

Administration of Telmisartan Reduced Systolic Blood Pressure and Oxidative Stress Probably Through the Activation of PI3K/Akt/eNOS Pathway and NO Release in Spontaneously Hypertensive Rats

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Summary

We investigated the effects of telmisartan, the blocker of angiotensin II receptor 1, on the regulation of systolic blood pressure (SBP) and oxidative stress through endothelial nitric oxide (NO) release in spontaneously hypertensive rats (SHRs). SHRs randomly received placebo, oral feeding of telmisartan (5 mg/kg or 10 mg/kg) every day and Wistar-Kyoto rats (WKYs) served as normotensive control. The SBP of rat was measured before and weekly thereafter. After a total of 8-week treatment, rats were killed for experimental measurements. Parameters that subject to measurements in isolated aorta endothelial cells include: NO concentration, protein expression levels of angiotensin II receptor 1, nitrotyrosine, 8-isoprostane, SOD, PI3K, Akt, AMPK and eNOS. In addition, L-NMMA, a general inhibitor of nitric oxide synthase, was also applied to test the inhibition of NO concentration. We found that SBPs were significantly lower in telmisartan therapy group than in placebo treated hypertensive rats and WKYs ($p < 0.05$). The NO concentration was significantly higher in telmisartan-treated group with increased activity of the PI3K/Akt pathway and activated eNOS signaling. Blockade of Akt activity reversed such effects. Activation of AMPK also contributed to the phosphorylation of eNOS. L-NMMA treatment reduced less NO concentration in SHR rats than the telmisartan co-treated groups. Oxidative stress in SHRs was also attenuated by telmisartan administration, shown by reduced formation of nitrotyrosine, 8-isoprostane, and recovered SOD protein level. Telmisartan enhanced NO release by activating the PI3K/Akt system, AMPK phosphorylation and eNOS expression, which attenuated the blood pressure and oxidative stress in SHRs.

Key words

Angiotensin • NO • Hypertension • Oxidative stress • Telmisartan

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Introduction

Blood pressure (BP) is regulated through the integration of cardiac, neuronal, humoral, and vascular mechanisms. The renin-angiotensin system is one of the most important regulators of blood pressure (Crowley and Coffman 2008). Studies have shown that chronic treatment of angiotensin II receptor blockers (ARBs) has beneficial effects in spontaneously hypertensive rats (SHRs) (Dupuis *et al.* 2005). Clinical studies have also reported that ARBs hold beneficial effects on cardiovascular morbidity and mortality in hypertensive patients (Pfeffer *et al.* 2003, Yusuf *et al.* 2003). Nitric oxide (NO) is a highly reactive gaseous signaling molecule with a short half-life (3-5 s). It can diffuse through the biological membrane due to its both water- and lipid-soluble features. NO is recognized as an endothelium-derived relaxing factor that is bio-synthesized endogenously from L-arginine and oxygen by nitric oxide synthases (NOS) (Marsh and Marsh 2000). Evidences have shown that rats treated with compounds that diminish NO bioavailability, such as pharmacologic inhibitors of endothelial nitric oxide

synthase (eNOS) including L-nitroarginine or L-N-arginine methyl ester, displayed reduced vascular responsiveness to normal vasodilatory stimuli (Sakuma *et al.* 1992). Knockout of eNOS in mice also confirmed the roles of NO in BP regulation (Liu and Huang 2008). In this study, we hypothesized that the angiotensin II receptor antagonist telmisartan, in addition to its effect on the RAAS, could enhance the NO release and reduce oxidative stress in aorta endothelial cells (ECs) by up-regulating the eNOS expression through activating PI3K/Akt pathway and AMPK pathway, resulting in attenuated blood pressure in SHR. s.

Material and Methods

Animal experiments

Ten-week-old male spontaneously hypertensive rats (SHRs, 220-240 g) were fed a standard chow diet. Rats were randomly separated to the following treatments: oral feeding of 5 mg/kg or 10 mg/kg telmisartan in drinking water purchased from Boehringer Ingelheim Inc. (Shanghai, China) per day and vehicle control SHRs (n=8). Selection of telmisartan dosages was based on preliminary studies in our laboratory and previous studies (Susic *et al.* 2013). Age-matched Wistar-Kyoto rats (WKYs, ~200 g) were used as normotensive controls (n=8). Systolic arterial pressure was measured by tail-cuff plethysmography once a week. After eight weeks treatments, all rats were anaesthetized with sodium urethane (1.5 g/kg i.p.) and exsanguinated. Aortic homogenates were obtained for following Western blot assay. All animal experiments are approved by the Animal Ethics Committee of Tianjin Medical University.

Isolation of the aorta endothelial cells from SHR rats and Wistar-Kyoto rats

The aorta endothelial cells were isolated using a modification of the murine EC isolation method of Kobayashi *et al.* (2005). Thoracic aortae were excised and placed in a phosphate buffered solution (PBS) at pH 7.4. Aortae were carefully cleaned of fat, connective tissue and blood, taking care not to touch the luminal surface. The tissue was rinsed with Hank's Balanced Salt Solution (HBSS) and clamped at one end. A solution of 2 mg/ml Type I collagenase (Invitrogen, Carlsbad, CA) in HBSS was injected into the lumen and the tissue was incubated at 37 °C for 15 minutes. The clamp was then removed and the lumen flushed with HBSS to collect the ECs. The ECs were then plated in a 60 mm tissue culture

dish containing human EC growth media (EGM-2, Lonza, Inc., Basel, Switzerland) for further investigations. To test the inhibitory effects on Akt, 0.5 μM MK2206 (ChemieTek, Indianapolis, IN) was dissolved in DMSO and then treated in cell culture medium for 24 h (Liu *et al.* 2011).

Measurement of NO concentration in the aorta endothelial cells

The fabrication and calibration of the NO electrode were made as described previous study with minor modifications (Tjong *et al.* 2007). In brief, a platinum wire insulated in a polyethylene tube was dipped with Nafion. The Nafion-coated electrode was further modified with palladium and iridium oxide particles for improving the sensitivity of the NO electrode. Then, a thin film of poly-o-aminophenol (POAP) was deposited in the outer layer to ameliorate the selectivity of the NO electrode and to avoid fouling by proteins. NO standards were prepared by serial dilution of a saturated NO solution. The saturated NO solution was prepared by bubbling PBS (pH 7.0) with pure nitrogen for 30 min to remove O₂, following by NO gas (Matheson Gas, Basking Ridge, NJ) for 30 min. Standards were kept in a glass flask with a rubber septum. Electrochemical experiments were performed with a CHI 660A electrochemical analyzer (CH Instruments, Austin, TX) in a three-compartment cell with an Ag/AgCl reference electrode, a Pt wire auxiliary electrode, and a chemically modified electrode as working electrode. The NO electrode was calibrated with successive injections of various concentrations of NO from 20 to 1000 nM to the artificial cerebrospinal fluid in the recording chamber. The current was measured at a voltage of 0.9 V. The current response to various NO concentrations in a nanomolar range was very close to linear with the coefficient of the linear equation ($y=a+bx$) not less than 0.95. The detection limit of our electrode was about 10 nM with signal to noise ratio of 3 (Jian *et al.* 2007).

The aorta endothelial cells were equilibrated in the perfusate for 15-30 min. The tip of the NO electrode was gently placed at the endothelial cells under visual guidance with a dissecting microscope and the level of NO in the extracellular space was then measured. To test the effect of nitric oxide synthase (NOS) inhibitor on NO concentration, cells were pre-treated with 100 μM L-NMMA (Sigma, St. Louise, MO) for 10 min before NO detection. NO concentration from Wistar-Kyoto rats was used as control.

Western blotting

Proteins from the aorta endothelial cells and aortic homogenates were extracted by using protein extraction kit from Invitrogen. Concentration for each protein sample was analyzed *via* bicinchoninic acid (BCA) protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins were mixed with Laemmi buffer containing lysis buffer, 10 % 2-mercaptoethanol, and 2 mg/ml bromophenol blue. Samples were incubated at 95 °C for 5 min and 20 µl of each sample was loaded in each well of a 10 % SDS-polyacrylamide mini-gel. Membranes were then transferred to polyvinylidenedifluoride membranes using a transblotting apparatus (Bio-Rad) for 60 min. Then membranes were incubated at room temperature for 2 h in TBS buffer with 5 % skimmed milk, followed by incubating with appropriate primary antibodies including eNOS (1:1000, Santa Cruz Biotechnology Inc. Santa Cruz, CA), p-eNOS (at Ser1177, 1:1000, Santa Cruz), PI3K (1:1000, Cell Signaling, Danvers, MA), p-PI3K (at Tyr508, 1:1000, Cell Signaling), AMPK (1:1000, Cell Signaling), p-AMPK (at Thr172, 1:1000, Cell Signaling), Akt (1:1000, Cell Signaling), p-Akt (at Ser473, 1:1000, Cell Signaling), nitrotyrosine (NTR, 1:1000, Cell Signaling), SOD (1:1000, Santa Cruz), Cytochrome P450 2E1 (CYP2E1, 1:1000, Abcam), and angiotensin II receptor 1 (1:1000, Abcam, Cambridge, MA) in TBS buffer with 5 % skimmed milk for overnight at 4 °C. After incubation, membranes were washed and incubated with second antibody, anti-mouse IgG conjugated to HRP for eNOS and p-eNOS (1:10000; Santa Cruz), anti-goat IgG conjugated to HRP for angiotensin II receptor 1 (1:10000; Santa Cruz), anti-rabbit for PI3K, p-PI3K, Akt, p-Akt, NTR, and SOD (1:10000; Santa Cruz) in TBS solution with 5 % skimmed milk for 1 h. Then blots were developed using chemiluminescence reagent (Pierce Biotechnology, Rockford, IL). Films were exposed and analyzed by using ImageJ software (National Institute of Health, Bethesda, MD). Results were expressed in relative optical density against parallel blotting of β-actin (Sigma, St. Louis, MO).

8-isoprostane measurement

To evaluate the oxidative stress in the primary cultured aorta endothelial cells of SHRs, the level of 8-isoprostane for each sample was measured using commercial kit from Cayman Chemical (Cayman Chemical Company, Ann Arbor, Michigan) and expressed as percentage of control level in Figure 4.

Statistics and data analysis

Graphpad Prism software (Graphpad Software, Inc., San Diego, CA) was used to analyze the statistics of the data. Results are presented as means ± SEM and statistical analyses between groups are one-way ANOVA with *post-hoc* tests for multiple comparisons (Bonferroni correction). Statistical significance was considered at $p < 0.05$.

Results

To determine the effect of telmisartan treatment on blood pressure in SHR rats, we measured the SBPs of all group rats every week. The baseline SBP in SHRs was 182 ± 2 mm Hg which was much higher than that in WKY (121 ± 1 mm Hg, $p < 0.001$). Administrations of telmisartan in the dose of 10 mg/kg concentration showed a significant decrease in SBP from week 2, and the administration of 5 mg/kg telmisartan showed a substantial decrease in SBP decrease from week 3 (Fig. 1, $p < 0.01$). At the end of week 8, the SBP of both telmisartan-treated groups showed significant reduction when compared with the vehicle control SHR rats.

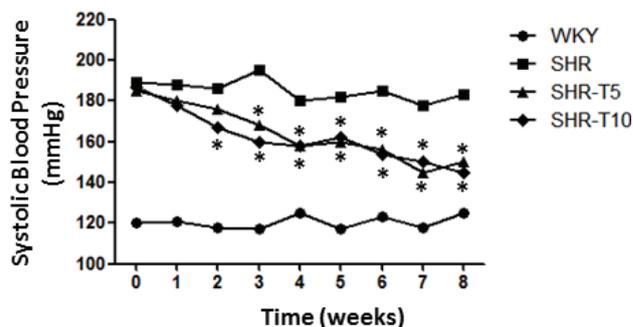


Fig. 1. Effect of telmisartan treatment on blood pressure of SHR rats with or without telmisartan co-treatment from week 1 to week 8. Results are presented as means ± SEM and statistical analyses between groups are one-way ANOVA with *post-hoc* tests for multiple comparisons. Statistical significance was considered at $p < 0.05$ ($n = 8$). Age-matched Wistar-Kyoto rats were used as normotensive controls. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats, SHR-T5, SHR with 5 mg/kg telmisartan; SHR-T10, SHR with 10 mg/kg telmisartan.

We then examined the endogenous NO bioactivity in the isolated endothelial cells. The NO concentration in the both telmisartan-treated groups (5 mg/kg and 10 mg/kg) increased significantly when compared with that in the vehicle control SHR rats (Fig. 2A, $p < 0.05$). The NO concentration reduced in all

groups of SHRs significantly after treatment with 100 μ M L-NMMA. The effect of L-NMMA on endogenous NO concentration in telmisartan-free group was significantly stronger than that in telmisartan treated groups (Fig. 2B, $p < 0.05$).

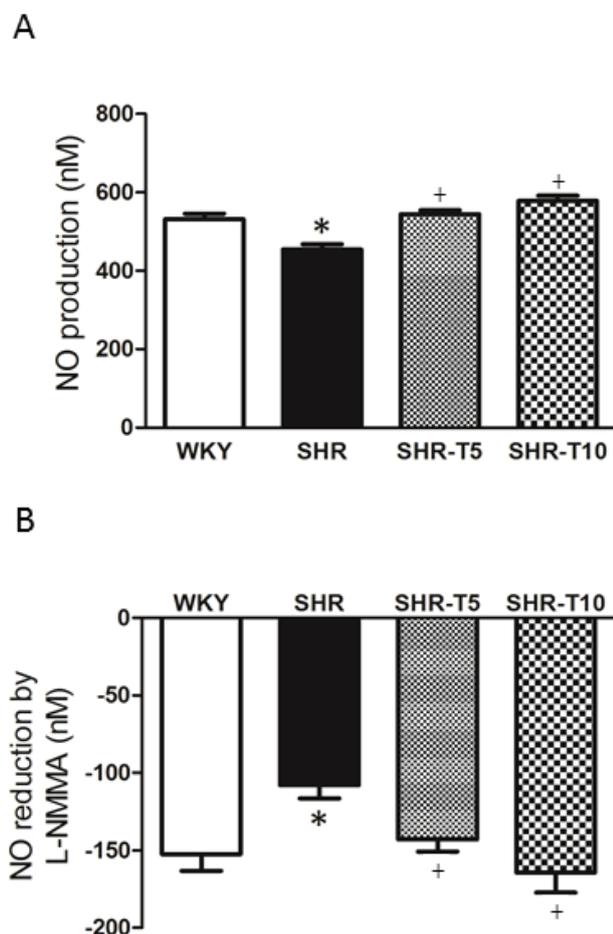


Fig. 2. Effect of telmisartan treatment on nitric oxide (NO) production from isolated endothelial cells of both SHR rats and Wistar-Kyoto rats (**A**). After pre-treatment with 100 μ M nitric oxide synthase (NOS) inhibitor L-NMMA, reduction of NO production was also measure in isolated endothelial cells (**B**). Results are presented as means \pm SEM and statistical analyses between groups are one-way ANOVA with *post-hoc* tests for multiple comparisons. Statistical significance was considered at $p < 0.05$ ($n = 8$). SHR, spontaneously hypertensive rats, SHR-T5, SHR with 5 mg/kg telmisartan; SHR-T10, SHR with 10 mg/kg telmisartan.

Both administrations of telmisartan (5 mg/kg and 10 mg/kg) significantly reduced the formation of nitrotyrosine and 8-isoprostane in the primary cultured aorta endothelial cells of SHR rats, indicating a reduction of oxidative stress in these cells (Fig. 3A and 3B, $p < 0.01$). This effect was accompanied by the restoration of endogenous protein level of antioxidant enzyme SOD (Fig. 3C, $p < 0.01$). In addition, as a key mediator in the

formation of oxidative stress, the protein expression level of CYP2E1 was also down-regulated through the action of telmisartan (Fig. 3D, $p < 0.01$).

The protein expression of eNOS and phosphorylated eNOS in the aorta endothelial cells were examined by Western blot. Results showed that total and phosphorylated eNOS were significantly lower in SHR rats than those in WKY rats (Fig. 4). The total eNOS expression was significantly increased in both telmisartan-treated groups when compared with the vehicle control SHR rats. The phosphorylation of eNOS also increased significantly in groups co-treated with telmisartan when compared with the vehicle control SHR rats (Fig. 4). When cells were treated with Akt-specific blocker, both levels of phosphorylated eNOS and total eNOS were partially blocked.

We then test the protein expression level of angiotensin II receptor 1 after the treatment of its specific blocker. As expected, our results showed that the receptor protein expression was significantly decreased in the telmisartan-treated groups when compared with the vehicle control SHR rats. Data suggesting that the telmisartan is effectively specific for blocking this receptor (Fig. 5A).

The protein expression of phosphorylation of PI3K and Akt in the endothelial cells were examined by Western blot study. Results showed that the phosphorylation forms of PI3K and Akt were significantly increased in telmisartan-treated groups than control SHR rats. However, the total protein expressions of PI3K and Akt did not show any change after the co-treatment with both telmisartan concentrations when compared with the vehicle control SHR rats (the exact levels of total proteins were not shown) (Fig. 5B and 5C). MK2206 treatment only blocked the phosphorylated form of Akt but did not influence its total form, as well as the expression of PI3K. We also found that the activity of AMPK was activated by the treatment of telmisartan, which probably contributed to the activation of eNOS (Fig. 5D).

To connect the findings from *in vitro* to *in vivo*, we then measured the levels of PI3K, Akt, and eNOS in the aortic homogenates from SHRs. After the co-treatments with telmisartan, the level changes of phosphorylated PI3K, Akt, and eNOS showed very similar trends with the *in vitro* results, indicating a consistent phenotype between *in vitro* and *in vivo* studies (Fig. 6).

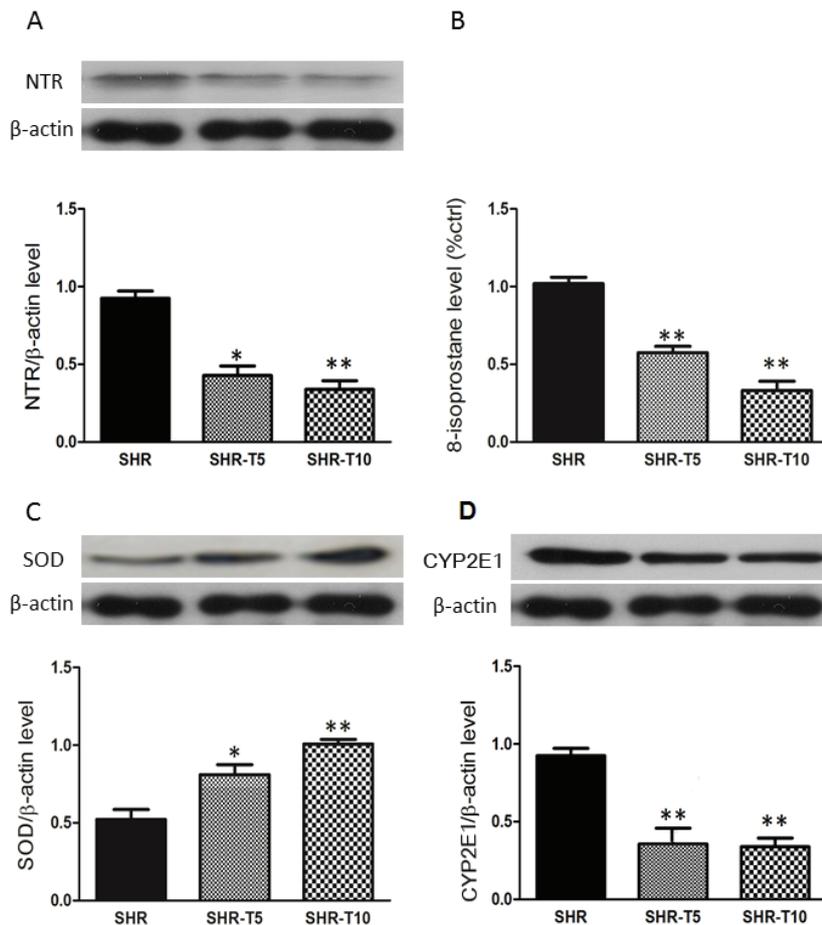
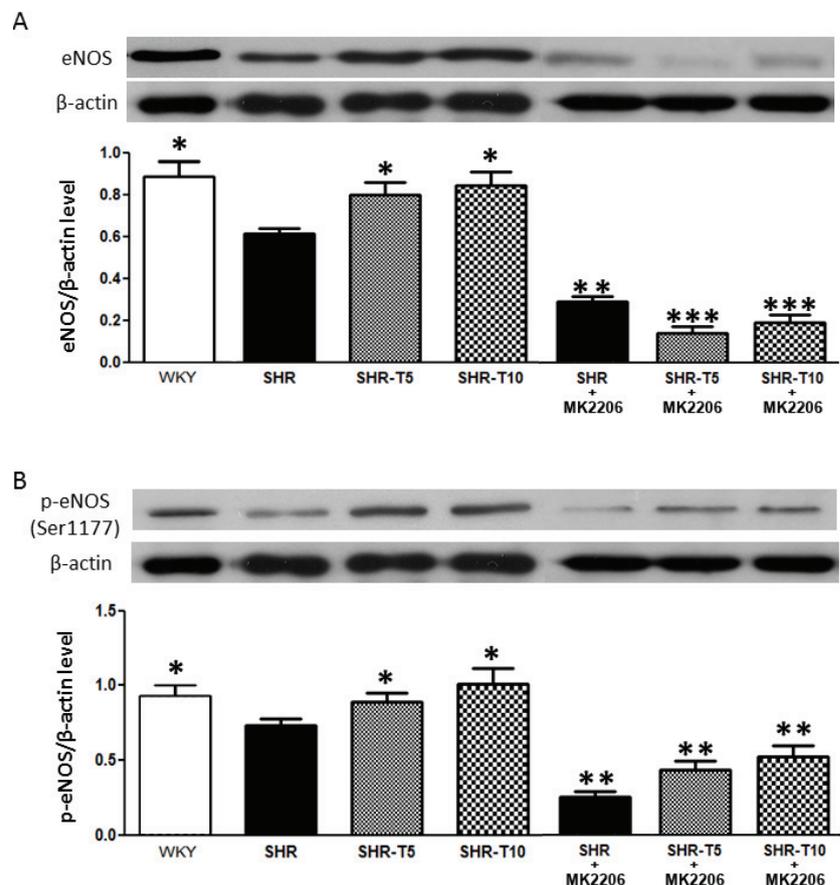


Fig. 3. Representative Western blot results for the formation of nitrotyrosine (NTR, **A**), SOD (**C**), and CYP2E1 (**D**) in SHR rats with or without telmisartan co-treatment. Level of 8-isoprostane was measured in aorta endothelial cells (**B**). Results are presented as means \pm SEM and statistical analyses between groups are one-way ANOVA with *post-hoc* tests for multiple comparisons. Statistical significance was considered at $p < 0.05$ ($n = 8$). SHR, spontaneously hypertensive rats; SHR-T5, SHR with 5 mg/kg telmisartan; SHR-T10, SHR with 10 mg/kg telmisartan.

Fig. 4. Representative Western blot results for (**A**) total eNOS and (**B**) phosphorylated eNOS in SHR rats with or without telmisartan co-treatment and WKY rats. For SHR rats endothelial cells, Akt specific blocker MK2206 was co-treated with or without telmisartan. Results are presented as means \pm SEM and statistical analyses between groups are one-way ANOVA with *post-hoc* tests for multiple comparisons. Statistical significance was considered at $p < 0.05$ ($n = 8$). SHR, spontaneously hypertensive rats; SHR-T5, SHR with 5 mg/kg telmisartan; SHR-T10, SHR with 10 mg/kg telmisartan.



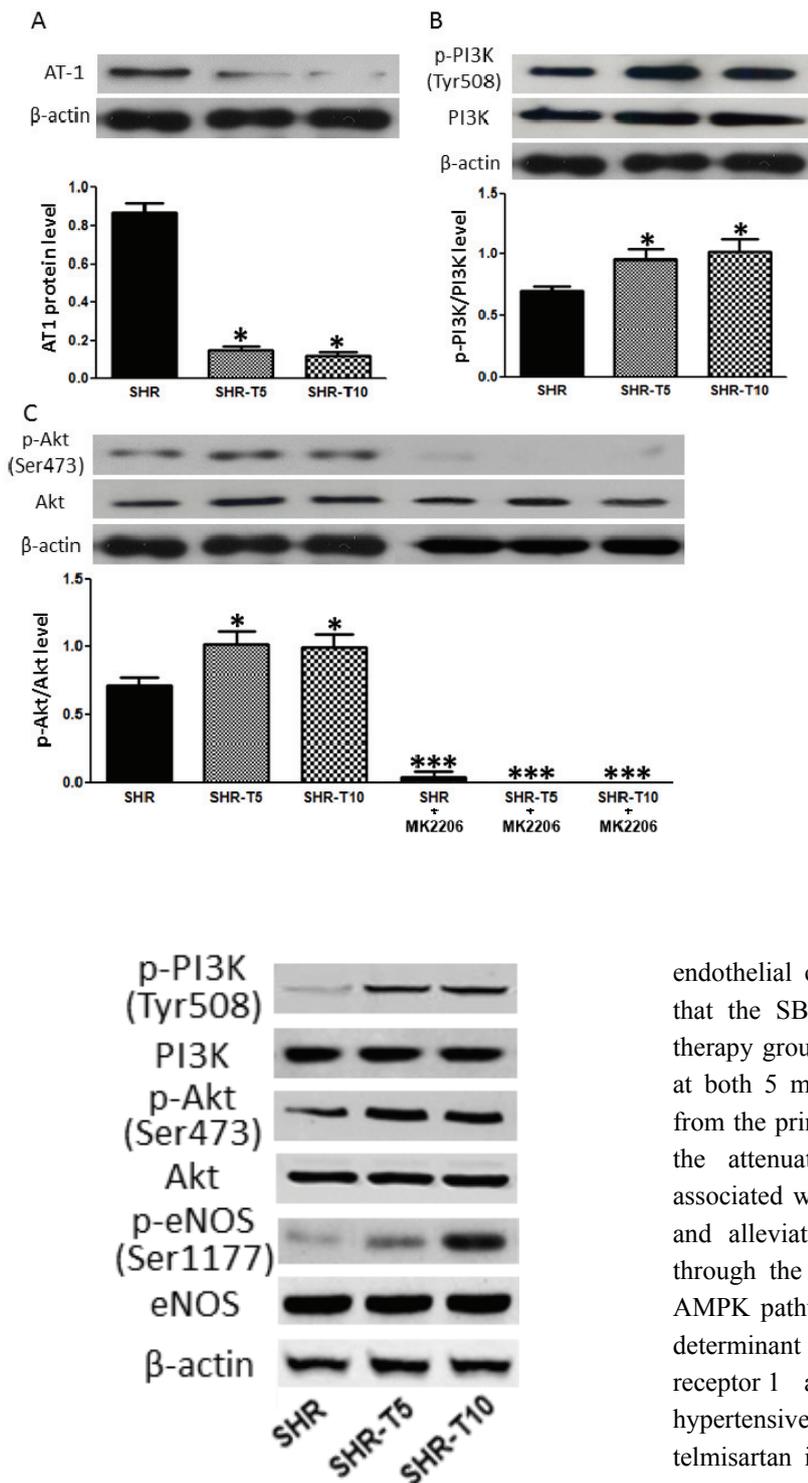
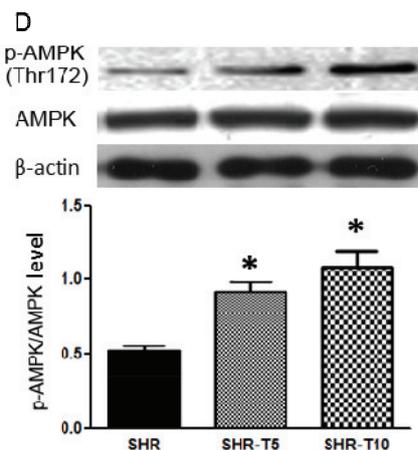


Fig. 6. Representative Western blot results for phosphorylated and total form of PI3K, Akt, and eNOS in the aortic homogenates of SHR rats with or without telmisartan co-treatment. SHR, spontaneously hypertensive rats, SHR-T5, SHR with 5 mg/kg telmisartan; SHR-T10, SHR with 10 mg/kg telmisartan.

Discussion

This is the first study reporting telmisartan increased NO bioactivity in the primary SHR rat aorta

Fig. 5. Representative Western blot results for (A) angiotensin II receptor 1 (AT 1), (B) phosphorylated and total PI3K, (C) phosphorylated and total Akt, and (D) phosphorylated and total AMPK in SHR rats with or without telmisartan. For SHR rats endothelial cells, Akt specific blocker MK2206 was co-treated with or without telmisartan. Results are presented as means ± SEM and statistical analyses between groups are one-way ANOVA with *post-hoc* tests for multiple comparisons. Statistical significance was considered at $p < 0.05$ ($n = 8$). SHR, spontaneously hypertensive rats, SHR-T5, SHR with 5 mg/kg telmisartan; SHR-T10, SHR with 10 mg/kg telmisartan.



endothelial cell. In the current study, we demonstrated that the SBPs were significantly lower in telmisartan therapy groups than in placebo-treated hypertensive rats, at both 5 mg/kg and 10 mg/kg concentrations. Results from the primary cultured aorta endothelial cells showed the attenuation of hypertension in SHR rats was associated with increased endogenous NO concentration and alleviated oxidative stress, which were probably through the activation of PI3k/Akt/eNOS pathway and AMPK pathway. Hypertension is considered as a major determinant of endothelial dysfunction and angiotensin II receptor 1 antagonists are shown to possess anti-hypertensive effect. Substantial evidences suggested that telmisartan is also a partial PPAR γ agonist and thus it may efficiently improve endothelial function (Benson *et al.* 2004, Kobayashi *et al.* 2008). Clinical studies also showed that telmisartan was well-tolerated and effective in lowering blood pressure in hypertensive patients (de Gasparo *et al.* 2000, Sharpe *et al.* 2001, Kulkarni *et al.* 2005). In this study, NO concentration in the SHR was reduced as compared to that in WKYs, which is in agreement with some recent studies (Yang *et al.* 2011a,b). However, other studies found elevated NO production and NOS expression in the aorta of SHRs

when compared with WKYs (Púzserová *et al.* 2007, Caniffi *et al.* 2011, Zheng and Yu 2012). The discrepancies among these studies might result from the temporal and spatial specificity of NOS expressions and other upstream pathways (e.g. PI3K/Akt and AMPK), which determine actual NO production in the aorta of these rat strains. Detail mechanism needs further research.

In the present study, telmisartan increased eNOS phosphorylation at Ser1177 as revealed by Western blot analysis on the rat aorta endothelial cells. In fact, eNOS is not only regulated at its expression level, but also its activity is modified by phosphorylation (Harris *et al.* 2001) and post-translational mechanisms including the interaction of eNOS with other regulatory proteins (Garcia-Cardena *et al.* 1997, Kone 2000). Increased eNOS phosphorylation may result from an increased eNOS expression by telmisartan and the elevated expression of other eNOS upstream pathways, e.g. PI3K/Akt pathway and AMPK pathway. From our results, the phosphorylation of both PI3K and Akt occurred after telmisartan treatment in the primary cell, indicating the activation of this pathway. It is interesting that this finding is opposite to a recent study showing that treatment with renin reduced hypertension through activating AT1/PI3K/Akt/eNOS signaling (Cheng *et al.* 2012). The discrepancy can be attributed to different cell types and mechanisms which need further investigation. The blockade of NOS activity with its general inhibitor L-NMMA largely decreased the production of NO in SHR rats, suggesting the NO concentration in the endothelial cells was specific to the NOS (e.g. eNOS), further confirmed the possible involvement of PI3K/Akt/eNOS pathway in the beneficial effects of telmisartan. We also found that AMPK was activated in the upstream of eNOS, which was consistent with a very recent study reporting that telmisartan activates the AMPK/SIRT1 pathway in skeletal muscle (Shiota *et al.* 2012). In addition, the activation of eNOS may also relate to eNOS-interacting proteins. Telmisartan was reported to improve endothelial function by augmenting the vascular

level of tetrahydrobiopterin (BH₄, an eNOS cofactor) in aortae of Dahl salt-sensitive rats (Satoh *et al.* 2010). Moreover, telmisartan up-regulates a BH₄-synthesizing enzyme GTP cyclohydrolase I, which reduces eNOS uncoupling in diabetic rats (Wenzel *et al.* 2008). Polikandriotis *et al.* (2005) showed that rosiglitazone elevates endothelial NO concentration by increasing heat shock protein 90 (hsp90) in HUVEC.30, while hsp90 was identified to strengthen eNOS activities by promoting eNOS-Ser1177 phosphorylation (Fontana *et al.* 2002). These observations may explain part of mechanisms by which telmisartan increases the eNOS activity in vasculatures. Furthermore, we should also consider the negative feedback regulation of NOS by NO. The elevation of NO production by telmisartan could result in its attenuation after longer telmisartan treatment. Thus, during long-term treatment, the effect of telmisartan on BP could be primarily associated with direct attenuation of AT1 signaling rather than with improved NO bioavailability (Kopincová *et al.* 2012). However, these possibilities indeed need further experimental verifications. Another limitation of the study is the lack of rat urinary excretion data, which demonstrates the sodium balance. It is also interesting that telmisartan treatment decreased the protein level of AT1. This finding is consistent with a recent study that telmisartan down-regulates AT1 mRNA and protein levels through activation of PPAR γ (Imayama *et al.* 2006).

As the summary, our results have showed that the SBPs were lowered by the treatment of 5 mg/kg and 10 mg/kg telmisartan treatments through blocking angiotensin II receptor 1, activating the PI3K/Akt/eNOS pathway and AMPK pathway, increasing NO release, and alleviating oxidative stress in SHR rats. Those results contributed novel knowledge to the anti-hypertensive properties of telmisartan. *In vivo* data using the aortic and kidney homogenates are needed to reproduce these findings in future studies.

Conflict of Interest

There is no conflict of interest.

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