Cardioprotective Mechanism of Telmisartan via PPAR-γ-eNOS Pathway in Dahl Salt-Sensitive Hypertensive Rats

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BACKGROUND
Recently, some investigators have shown that telmisartan, an angiotensin II (Ang II)-receptor blocker (ARB), is a partial agonist of the peroxisome proliferator–activated receptor-γ (PPAR-γ). We investigate whether telmisartan improves cardiovascular remodeling associated with the production of endothelial nitric oxide synthase (eNOS) through PPAR-γ, inhibits the Rho-kinase pathway, and suppresses oxidative stress in Dahl salt-sensitive (DS) hypertensive rats.

METHODS
Telmisartan (1 mg/kg per day) or telmisartan plus PPAR-γ inhibitor, GW9662 (1 mg/kg per day) was administered from the age of 6–11 weeks. Age-matched male Dahl salt-resistant (DR) rats served as a control group.

RESULTS
The levels of eNOS and PPAR-γ expression, and eNOS phosphorylation were significantly lower in DS rats than in DR rats. Chronic telmisartan treatment in DS rats significantly increased these parameters, but not telmisartan plus GW9662. Telmisartan effectively inhibited the vascular lesion formation such as medial thickness and perivascular fibrosis, but not telmisartan plus GW9662. Moreover, upregulated RhoA protein, Rho-kinase mRNA, and myosin light-chain phosphorylation in DS rats was decreased by telmisartan to a similar degree as observed after treatment with Y-27632, a selective Rho-kinase inhibitor. In addition, NAD(P)H oxidase p22phox, p47phox, gp91phox expression, and mitogen-activated protein kinase and its downstream effector p70 S6 kinase phosphorylation in DS rats was also inhibited by telmisartan.

CONCLUSIONS
These results suggest that the cardioprotective mechanism of telmisartan may be partly due to improvement of endothelial function associated with PPAR-γ-eNOS, oxidative stress, and Rho-kinase pathway.

The peroxisome proliferator–activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand–activated transcription factors,1 and the most abundant isoform in adipose tissue. PPAR-γ plays a critical role in regulating carbohydrate and lipid metabolisms. In addition, PPAR-γ ligands have modest antihypertensive effects related at least in part to their ability to promote peripheral vasodilation, improve insulin sensitivity, and decrease the risk for atherosclerosis.2,3 Telmisartan, an angiotensin II (Ang II)-receptor blocker (ARB) recently approved for the treatment of hypertension, is structurally similar to a PPAR-γ agonist.4 In fact, telmisartan treatment in vitro augmented the PPAR-γ activity.4 This dual ARB/PPAR-γ agonist is capable of integrating and modulating two major metabolic pathways, one through activation of PPAR-γ pathway,5 and the second by selectively blocking the Ang II type 1 (AT₁) receptor–dependent proinflammatory, proatherogenic pathway.6 Substantial evidence indicates that activation of PPAR-γ leads to suppression of proinflammatory and proatherogenic molecules and related signaling pathways. Therefore, combined AT₁ receptor antagonism and PPAR-γ activation would constitute a powerful method of mitigating the metabolic and inflammatory derangements, which contribute to the development of atherosclerosis, vascular restenosis, and myocardial fibrosis.7,8 Recently, Cho et al.9 showed that troglitazone stimulated nitric oxide (NO) production by increasing endothelial NO synthase (eNOS) phosphorylation via a PPAR-γ-dependent pathway. However, the interactions between PPAR-γ and eNOS under chronic treatment of telmisartan in Dahl salt-sensitive hypertensive (DS) rats remain unknown. The aim of this study was to evaluate whether the dual ARB/PPAR-γ agonist telmisartan improves cardiovascular remodeling associated with eNOS production through PPAR-γ. Moreover, we examined whether telmisartan inhibits the Rho-kinase pathway, and suppresses oxidative stress in the left ventricle (LV) of DS rats.

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METHODS

Animals. Male inbred DS and Dahl salt-resistant (DR) rats (Eisai, Tokyo, Japan) were weaned and fed a diet containing 0.3% NaCl until 6 weeks of age. Thereafter, they were fed a diet containing 8% NaCl until 11 weeks of age. Systolic blood pressure was measured by the tail-cuff method at the start of the 8% NaCl diet and at 1-week intervals thereafter. All of the procedures were in accordance with our institutional guidelines for animal research and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental design. When the rats were 6 weeks old, DS rats (n = 23) were randomly divided into three groups and treated with one of the following drug combinations for 5 weeks, from 6 weeks of age to LVH stage: group 1, vehicle (DS-V; n = 9); group 2, telmisartan (1 mg/kg per day; Boehringer Ingelheim, Ingelheim, Germany; DS-TS; n = 8); and group 3, telmisartan plus PPAR-γ inhibitor, GW9662 (1 mg/kg per day (gastric gavage); DS-TS + GW; n = 6). Telmisartan (1 mg/kg per day PO, dissolved in dimethyl sulfoxide with dH2O) was administered to the rats by feeding tubes. Age-matched male DR rats served as a control group (DR-C; n = 9).

Moreover, to elucidate whether Rho-kinase pathway is involved in DS and DR rats, and whether Rho-kinase pathway is associated with AT1 receptor, we evaluated the effect of telmisartan and selective Rho-kinase inhibitor, Y-27632, on Rho-kinase. In addition, to evaluate the mechanisms of the beneficial effect of inhibiting the AT1 receptor and the Rho-kinase pathway, expression of eNOS mRNA was measured. The osmotic mini-pump (model 2ML2; Alzet) containing Y-27632, a selective Rho-kinase inhibitor, saline solution was implanted subcutaneously. Subpressor dose of Y-27632 in DS rats (3 mg/kg per day; DS-Y; n = 7) was continuously infused from 6 to 11 weeks.

Quantification of mRNA using reverse transcription–PCR. All of the procedures used for the mRNA extraction, cDNA synthesis, PCR, and quantification of PCR product were described in detail in our previous report.10–11 PCR was done using synthetic oligonucleotide primers as reported previously.11–13 The numbers of PCR cycles for the 12 genes examined were as follows: eNOS, 30; PPAR-γ, 32; Rho-kinase, 30; NAD(P)H oxidase p22phox, 36; p47phox, 32; gp91phox, 33; LOX-1, 38; transforming growth factor-β1 (TGF-β1), 32; type I collagen, 27; plasminogen activator inhibitor-1 (PAI-1), 32; tumor necrosis factor-α (TNF-α), 30; and GAPDH, 22.

Western blot analysis. The eNOS, PPAR-γ, RhoA, NAD(P)H oxidase p22phox, p47phox, gp91phox, and LOX-1 proteins were measured as described previously.10–13 Moreover, eNOS, myosin light chain, p65 nuclear factor-κB (p65NF-κB), protein kinase C-ε (PKC-ε), p44/p42 extracellular signal-regulated kinase (p44/p42ERK), and p70 S6 kinase (p70S6K) phosphorylation was measured as described in detail previously.10–13

Detection of superoxide anion and determination of NADPH oxidase activity. Histological detection of superoxide anion in the LV was performed using dihydroethidium as described previously.13 The NADPH oxidase activity in the LV was assessed by the measurement of superoxide-enhanced lucigenin chemiluminescence as described previously10,13 with some modifications.

Histological examination and evaluation of cardiovascular remodeling. At 11 weeks, histological examination was performed as described in detail previously.10,12,13 In brief, the wall-to-lumen ratio (the area of the vessel wall divided by the area of the total blood vessel lumen) was determined. The area of fibrosis immediately surrounding the blood vessels was calculated, and perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total area of the vessel in the microscopic field of each Masson’s trichrome–stained section.

Statistical analysis. All of the values are expressed as mean ± s.e.m. Mean values were compared among the four groups by analysis of variance and the Bonferroni post hoc test for multiple comparisons. P < 0.05 was considered statistically significant.

RESULTS

Physiologic profiles after 5 weeks of telmisartan treatment

Body weight was significantly lower in DS rats than in DR rats, and was not changed by the administration of telmisartan or telmisartan plus GW9662. In contrast, DS rats had higher left ventricular weight/body weight than DR rats. Telmisartan and telmisartan plus GW9662 significantly decreased left ventricular weight/body weight than nontreated DS rats. However, this ratio was significantly lower in telmisartan than in telmisartan plus GW9662. DS rats had markedly higher systolic blood pressure using the tail-cuff method than DR rats. None of long-term telmisartan or telmisartan plus GW9662 affected systolic blood pressure. There were no significant differences in heart rate among the four groups (Table 1).

Cardiovascular remodeling

Wall-to-lumen ratio and perivascular fibrosis were significantly higher in DS rats than in DR rats. Telmisartan and telmisartan plus GW9662 significantly decreased wall-to-lumen ratio and perivascular fibrosis compared with DS rats. However, these ratios were significantly lower in telmisartan than in telmisartan plus GW9662 (Figure 1a–d, Table 1).

Superoxide anion production and NADPH oxidase activity

To assess the involvement of oxidative stress in DS rats, superoxide anion production and NADPH oxidase activity were evaluated. Superoxide anion production was higher in DS rats than in DR rats. Chronic telmisartan therapy in DS rats significantly reduced superoxide anion production, but not telmisartan plus GW9662 (Figure 1e–h). Moreover, NADPH oxidase activity was significantly higher in DS rats than in DR rats. Long-term telmisartan treatment in DS rats significantly reduced the NADPH oxidase activity, but not telmisartan plus GW9662 (Table 1).
Table 1 | General characteristics and cardiovascular remodeling in DS rats, telmisartan-treated DS rats, and telmisartan plus GW9662–treated DS rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DR-C</th>
<th>DS-V</th>
<th>DS-TS</th>
<th>DS-TS + GW</th>
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<tr>
<td>Number</td>
<td>9</td>
<td>9</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>BW (g)</td>
<td>356 ± 8</td>
<td>314 ± 11*</td>
<td>341 ± 10</td>
<td>337 ± 12</td>
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<tr>
<td>SBP (mm Hg)</td>
<td>131 ± 4</td>
<td>238 ± 7*</td>
<td>236 ± 6*</td>
<td>235 ± 8*</td>
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<tr>
<td>LVW/BW (mg/g)</td>
<td>2.19 ± 0.06</td>
<td>3.41 ± 0.09*</td>
<td>2.61 ± 0.07*</td>
<td>3.01 ± 0.08*</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>460 ± 10</td>
<td>468 ± 12</td>
<td>453 ± 11</td>
<td>449 ± 12</td>
</tr>
<tr>
<td>Wall-to-lumen ratio</td>
<td>0.11 ± 0.01</td>
<td>0.34 ± 0.02*</td>
<td>0.18 ± 0.01*</td>
<td>0.26 ± 0.02*</td>
</tr>
<tr>
<td>Perivascular fibrosis (%)</td>
<td>0.25 ± 0.02</td>
<td>0.79 ± 0.06*</td>
<td>0.43 ± 0.04*</td>
<td>0.59 ± 0.05*</td>
</tr>
<tr>
<td>NAD(P)H oxidase (%)</td>
<td>100 ± 5.5</td>
<td>211.7 ± 11.4*</td>
<td>142.7 ± 6.3*</td>
<td>173.1 ± 7.3*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± s.e.m. BW, indicates body weight; DR-C, Dahl salt-resistant served as a control group; DS-TS, Dahl salt-sensitive hypertensive rats treated with telmisartan; DS-V, Dahl salt-sensitive hypertensive rats treated with vehicle; GW, GW9662; HR, heart rate; LVW, left ventricular weight; SBP, systolic blood pressure. *P < 0.01 vs. DR-C; †P < 0.01 vs. DR-C; ‡P < 0.01 vs. DS-V; ††P < 0.01 vs. DS-V; †‡P < 0.01 vs. DS-TS; DR-C, Dahl salt-resistant rats; DS-V, Dahl salt-sensitive hypertensive rats treated with vehicle; DS-TS, Dahl salt-sensitive hypertensive rats treated with telmisartan; and DS-TS + GW, Dahl salt-sensitive hypertensive rats treated with telmisartan plus GW9662.

Expression of eNOS and PPAR-γ, and eNOS phosphorylation

Figure 2 shows eNOS and PPAR-γ mRNA and protein, and eNOS phosphorylation in each group of DS rats subjected to 5 weeks of drug treatment. The levels of eNOS and PPAR-γ expression, and eNOS phosphorylation were significantly lower in DS rats than in DR rats. Chronic telmisartan treatment in DS rats significantly increased these parameters, but not telmisartan plus GW9662 (Figure 2a,b).

Expression of NAD(P)H oxidase subunit and LOX-1

Figure 3 shows levels of expression of NAD(P)H oxidase p22phox, p47phox, gp91phox, and LOX-1 in each group after 5 weeks of drug treatment. NAD(P)H oxidase p22phox, p47phox, gp91phox, and LOX-1 mRNA and protein levels in the LV were significantly higher in DS rats than in DR rats. NAD(P)H oxidase subunits and LOX-1 expression levels were significantly decreased by telmisartan, but not telmisartan plus GW9662 (Figure 3a–h).

Phosphorylation of p65NF-κb, PKC-ε, p44/p42ERK, p70S6K, and expression of TGF-β1, type I collagen, PAI-1, and TNF-α

Figure 4 shows phosphorylation of p65NF-κb, PKC-ε, p44/p42ERK, p70S6K, and expression of TGF-β1, type I collagen, PAI-1, and TNF-α in each group of DS rats subjected to 5 weeks of drug treatment. The phosphorylation of p65NF-κb, PKC-ε, p44/p42ERK, and p70S6K in the LV was significantly higher in DS rats than in DR rats. Chronic telmisartan therapy in DS rats significantly decreased the phosphorylation of p65NF-κb, PKC-ε, p44/p42ERK, and p70S6K, but not telmisartan plus GW9662 (Figure 4a–d). Moreover, expression of TGF-β1, type I collagen, PAI-1, and TNF-α in the LV was significantly higher in DS rats than in DR rats. The levels of these ratios were

![Micrographs](image)
In this study, we show that administration of telmisartan to DS rats ameliorated cardiac hypertrophy, cardiovascular remodeling, and suppressed expression of the genes coding for growth factors. In addition, telmisartan stimulates eNOS production through PPAR-γ and Rho-kinase pathway, suppresses NF-κB from NAD(P)H oxidative/LOX-1 pathway, and inhibits phosphorylation of intracellular signal transduction via activated mitogen-activated protein kinase and its downstream effector p70S6K through PKC-ε pathway. These findings indicate that these blood pressure–independent cardioprotective mechanisms may be related to improvement of endothelial function by PPAR-γ/Rho-kinase pathway, reduction of oxidative stress pathway, and suppression of PKC/ERK pathway.

Figure 4 | Effects of chronic telmisartan treatment on phosphorylation of (a) p65NF-κB, (b) PKC-ε, (c) p44/p42ERK, (d) p70S6K, and (e) expression of TGF-β1, (f) type I collagen, (g) PAI-1, and (h) TNF-α. Values are means ± s.e.m. n = 3–5 per group. *P < 0.05, **P < 0.01 vs. DR-C; †P < 0.05 vs. DS-V; ††P < 0.05 vs. DS-TS. DR-C, Dahl salt-resistant rats; DS-V, Dahl salt-sensitive hypertensive rats treated with vehicle; DS-TS, Dahl salt-sensitive hypertensive rats treated with telmisartan; and DS-TS + GW, Dahl salt-sensitive hypertensive rats treated with telmisartan plus GW9662.

Figure 5 | Effects of chronic telmisartan treatment on RhoA protein, Rho-kinase mRNA, MLC phosphorylation, and eNOS and mRNA. (a) Typical reverse transcriptase-PCR and western blot bands. (b) Mean densities of the Rho-kinase and eNOS mRNA band in relation to GAPDH, and percent of control of RhoA protein and MLC phosphorylation. Values are means ± s.e.m. n = 3–5 per group. *P < 0.05 vs. DR-C; †P < 0.05 vs. DS-V; ††P < 0.01 vs. DR-C; Dahl salt-resistant rats; DS-V, Dahl salt-sensitive hypertensive rats treated with vehicle; DS-TS, Dahl salt-sensitive hypertensive rats treated with telmisartan; DS-Y, Dahl salt-sensitive hypertensive rats treated with Y-27632; and MLC, myosin light chain.

**Suppressive effect of NAD(P)H oxidase**

This study showed that AT1 receptor blocker, telmisartan, inhibits the production of reactive oxygen species, which is associated with suppression of NAD(P)H oxidase subunit expression. Ang II stimulation produces superoxide via AT1 receptors and activation of NAD(P)H oxidases in endothelial and vascular smooth muscle cells. Superoxide production by vascular tissues and its interaction with NO play a crucial role in vascular pathophysiology. Indeed, Zhang et al. have shown that a pathophysiological level of Ang II impairs NO-dependent dilation in isolated porcine coronary arteries, which is a result of elevated superoxide production via AT1 receptor activation of NAD(P)H oxidase. In this study,
we demonstrated that telmisartan improved cardiovascular remodeling in association with the suppression of oxidative stress via the inhibition of NAD(P)H oxidase subunits expression, NAD(P)H oxidase activity by lucigenin-enhanced chemiluminescence method, and superoxide production by dihydroethidium staining. These actions were independent of the blood pressure lowering effect. Reactive oxygen species from the vessel wall are thought to play a pivotal role in atherogenesis. In addition, superoxide anion is produced via the activation of NAD(P)H oxidase in vessel wall cells and plays a critical role as the intracellular transmission factor in the Ang II–signaling system. Recently, Takaya et al. showed that in apolipoprotein E–deficient (apoE-KO) mice clinically relevant doses of telmisartan reduced atherosclerosis in association with suppressions of superoxide production by reducing NAD(P)H oxidase activity. These findings suggest that oxidative stress may play an important role in the involvement of impaired endothelial function and the pathogenesis of cardiovascular remodeling in DS rats, and that the increment of superoxide production was reduced by inactivation of NAD(P)H-dependent oxidase activity due to telmisartan.

Beyond blood pressure lowering effects
Recent reports indicated that ARB might contribute to the renoprotection observed beyond blood pressure lowering. We have demonstrated that there are major improvements of cardiovascular remodeling as well as endothelial function with dose of telmisartan despite the absence of blood pressure reduction. There are several recent publications that document the blood pressure–independent beneficial effects of telmisartan in the heart of several animal models. Takenaka et al. evaluated the role of telmisartan in mediating the transition from hypertrophy to heart failure in DS rats. The cardioprotective properties of telmisartan occurred in the absence of a decrease in blood pressure but were associated with a decrease in myocardial oxidative stress and extracellular matrix, which may be involved in mediating the adverse effects of Ang II system. Therefore, these findings suggest that there is a clear role for AT1 receptor in the pathogenesis of cardiovascular remodeling and that telmisartan may have direct cardioprotective effects. Thus, these blood pressure–independent cardioprotective mechanisms may be related to improvement of endothelial function associated with PPAR-γ-eNOS, oxidative stress, and Rho-kinase pathway.

Study limitation
A study limitation was that we did not observe 24-h blood pressure changes in this study because we used tail-cuff method. Because of this study limitation, we have to perform further investigations to elucidate whether the cardioprotective effect of telmisartan that was shown in this study is completely independent from blood pressure control. Moreover, the other study limitation was that we assessed the activity of NADPH oxidase in the LV by the measurement of superoxide-enhanced lucigenin chemiluminescence. Many previous studies have investigated NADPH oxidase activity using lucigenin-enhanced chemiluminescence. However, lucigenin has been recently criticized for its ability to redox cycle and its propensity to measure cellular reductase activity independent from NADPH oxidase. In addition, this technique does not provide information concerning the location of superoxide production in situ. For this purpose, we used staining with dihydroethidium, which is specific for superoxide production.

Conclusions
Telmisartan increases eNOS production by stimulation of the PPAR-γ signaling pathway. In addition, telmisartan inhibits the production of reactive oxygen species via AT1 receptor, which is associated with suppression of NAD(P)H oxidase subunit expression. Moreover, these actions lead to an improvement in cardiovascular remodeling. Therefore, the dual combined PPAR-γ activation and AT1 receptor antagonism would constitute powerful methods for beneficial cardioprotection. Thus, the dual ARB/PPAR-γ agonists telmisartan might have significant therapeutic potential in the treatment of atherosclerotic hypertension.

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