol), markedly attenuated renal injury in DOCA-salt hypertensive rats and Dahl salt-sensitive hypertensive rats. Interestingly, recent studies also indicate that aldosterone increases ROS production. Virdis et al showed that vascular NAD(P)H oxidase activity and ROS production were increased in aldosterone/salt-treated hypertensive rats. It was also shown that treatment with eplerenone improved endothelial dysfunction and reduced vascular superoxide anion (O$_2^-$) generation in diet-induced atherosclerosis. Similarly, eplerenone reduced aortic atherosclerotic lesion and O$_2^-$ generation in peritoneal macrophages in apolipoprotein E-deficient mice.

The purpose of this study was to investigate the role of ROS in the pathogenesis of aldosterone/salt-induced renal injury. Therefore, the effects of tempol, which exhibit antioxidant activity against O$_2^-$ as well as hydroxy radicals, on renal injury and ROS levels were examined in aldosterone/salt hypertensive rats. To evaluate the possible contribution of NAD(P)H oxidase to ROS generation, we also measured renal cortical mRNA expression of p22phox, Nox-1, and gp91phox, which are essential membrane components of NAD(P)H oxidase. Because ROS-induced cell growth and differentiation have been implicated, at least in part, in the activation of mitogen-activated protein kinases (MAPKs), the effects of tempol on kidney MAPKs activities were also examined. We measured the activities of the classical MAPKs, including extracellular signal-regulated kinases (ERK1/2), c-Jun-NH$_2$-terminal kinases (JNK), and p38 MAPK, and a new MAPK family member, BigMAPK-1 (BMK1).

**Methods**

**Animal Preparation**

All experimental procedures were performed according to the guidelines for the care and use of animals established by the Kagawa Medical University. Male Sprague-Dawley rats (Clea, Japan), weighing 220 to 258 g at the beginning of the experiments, were randomly treated with one of the following combinations for 6 weeks: group 1, tap water plus vehicle (0.5% ethanol, SC, n=6); group 2, 1% NaCl in the drinking solution plus vehicle (n=8); group 3, 1% NaCl plus aldosterone (0.75 µg/H, SC, n=8); group 4, 1% NaCl plus aldosterone plus eplerenone (0.125% in chow, n=8); and group 5, 1% NaCl plus aldosterone plus tempol (3 mmol/L in drinking solution, n=8). Rats were anesthetized with sodium pentobarbital (50 mg/kg, IP), and an osmotic minipump (model 2002; Alza Co, Palo Alto, Calif) was implanted subcutaneously at the dorsum of the neck to infuse vehicle or aldosterone. The doses of aldosterone, eplerenone, and tempol were determined on the basis of results from previous studies in rats.

Systolic blood pressure (SBP) was measured in conscious rats by tail-cuff plethysmography (BP-98A; Softron Co, Tokyo, Japan) and 24-hour urine samples were collected at 1, 3, and 6 weeks. Blood and kidney samples were harvested at the end of week 6. After decapitation, the left kidney was removed, snap-frozen in liquid nitrogen, and stored at -80°C until processing for protein or RNA extraction and analysis of thiobarbituric acid reactive substances (TBARS) contents. The right kidney was perfused with chilled saline solution and fixed in 10% buffered paraformaldehyde for histological examination. The heart was also excised and weighed.

**mRNA Expression of NAD(P)H Oxidase Components and MAPK Activities in Renal Cortical Tissues**

p22phox, Nox-1, and gp91phox mRNA expression levels in the renal cortical tissues were quantitatively analyzed by real-time transcription polymerase chain reaction (PCR), as described previously in detail. Data are expressed as the relative differences in vehicle/1% NaCl-treated, 1% NaCl/aldosterone-treated, 1% NaCl/aldosterone/eplerenone-treated, or 1% NaCl/aldosterone/tempol-treated rats compared with vehicle-infused rats after normalization to the expression of GAPDH. Oligonucleotide primers for p22phox, Nox-1, and gp91phox were synthesized based on published sequences.

We used immunoblotting with antibodies against phospho-ERK1/2 and phospho-p38 MAPK (Cell Signaling Technology) to evaluate ERK1/2 and p38 MAPK activation, as described previously. JNK activity was measured using a commercially available kit based on the phosphorylation of recombinant c-Jun. Immunoblotting was performed with antibodies against phospho-c-Jun (Cell Signaling Technology). BMK1 activity was measured by Western blotting analysis with a phospho-specific antibody for ERK5 (Cell Signaling Technology), as previously described. We also evaluated total ERK1/2, JNK, p38 MAPK, and BMK1 protein expression using pan-ERK1/2, JNK (c-Jun), p38 MAPK, and BMK1 (ERK5) antibodies (Cell Signaling Technology Inc). All values were normalized by arbitrarily setting the densitometry of vehicle-infused rats to 1.0.

**Histological Examination**

Kidneys were fixed with 10% formalin (pH 7.4), embedded in paraffin, sectioned into 4-μm slices, and stained with periodic acid-Schiff reagent. Thereafter, glomerular cellularity was determined by counting total nuclear cells in each glomerulus using light microscopy. The diameters of glomeruli in each experimental group were also measured using a visual caliper (SVS 30000; Showa Electric Laboratory). For an index of cellular proliferative activity, the glomerular cells expressing proliferating cell nuclear antigen (PCNA) were assessed. The glomerular cells immunoreactive for PCNA were counted. The data were expressed as the number of positive cells per glomerulus cross section, as described previously. For all measurements, 240 to 320 randomly selected glomeruli were examined.

**Analytical Procedures**

$U_{crea}$ was determined using a protein assay kit (microTP-test; Wako Co, Tokyo, Japan). We determined the degree of lipid peroxidation using biochemical assays of TBARS in renal cortical tissues and urine, as described previously. Renal cortical tissue collagen content was determined on the basis of the hydroxyproline concentration.

**Statistical Analysis**

The values are presented as means±SE. Statistical comparisons of the differences were performed using one-way analysis of variance combined with Newman-Keuls post hoc test. $P<0.05$ was considered statistically significant.
Results

Blood Pressure and Body, Kidney, and Heart Weights
The temporal profile of SBP is depicted in Figure 1A. SBP was identical among the 5 groups at the beginning of the protocol. SBP was unaltered during the protocol in vehicle- or vehicle/1% NaCl-treated rats (124 ± 1 and 118 ± 3 mm Hg, respectively, at week 6). However, aldosterone/1% NaCl-treated rats had progressive development of hypertension (165 ± 5 mm Hg at week 6). Concurrent administration of eplerenone or tempol prevented the development of hypertension in these animals (127 ± 2 and 125 ± 5 mm Hg, respectively, at week 6).

Body, kidney and heart weights are summarized in the Table. Treatment with vehicle/1% NaCl for 6 weeks significantly increased body and kidney weights. Kidney-to-body weight ratios were similar between vehicle- and vehicle/1% NaCl-treated rats. Aldosterone infusion to 1% NaCl-treated rats significantly increased kidney weight and the kidney/body weight ratio. Concurrent administrations of eplerenone and tempol resulted in similar decreases in the kidney weights and kidney/body weight ratios of aldosterone/1% NaCl-treated rats. However, these values were significantly higher than those of vehicle- or vehicle/1% NaCl-treated rats. Similar results were observed in heart weights and heart/body weight ratios (Table).

U protein V and Renal Cortical Collagen Content
The temporal profile ratio of U protein V is depicted in Figure 1B. Treatment with 1% NaCl alone did not alter U protein V. However, aldosterone/1% NaCl-treated rats showed a markedly higher U protein V (101 ± 24 mg/d at 6 week). Treatment with eplerenone or tempol to aldosterone/1% NaCl-treated rats prevented the increases in U protein V (10 ± 2 and 9 ± 2 mg/d, respectively). The hydroxyproline concentration in the renal cortical tissue of aldosterone/1% NaCl-treated rats was 14.5 ± 1.2 nmol/mg. The calculated cortical collagen content in aldosterone/1% NaCl-treated rats was 16.0 ± 0.7 g/mg, which was significantly higher than those of vehicle- and vehicle/1% NaCl-treated rats. In aldosterone/1% NaCl-treated rats, eplerenone and tempol normalized the collagen contents in renal cortex (Table).

| Effects of 6 Weeks of 1% NaCl, Aldosterone, Eplerenone, and Tempol on Body Weight (BW), Left Kidney Weight (LKW), Heart Weight (HW), and Renal Cortical Collagen Content in Rats |
|---|---|---|---|---|---|
| Vehicle | Vehicle + 1% NaCl | 1% NaCl + Aldosterone | 1% NaCl + Aldosterone + Eplerenone | 1% NaCl + Aldosterone + Tempol |
| Initial BW (g) | 234 ± 9 | 235 ± 8 | 247 ± 7 | 245 ± 8 | 242 ± 7 |
| Final BW (g) | 425 ± 15 | 462 ± 18* | 483 ± 10* | 503 ± 21* | 484 ± 12* |
| LKW (g) | 1.52 ± 0.04 | 1.62 ± 0.08 | 2.58 ± 0.09† | 1.94 ± 0.05†‡ | 2.06 ± 0.08†‡ |
| LKW/BW (%) | 3.55 ± 0.28 | 3.57 ± 0.23 | 5.39 ± 0.28* | 3.92 ± 0.17† | 4.05 ± 0.21†‡ |
| HW (g) | 1.35 ± 0.08 | 1.41 ± 0.03 | 1.73 ± 0.03* | 1.46 ± 0.04‡ | 1.47 ± 0.04‡ |
| HW/BW (%) | 3.10 ± 0.08 | 3.00 ± 0.05 | 3.59 ± 0.11* | 3.04 ± 0.17† | 3.09 ± 0.07† |
| Renal cortical collagen content (µg/g) | 11.9 ± 0.4 | 13.1 ± 1.1 | 16.0 ± 0.7*† | 11.7 ± 0.7‡ | 12.0 ± 0.6‡ |

Values are means ± SE.

*P < 0.05 vs vehicle; †P < 0.05: vehicle + 1% NaCl vs 1% NaCl + aldosterone, eplerenone, or tempol; ‡P < 0.05: 1% NaCl + aldosterone vs 1% NaCl + aldosterone + eplerenone or tempol.
Histological Findings
Vehicle- or vehicle/1% NaCl-treated rats showed normal glomeruli. However, aldosterone/1% NaCl-treated rats exhibited damaged glomeruli characterized by cell proliferation and mesangial matrix expansion (Figure 2A). Glomerular cellularity (nuclear cells/glomerulus) was elevated in aldosterone/1% NaCl-treated rats (67±1) compared with vehicle-treated (52±1) or vehicle/1% NaCl-treated rats (54±1) (Figure 2B). Figure 2C shows glomerular size distribution; glomeruli in aldosterone/1% NaCl-treated rats tended to be larger than vehicle- or vehicle/1% NaCl-treated rats. In aldosterone/1% NaCl-treated rats, concurrent administration of eplerenone or tempol normalized glomerular changes and improved both indicators (Figure 2A to 2C). Glomerular cells expressing PCNA were significantly increased in aldosterone/1% NaCl-treated rats (3.12±1.08 cells/glomerular cross section) compared with vehicle- or vehicle/1% NaCl-treated rats (0.84±0.13 and 0.96±0.21 cells/glomerular cross section, P<0.05, respectively). In aldosterone/1% NaCl-treated rats, PCNA positive cells in glomeruli were significantly decreased by concurrent administration of eplerenone or tempol (1.28±0.32 and 1.34±0.39 cells/glomerular cross section, P<0.05, respectively).

Renal Cortical TBARS Contents, Urinary Excretion Rate of TBARS (UTBARS V), and mRNA Expression of NAD(P)H Oxidase Components
Aldosterone/1% NaCl-treated rats showed significantly higher renal cortical TBARS contents (0.23±0.02 nmol/mg protein) than those of vehicle- and vehicle/1% NaCl-treated rats (0.10±0.01 and 0.09±0.01 nmol/mg protein, respectively; Figure 3A). Concurrent administration of eplerenone or tempol prevented the increases in TBARS contents of aldosterone/1% NaCl-treated rats (0.08±0.01 and 0.11±0.03 nmol/mg protein, respectively; Figure 3A). Similar results were observed in UTBARS V (Figure 3B). Namely, aldosterone...
infusion to 1% NaCl-treated rats significantly increased UTBARS V, which was prevented by concurrent administration of eplerenone or tempol (Figure 3B).

Expression of p22phox and Nox-4 mRNA in renal cortical tissues were similar between vehicle- and vehicle/1% NaCl-treated rats. However, vehicle/1% NaCl-treated rats showed approximately 3-times higher gp91phox mRNA expression compared with untreated vehicle-infused rats (Figure 4). Aldosterone/1% NaCl-treated rats showed significantly higher p22phox, Nox-4, and gp91phox expression than those of vehicle-treated (2.9 ± 0.3-fold, 3.9 ± 0.7-fold, and 10.0 ± 1.2-fold, respectively) and vehicle/1% NaCl-treated rats (2.3 ± 0.2-fold, 4.3 ± 0.8-fold, and 3.0 ± 0.3 fold, respectively). Treatment of eplerenone prevented aldosterone-induced increases in p22phox, Nox-4, and gp91phox mRNA expression. Similarly, tempol significantly decreased p22phox mRNA expression. However, Nox-4 and gp91phox mRNA expression were not altered by treatment with tempol (Figure 4).

Renal Cortical ERK1/2, JNK, p38 MAPK, and BMK1 Activities

The activities of ERK1/2, JNK, p38 MAPK, and BMK1 in renal cortical tissues were similar between vehicle-treated and vehicle/1% NaCl-treated rats. However, ERK1/2, JNK, and BMK1 activities in the renal cortical tissues of aldosterone/1% NaCl-treated rats were 3.7 ± 0.2-fold, 2.0 ± 0.3-fold, and 3.3 ± 0.2-fold higher, respectively, than those of vehicle-infused rats (Figure 5). Similarly, aldosterone/1% NaCl-treated rats showed higher ERK1/2, JNK, and BMK1 activities than vehicle/1% NaCl-treated rats (3.3 ± 0.3-fold, 2.3 ± 0.3-fold, and 3.0 ± 0.2-fold, respectively). In aldosterone/1% NaCl-treated rats, concurrent administration of eplerenone or tempol normalized these MAPK activities. However, renal cortical p38 MAPK activity was not different among all animal groups. No differences in the amounts of ERK1/2, JNK (c-Jun), p38 MAPK, and BMK1 (ERK 5) antibodies were observed in samples by Western blotting analyses using pan-ERK1/2, JNK (c-Jun), p38 MAPK, and BMK1 (ERK 5) antibodies.

Discussion

In agreement with previous studies performed in uninephrectomized rats,4,5 the present study showed that chronic aldosterone/salt treatment to non-nephrectomized rats induced hypertension and renal injury, characterized by glomerular changes, proteinuria, and collagen accumulation. The present study also provides evidence that aldosterone/salt-induced renal injury is associated with increases in renal cortical TBARS levels and UTBARS V. Furthermore, treatment with an antioxidant, tempol, normalized renal cortical TBARS contents and UTBARS V, and it prevented the development of renal injury in these animals. These data suggest that ROS are involved in the progression of renal injury induced by aldosterone/salt.

Many studies indicate that Ang II, the principal effector of the renin-angiotensin-aldosterone system, induces cellular changes through NAD(P)H oxidase-mediated ROS production.21–23 Recent studies by Weber et al 32,33 showed that immunohistochemical staining for gp91phox and 3-nitrotyrosine (a marker of nitrosative stress) were significantly increased in the heart of aldosterone/salt-treated uninephrectomized rats. In the present study, increased renal ROS levels in aldosterone/salt-treated rats are associated with increased mRNA expression of p22phox, Nox-4, and gp91phox. Thus, these data suggest the possibility that at least some of the aldosterone/salt-induced ROS production in the kidney are mediated through the NAD(P)H oxidase pathway. To support this possibility further, future studies will be needed to measure renal NAD(P)H oxidase activity in aldosterone/salt-treated rats. The present study also showed that increased intrarenal ROS levels and NAD(P)H oxidase expression induced by aldosterone/salt were prevented by treatment with eplerenone. These data are consistent with those of previous studies17,18 that increased vascular NAD(P)H oxidase activity and ROS production observed in
pathological conditions are reduced by treatment with spironolactone. In the present study, we also observed that tempol significantly reduced p22phox mRNA expression in the renal cortical tissues of aldosterone/salt-treated rats. Nox-4 and gp91phox expression were not altered by treatment with tempol. At this time, we have no satisfactory explanation for the decreased expression of p22phox by tempol. Further in vitro studies are required to determine the precise mechanisms responsible for tempol-induced alterations in the expression of NAD(P)H components.

ROS have been recognized as important mediators that regulate signal transduction pathways, including MAPK.21–25 The present study showed that in aldosterone/salt hypertensive rats, renal injury was associated with increases in ERK1/2, JNK, and BMK1 activities in the renal cortical tissues. However, renal p38 MAPK activity was unchanged in these animals, suggesting differential activations for each of the MAPK subfamily. As expected, MR blockade with eplerenone prevented aldosterone/salt-induced ERK1/2, JNK, and BMK1 activation. Interestingly, tempol also normalized these MAPKs activities in the kidneys of aldosterone/salt-treated rats. These data suggest that chronic treatment with aldosterone/salt induces MAPK activation via a ROS-dependent pathway. Although ROS also regulate other signaling molecules,21–25 it is possible that ROS-mediated MAPK activation is involved, at least in part, in the progression of renal injury observed in aldosterone/salt hypertensive rats.

Consistent with previous observations in hypertensive animals,13–15,17,25,34–35 tempol significantly decreased arterial pressure in aldosterone/salt-treated rats. Therefore, the possibility exists that the effects of tempol on intrarenal ROS.
levels and MAPKs activities may be dependent on arterial pressure changes. There are many studies that have documented that tempol reduces $\mathrm{O}_2^-$ levels in vitro.\textsuperscript{34-38} It has also been demonstrated that tempol reduces the formation of hydroxyl radicals generated in the Fenton reaction by reducing the intracellular levels of ferrous iron.\textsuperscript{16,38,39} In addition, tempol can reduce ROS levels and ameliorate ROS-related tissue injury in inflammation,\textsuperscript{40} ischemia/reperfusion,\textsuperscript{41,42} and radiation\textsuperscript{43} in the absence of blood pressure reduction. Recently, we have demonstrated that tempol prevents ROS generation and MAPK activation in aortic and heart tissues induced by acute infusion of Ang II in conscious rats.\textsuperscript{44} Interestingly, we also observed that the hypertensive response to acute Ang II infusion was not affected by treatment with tempol in conscious rats, suggesting that the effects of tempol on ROS levels and MAPKs activities are not sole consequences of blood pressure changes.\textsuperscript{44} Nevertheless, the aldosterone/salt hypertension model may not allow for this distinction; thus, the possibility for blood pressure-dependent effects of tempol on ROS levels and MAPKs activities cannot be ruled out in the present study. Further studies will be performed to investigate blood pressure-dependent and independent effects of tempol in aldosterone/salt-treated rats.

### Perspectives

The present study demonstrates that renal injury is associated with increases in renal cortical ROS levels and the activation of MAPKs in aldosterone/salt hypertensive rats. It has also been shown that p22phox, Nox-4, and gp91phox mRNA were upregulated in the kidneys of these animals. Furthermore, tempol treatment prevents the elevation of ROS levels and MAPKs activities and ameliorates renal injury. These findings might provide novel insights into the roles of ROS and MAPK in the pathogenesis of aldosterone/salt-induced renal injury. Based on the results from the present study, it can be speculated that some of the reno-protective effects of MR antagonists reported in recent clinical studies\textsuperscript{10,11} are mediated through their antioxidative actions.

### Acknowledgments

This work was supported by Pfizer Inc. (Pharmacia K.K.), a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan, the Research Foundation for Pharmaceutical Sciences, and the Mitsui Life Social Welfare Foundation (to Akira Nishiyama). We gratefully thank Akira Miyatake (Kagawa Medical University) and Hitotoshi Kakari (Fujisangyo Co., Kagawa, Japan) for excellent technical assistance.

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Hypertension. 2004;43:841-848; originally published online February 9, 2004; doi: 10.1161/01.HYP.0000118519.66430.22

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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