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Chronic Production of Peroxynitrite in the Vascular Wall Impairs Vasorelaxation Function in SHR/NDmcr-cp Rats, an Animal Model of Metabolic Syndrome

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Abstract. We have previously reported that peroxynitrite is involved in dysfunction of nitric oxide (NO)-mediated vasorelaxation in SHR/NDmcr-cp rats (SHR-cp), which display typical symptoms of metabolic syndrome. This study investigated whether peroxynitrite is actually generated in the vascular wall with angiotensin II–induced NADPH-oxidase activation, thus contributing to the dysfunction. In isolated mesenteric arteries of male 18-week-old SHR-cp, relaxations in response to acetylcholine and sodium nitroprusside were impaired compared with that in Wistar-Kyoto rats. This impaired relaxation was not restored by treatment with apocynin, an NADPH-oxidase inhibitor. Protein expression of endothelial NO synthase increased while that of soluble guanylyl cyclase (sGC) decreased in the artery. We observed increased production of superoxide anions and peroxynitrite from the artery and their inhibition by apocynin, and also increased contents of nitrotyrosine, a biomarker of peroxynitrite, in mesenteric arteries and angiotensin II in aortas. Long-term (8 weeks) administration of telmisartan, an angiotensin II type 1–receptor antagonist, prevented the impaired vasorelaxation, decreased sGC expression and increased nitrotyrosine content in mesenteric arteries. These findings suggest that in the vascular wall of SHR-cp, peroxynitrite is continually produced by the reaction of NO with NADPH oxidase–derived superoxide via angiotensin II and gradually denatures sGC protein, leading to vasorelaxation dysfunction.

Keywords: peroxynitrite, angiotensin II, metabolic syndrome, NADPH oxidase, oxidative stress

Introduction

Metabolic syndrome is a group of metabolic risk factors that occur in an individual, which is basically associated with obesity and insulin resistance. This syndrome is known to accelerate the atherogenic process. Thus, people with metabolic syndrome have the risk of developing diseases related to atherosclerosis, that is, heart attack or stroke (1). An initial event in the atherogenic process is considered to be vascular endothelial dysfunction. The vascular endothelium regulates the tonus of underlying smooth muscle cells by releasing vasorelaxing factors such as nitric oxide (NO). NO also plays an important role in protection against the onset of atherosclerosis via inhibition of smooth muscle cell growth and migration, platelet aggregation, monocyte adhesion, inflammation, lipoprotein oxidation, and remodeling (2). Therefore, a decline in NO bioavailability is commonly thought to be one of the factors leading to the development of atherosclerosis (3).

A decline in NO bioavailability may be caused by decreased NO production from the endothelium, accelerated NO degradation by reactive oxygen species (ROS), and dysfunction in response to NO in effector cells, that is, vascular smooth muscle cells. In these processes, accelerated NO degradation by ROS has been focused upon as a remarkable mechanism because up-regulated and activated ROS are considered to play a

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critical role in the development of several pathophysiological conditions, including atherosclerosis and hypertension, in animals and patients (4 – 6). In blood vessels, a major ROS is superoxide anion produced by angiotensin II–induced NADPH-oxidase activation (7, 8). Superoxide anions are associated with vascular endothelial dysfunction in experimental animals and patients with metabolic disorders (4, 6, 9, 10). The superoxide anion itself is widely accepted as a powerful oxidant, but recent attention has been focused on peroxynitrite, which is formed by rapid reaction of NO with superoxide anion. Peroxynitrite is known to be much more reactive than its parent molecules NO and superoxide anion, although it does not exist as a free radical in nature (11). Peroxynitrite can cause profound cellular injury and cell death by interacting with lipids, DNA, and proteins via oxidative and nitrosative reactions; and peroxynitrite generation represents a crucial pathogenic mechanism in conditions such as stroke, myocardial infarction, chronic heart failure, diabetes, and chronic inflammatory diseases (11 – 15). Therefore, peroxynitrite may play an important role in modulating vascular injury in patients and animals with metabolic syndrome.

We have previously demonstrated that systemic oxidative–nitrosative stress is increased in SHR/NDmcr-cp rats (SHR-cp), which display typical symptoms of metabolic syndrome (16), and proposed that peroxynitrite is involved in dysfunction of vasorelaxation in aortas of SHR-cp (17, 18). However, it is currently unclear whether peroxynitrite is actually generated in the vascular walls per se via angiotensin II–induced NADPH-oxidase activation and contributes to dysfunction of vasodilation in SHR-cp. Therefore, in the present study, we examined peroxynitrite production from mesenteric arteries of SHR-cp. We also investigated whether angiotensin II is involved in peroxynitrite production and how peroxynitrite leads to impairment of the vasorelaxation response in SHR-cp.

Materials and Methods

Experimental animals

Eighteen-week-old male SHR-cp and Wister-Kyoto rats (WKY) as controls (n = 12 each) were purchased from Japan SLC, Inc. (Hamamatsu) and used for a comparative experiment. Rats were established by the Disease Model Cooperative Research Association (Hamamatsu). In another experiment, 10-week-old male SHR-cp were divided into two groups (n = 6 each): telmisartan-treated group (Telm group) and control group. The Telm group was orally given telmisartan suspended in 0.5% CMC at a dose of 10 mg/kg once a day for 8 weeks. The control group was orally given 0.5% CMC alone (0.1 ml/100 g body weight). The dosage schedule was decided from previous data where telmisartan had effectively prevented hypertension and vascular dysfunction in aortas of SHR-cp (18).

The systolic blood pressure was determined in conscious rats by the tail-cuff method (MK-2000; Muromachi, Tokyo). In the experiments, blood was drawn from the abdominal aorta under anesthesia with pentobarbital sodium (60 mg/kg, i.p.), and the serum was separated by centrifugation at 3000 × *g* for 10 min. The following serum parameters were determined by commercial kits: triglyceride, total cholesterol, and glucose levels (Triglyceride E-test Wako, Cholesterol E-test Wako, and Glucose C II-test Wako, respectively; Wako Pure Chemical Ind., Ltd., Osaka) and insulin level (ultra-sensitive rat insulin kit; Morinaga Biochemistry Lab., Tokyo).

All rats were maintained on a standard diet and given water ad libitum in an air-conditioned room. The study protocols were performed according to the Guideline Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Relaxation studies

The mesenteric arteries were removed and immediately placed in Krebs-Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25.0 mM $NaHCO_3$, and 11.1 mM glucose). The second branch of the mesenteric artery was cleaned of adherent tissue and cut into 3-mm rings. The rings were mounted isometrically at an optimal resting tension of 0.3 g in a 10-ml organ bath filled with the solution (37°C, pH 7.4) described above. The bath solution was continuously aerated with a gas mixture of 95% O_2 and 5% CO_2. Isometric tension change was measured with a force-displacement transducer (Model t-7; NEC San-Ei, Tokyo) coupled to a dual channel chart recorder (Model 8K21, NEC San-Ei). The rings were preconstricted with 1 – 3 μM phenylephrine to generate approximately 80% of the maximal contraction. Once stable contraction was obtained, acetylcholine (0.1 nM – 1 μM) or sodium nitroprusside (0.1 nM – 1 μM) was cumulatively added to the bath. In some experiments, the contraction–relaxation procedure described above was repeated after the rings had been preincubated for 20 min with apocynin (300 μM), an NADPH oxidase inhibitor, and Mn-TBAP (100 μM), a cell-permeable superoxide dismutase mimetic. The relaxation response obtained was expressed as a percentage of the maximal relaxation caused by 100 μM papaverine.

Western blot analysis

The mesenteric arteries were homogenized in a glass

/glass homogenizer in lysis buffer [50 mM Tris-HCl buffer (pH 7.5), containing 0.15 M NaCl, 10 mM EDTA, 0.1% Tween-20, 0.01% (v/v) protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA) and 1 mM dithiothreitol]. The protein concentration was determined for each sample using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Equivalent amounts of total arterial protein (20 μg) were loaded on the SDS-PAGE (MULTIGEL II Mini 8/16; Cosmo Bio Co., Ltd., Tokyo), electrophoresed, and then blotted onto nitrocellulose. Proteins, endothelial NO synthase (eNOS), soluble guanylyl cyclase (sGC), cGMP-dependent protein kinase (PKG), alpha-actin, and beta-actin, were detected with their respective antibodies linked with the appropriate horseradish peroxidase–coupled secondary antibody. Alpha-actin or beta-actin was used as an internal control. The data were expressed as the following ratios with respect to the internal control proteins: eNOS/beta-actin, sGC/alpha-actin, and PKG/alpha-actin.

The antibodies used in the present experiments were as follows: antibody to eNOS and rabbit IgG conjugated to horseradish peroxidase (BD Transduction Laboratories, Lexington, KY, USA); antibody to beta-1 subunit of sGC (Alexis Biochemicals, Lausen, Switzerland); antibody to alpha-actin (Progen Biotechnik GMBH, Heidelberg, Germany); antibody to beta-actin (Sigma Chemical Co.); antibody to PKG 1 alpha and 1 beta (Carbiochem-Novabiochem Co., San Diego, CA, USA); antibody to mouse IgG (H + L) conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA).

Determination of superoxide production from mesenteric arteries: chemiluminescence assay

The mesenteric arterial ring preparations were opened and placed in plate wells (NUNC-IMMUNO PLATE; NUNC A/C, Roskilde, Denmark) containing the Krebs-Henseleit solution described above. Ten micromolar 2-metyl-6*p*-metoxyphenyle-thynyl-imidazopirazinon (ATTO Co., Tokyo), a superoxide anion-selective probe, was added to each well, and fluorescence images were acquired with a photon counting camera (Hamamatsu Photonics, Hamamatsu) every 1 min for 5 min. The chemiluminescence intensity was directly determined from the values obtained. The spontaneous release level of superoxide anion was expressed as the integrated value for 5 min normalized to tissue weight. In some experiments, the assay was performed after the rings had been preincubated for 20 min with apocynin (300 μM).

Determination of peroxynitrite production from mesenteric arteries: fluorescence assay

To detect peroxynitrite production from the mesenteric arteries of SHR-cp, we used aminophenyl fluoscein (APF), 2-[6-(4'-amino)phenoxy-3*H*-xanthen-3-on-9-yl] benzoic acid], (Daiichi Pure Chemicals, Co., Ltd., Tokyo). APF immediately reacts with highly reactive oxygen species such as peroxynitrite, and the fluorescence intensity greatly increases (19). Peroxynitrite can be distinguished from NO and superoxide because APF does not react with NO, superoxide, and hydrogen peroxide (19).

The mesenteric arterial ring preparation was opened and placed in a glass-bottomed dish (Non-Coat 35-mm Dish; Matsunami Glass Ind., Ltd., Osaka) containing the Krebs-Henseleit solution described above. The dish was placed on the stage of an inverted microscope coupled to a Nipkow disk conforcal scanner (CSU10; Yokokawa Electric, Tokyo) and a Lumina Vision Light (Mitani, Tokyo). An argon-krypton laser (Omnichrome, Chino, CA, USA) was used to emit an excitation wavelength of 488 nm. The emitted light was collected with a 510-nm long-pass dichroic reflector and a 515-nm long-pass emission filter through a planfluor objective. To the chamber, 5 μM APF was added, and then the fluorescence images were acquired with an ICCD camera (Hamamatsu Photonics) every 20 s for 5 min. The fluorescence intensity was directly obtained from the values recorded. The spontaneous release level of peroxynitrite was expressed as F/F0, namely, the fluorescence intensity at each point (F) was divided by the fluorescence intensity at the beginning of each experiment (F0). In some experiments, the assay was performed after the rings had been preincubated for 20 min with apocynin (300 μM).

Determination of nitrotyrosine content in mesenteric arteries: dot-blot assay

The mesenteric arteries were homogenized in a glass /glass homogenizer in ice-cold lysis buffer [50 mM Tris-HCl buffer (pH 7.5), containing 0.15 M NaCl, 1% Triton X-100, 10 mM EDTA, and 0.01% (v/v) protease inhibitor cocktail (Sigma Chemical Co.)] and then centrifuged to remove the insoluble material. The protein concentration in the supernatant was determined for each sample by using a bicinchoninic acid protein assay kit (Pierce). Triplicate samples of the protein (1 μl) were blotted onto nitrocellulose membranes. A dilution series of nitrated BSA (Sigma Chemical Co.) was loaded in triplicate to generate a standard curve. After blocking, the membrane was allowed to react with an anti-nitrotyrosine antibody (Upstate, Temecula, CA, USA) followed by horseradish peroxidase–coupled secondary

antibody (Vector Lab). Subsequent detection of the specific proteins was done by enhanced chemiluminescence (Chemi-Lumi One; Nacalai Tesque, Inc., Kyoto) on x-ray film for 1 – 3 min. The x-ray film was scanned into an HP Scan Pro program (Ver. 7.04; Hewlett-Packard Japan, Ltd., Tokyo) with an HP scanner (C4175). The density of the bands was measured using the Macintosh NIH-Image program (Ver. 1.63) and normalized to each protein concentration.

Determination of angiotensin II content in aortas: enzyme immunoassay

According to the manufacturer's instructions, the angiotensin II concentration in aortas was determined by using an angiotensin II EIA kit (SPIbio, Montigny Le Bretonneux, France).

Data analyses

Data are expressed as means ± S.E.M. Individual concentration–response curves for acetylcholine and sodium nitroprusside were analyzed to determine the pEC_{50} (negative logarithm molar concentration of agonist required to produce 50% of the maximal response) and the R_{max} (maximum relaxation response). The pEC_{50} values and R_{max} were calculated by Graph Pad Prism software (Ver. 4; GraphPad, La Jolla, CA, USA). Statistical analyses were performed with the Student *t*-test (Stat View software, Ver. 5.0; SAS, CA, USA). Differences were considered significant at $P<0.05$.

Results

Vasorelaxation and protein expression of eNOS and sGC in mesenteric arteries

SHR-cp developed spontaneous obesity, hypertension, hyperlipidemia, hyperglycemia, and hyperinsulinemia at 18 weeks of age (Table 1). Figure 1, A and B shows the relaxations in response to acetylcholine and sodium nitroprusside in mesenteric arterial rings isolated from SHR-cp and WKY, respectively. The pEC_{50} value of the relaxation responses to acetylcholine in SHR-cp was significantly smaller than that in WKY (SHR-cp, 7.30 ± 0.17; WKY, 8.01 ± 0.11). The R_{max} value of the relaxation in SHR-cp was significantly smaller than that in WKY (SHR-cp, 80.9 ± 6.4; WKY, 90.0 ± 2.6).

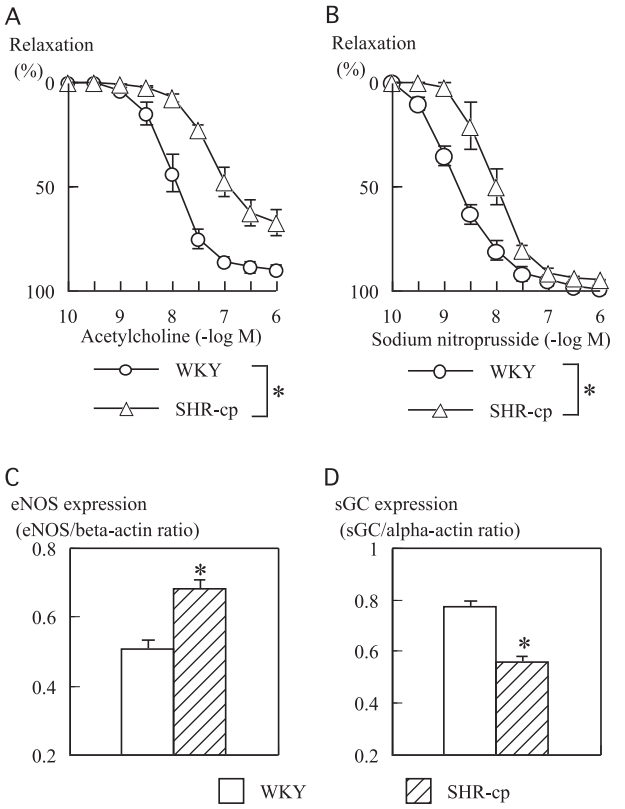

Fig. 1. Changes in relaxations in response to acetylcholine (A) and sodium nitroprusside (B) and protein levels of endothelial nitric oxide synthase (eNOS) (C) and soluble guanylyl cyclase (sGC) (D) in mesenteric arteries of SHR-cp and WKY. Results are expressed as the mean ± S.E.M. (n = 6 for each group). *$P<0.05$, as compared with the WKY group.

Table 1. Changes in body weight, systolic blood pressure, and serum levels of triglyceride, total cholesterol, glucose, and insulin in SHR-cp

Group	Body weight (g)	Systolic blood pressure (mmHg)	Triglyceride (mg/100 mL)	Total cholesterol (mg/100 mL)	Glucose (mg/100 mL)	Insulin (ng/mL)
Experiment 1						
WKY	406 ± 7	137 ± 3	69.4 ± 3.5	96.7 ± 3.2	123 ± 9	3.89 ± 1.31
SHR-cp	522 ± 3*	160 ± 4*	522 ± 30*	177 ± 10*	184 ± 8*	39.0 ± 3.8*
Experiment 2						
Control (SHR-cp)	496 ± 11	170 ± 7	454 ± 62	147 ± 6	184 ± 15	31.0 ± 6.1
Telm	466 ± 11	146 ± 7*	491 ± 70	150 ± 9	199 ± 13	37.0 ± 7.0

SHR-cp were treated with telmisartan (10 mg/kg per day, p.o.) for 8 weeks. Results are each expressed as the mean ± S.E.M. *$P<0.05$, as compared with the WKY or non-treated group.

Preincubation with apocynin, an NADPH oxidase inhibitor, and Mn-TBAP, a cell-permeable superoxide dismutase mimetic, did not restore the impaired acetylcholine-induced relaxation in SHR-cp (data not shown). On the other hand, the relaxation response to sodium nitroprusside was significantly impaired in SHR-cp compared with WKY (pEC_{50}: SHR-cp, 8.26 ± 0.12; WKY, 8.93 ± 0.09). The contractile response to phenylepherine, that is, the level of preconstruction of each ring, was not significantly different between rats of the two strains (data not shown).

As shown in Fig. 1C, the protein level of eNOS in mesenteric arteries significantly increased by approximately 1.34-fold in SHR-cp compared with WKY. On the other hand, that of sGC significantly decreased by approximately 0.70-fold in SHR-cp compared with WKY (Fig. 1D). There was no change in the protein level of PKG in mesenteric arteries (data not shown).

Superoxide and peroxynitrite production from mesenteric arteries

The degree of spontaneous superoxide production significantly increased in mesenteric arterial rings isolated from SHR-cp compared with WKY (Fig. 2A). Since NADPH oxidases are widely accepted to be a major source of superoxide anion in vascular walls (7), we examined the effect of apocynin, an inhibitor of NADPH oxidase, on superoxide production. The increased superoxide production in SHR-cp was significantly attenuated by pretreatment with apocynin (Fig. 2B).

Superoxide anion reacts with NO to form peroxynitrite. We therefore tried to determine peroxynitrite production using a fluorescent reagent for detection of highly reactive oxygen species such as peroxynitrite (19). The production levels of highly reactive oxygen species, perhaps peroxynitrite, in mesenteric arterial rings were significantly higher by approximately 3.0-fold in SHR-cp than WKY (Fig. 2C). This increased production was significantly attenuated by pretreatment with apocynin (Fig. 2D).

Contents of nitrotyrosine in mesenteric arteries and angiotensin II in aortas

Nitrotyrosine formation has been claimed to be a "footprint" of peroxynitrite, and therefore we tried to measure nitrotyrosine levels in mesenteric arteries using the Dot-blot assay. As shown in Fig. 3A, the mesenteric arterial nitrotyrosine level was significantly increased in SHR-cp compared with WKY.

Angiotensin II has been found to induce enhanced activity and increase in the mRNA expression of vascular NADPH oxidases in both in vitro and in vivo experiments (20, 21). Therefore, we measured angiotensin II contents in aortas of SHR-cp and WKY. The angiotensin II content significantly increased in aortas of SHR-cp compared with WKY (Fig. 3B).

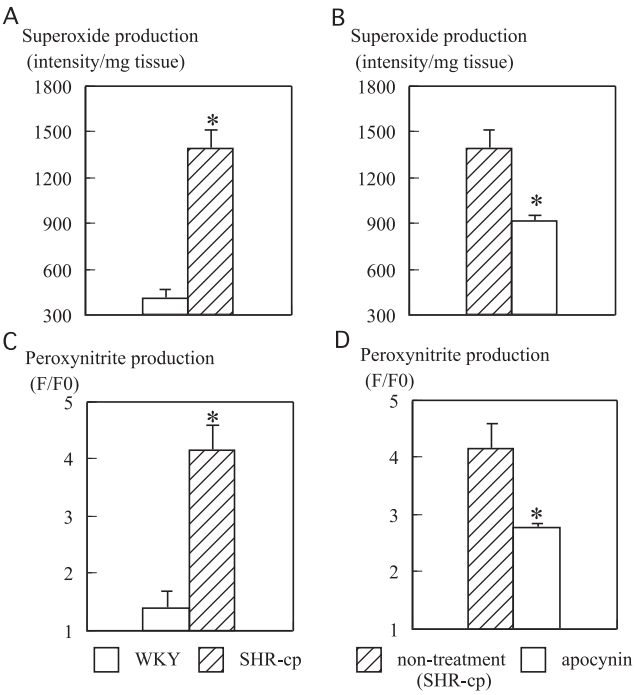

Fig. 2. Changes in production of superoxide (A) and peroxynitrite (C) from mesenteric arteries of SHR-cp and WKY and effects of apocynin, a NADPH oxidase inhibitor, on these changes in SHR-cp (B, D, respectively). Results are expressed as the mean ± S.E.M. (n = 6 for each group). *P<0.05, as compared with the WKY (A and B) or non-treated group (C and D).

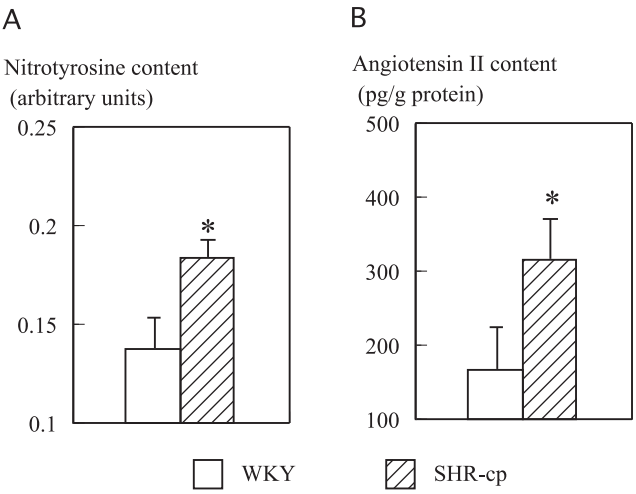

Fig. 3. Changes in nitrotyrosine contents in mesenteric arteries (A) and angiotensin II in aortas (B) of SHR-cp and WKY. Results are expressed as the mean ± S.E.M. (n = 6 for each group). *P<0.05, as compared with the WKY group.

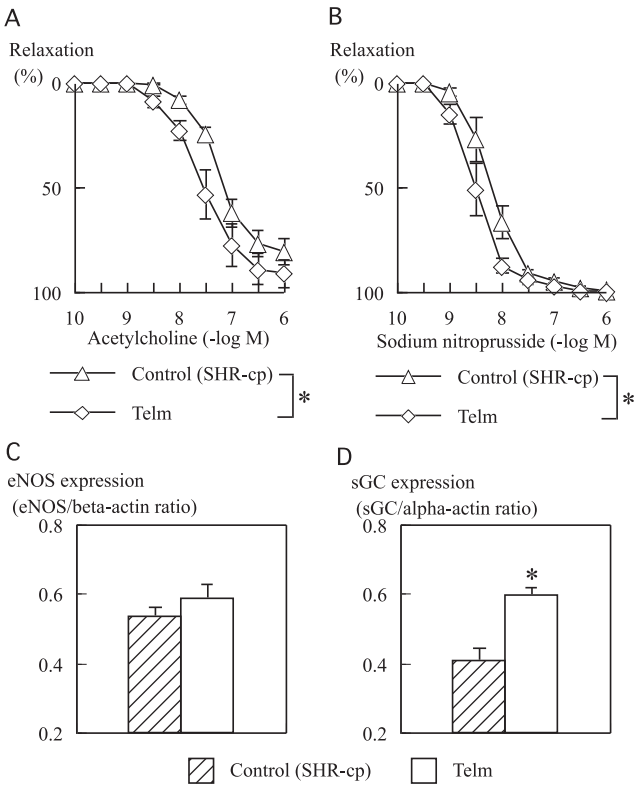

Fig. 4. Effects of long-term treatment with telmisartan (10 mg/kg per day, p.o., for 8 weeks) on relaxations in response to acetylcholine (A) and sodium nitroprusside (B) and protein levels of endothelial nitric oxide synthase (eNOS) (C) and soluble guanylyl cyclase (sGC) (D) in mesenteric arteries of SHR-cp. Results are expressed as the mean ± S.E.M. (n = 6 for each group). *P<0.05, as compared with the control group.

Effects of long-term treatment with telmisartan in SHR-cp

Angiotensin II–induced vascular NADPH oxidase activation has been found to be inhibited by in vivo treatment with angiotensin II type 1–receptor antagonists (21, 22). Thus, we investigated the effects of telmisartan, one of the antagonists, on abnormal events developing in mesenteric arteries of SHR-cp. Telmisartan showed antihypertensive effects, but could not improve the metabolic abnormalities (Table 1). The impaired relaxations in response to acetylcholine and sodium nitroprusside in mesenteric arteries of SHR-cp were significantly improved by long-term treatment with telmisartan (Fig. 4: A and B). The increased expression of eNOS was not significantly affected, but the decreased expression of sGC was significantly relieved by telmisartan (Fig. 4: C and D). Furthermore, the increased nitrotyrosine level was prevented by telmisartan (Fig. 5).

Discussion

The results of the present study demonstrate that in vascular walls of SHR-cp, a model of metabolic syndrome, peroxynitrite is gradually and persistently formed by the reaction of endothelial NO and angiotensin II–mediated NADPH oxidase–derived superoxide anion and that the chronic peroxynitrite production causes impairment of vasorelaxation due to a decrease in sGC in smooth muscle cells.

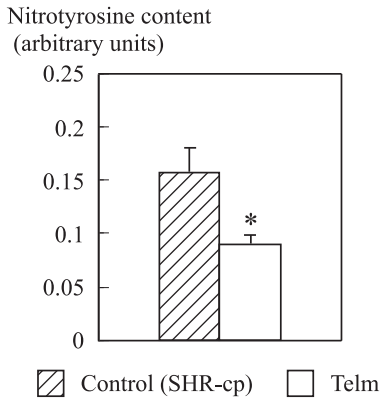

Fig. 5. Effects of long-term treatment with telmisartan (10 mg/kg per day, p.o., for 8 weeks) on nitrotyrosine contents in mesenteric arteries of SHR-cp. Results are expressed as the mean ± S.E.M. (n = 6 for each group). *P<0.05, as compared with the control group.

In spite of the relaxation dysfunction in mesenteric arteries of SHR-cp, we found an increase in protein expression of eNOS. Upregulation of eNOS expression by ROS and angiotensin II has been reported (23, 24). However, the present study could not clarify whether the increased eNOS leads to an increase in NO production/release from the endothelium or uncoupling of the enzyme. Angiotensin II–dependent generation of ROS, such as superoxide and peroxynitrite, is thought to reduce NO bioavailability because angiotensin II; superoxide; and peroxynitrite, which is formed by reaction of superoxide with NO, greatly increased in the arterial wall of SHR-cp. It has been reported that NADPH oxidase activation by angiotensin II, which generates superoxide, induces eNOS uncoupling (25). Endothelial NOS produces superoxide under uncoupling conditions where its substrate L-arginine and/or cofactor tetrahydrobiopterin (BH_4) is limited (26). Furthermore, an elevated concentration of peroxynitrite leads to eNOS uncoupling (27). Therefore, peroxynitrite may play a pivotal role in decreasing NO bioavailability. We also found an increased nitrotyrosine content in mesenteric arteries of SHR-cp. This finding supports the presence of peroxynitrite in the mesenteric arteries.

To investigate whether peroxynitrite formation in the vascular wall of SHR-cp is mediated by NADPH oxidase–derived superoxide, we used apocynin as an inhibitor of NADPH oxidase (28). A recent paper has

reported that in cell culture experiments using endothelial cells or vascular smooth muscle cells, apocynin predominantly acts as an antioxidant, not as an inhibitor of NADPH oxidase (29). However, Touyz comments on this report, noting that in vascular walls, various peroxidases could activate (oxidize) apocynin to inhibit vascular NADPH oxidase under physiological and pathophysiological conditions (30). Hence, we considered that apocynin acts as an inhibitor of NADPH oxidase under the conditions of the present study. In our study, apocynin could attenuate superoxide and peroxynitrite production from mesenteric arteries of SHR-cp. Similar results were obtained when Mn-TBAP, a cell-permeable superoxide dismutase, was obtained. This indicates that NADPH oxidase seems to be involved in peroxynitrite production via superoxide production. Nonetheless, these two agents could not restore the impaired relaxations in mesenteric arteries of SHR-cp. This finding suggests that the peroxynitrite-induced dysfunction is due to an abnormal functional event(s) in the vascular walls, that is, a decrease in sGC expression, rather than decreased NO availability due to elevated superoxide species in SHR-cp. Peroxynitrite may cause slow injury to the tissues over a long period of time; vascular dysfunction is observed in the mesenteric arteries of SHR-cp at 18 weeks of age.

sGC is an important NO effector enzyme and plays a critical role in vasorelaxation. Impaired vasorelaxation in response to nitroglycerin and a decrease in sGC expression have been found in angiotensin II–treated rats (22, 24). Furthermore, angiotensin II has been demonstrated to be an important vasoactive agonist for increasing the activity and expression of NADPH oxidase (9, 10, 20 – 22). We observed increased angiotensin II contents in arteries of SHR-cp. We could not evaluate its localization in vascular beds, but in rabbit mesenteric arteries, angiotensin II localized within smooth muscle cells has been reported to inhibit the relaxation via activation of angiotensin type 1 receptors (31). We also found decreased sGC expression followed by an impaired relaxation response to acetylcholine (endothelial-dependent responses) and sodium nitroprusside (NO responses to smooth muscle cells). Peroxynitrite is known to modify proteins containing a heme prosthetic group, such as hemoglobin, myoglobin, or cytochrome C, oxidizing ferrous heme into the corresponding ferric forms (11). In fact, in in vitro experiments, peroxynitrite has been reported to reduce the NO-stimulated catalytic activity of sGC, the heme-containing NO receptor (32). Furthermore, oxidation of sGC heme has been demonstrated to result not only in the loss of NO-sensitive sGC activity but also in acceleration of the protein degradation (33). From the above-mentioned results, it is very likely that sGC is a potential biological target for peroxynitrite. We have previously demonstrated that in aortas of SHR-cp, a decrease in sGC expression is accompanied by a decrease in cGMP levels (17). In the present study, unchanged PKG protein expression was also observed. These findings suggest that a decrease in sGC expression, leading to decreased cGMP levels, may be mainly due to the impaired vasorelaxation induced by peroxynitrite in the mesenteric arteries of SHR-cp.

To evaluate the above-mentioned hypothesis, we examined the effects of long-term administration (8 weeks) of telmisartan, an angiotensin II type 1–receptor antagonist, to SHR-cp. We found that telmisartan prevented not only the increase in nitrotyrosine contents and decrease in sGC expression, but also impaired relaxations in mesenteric arteries of SHR-cp. We have previously found similar results with aortas of SHR-cp and suggested that the protective effects of telmisartan are linked to decreased oxidative-nitrosative stress via reduction of a component of NADPH oxidase, independently of its antihypertensive effect (18). Furthermore, long-term administration of Coenzyme Q10, a strong antioxidant, to SHR-cp has been reported to prevent both increased oxidative-nitrative stress and vascular dysfunction in mesenteric arteries (34). Taken together, continuous production of peroxynitrite formed by the reaction of NO with superoxide via angiotension II in blood vessels may lead to gradual development of functional damage in SHR-cp.

Telmisartan is also known to have an agonistic effect on peroxisome proliferators-activated receptor-gamma (PPAR-γ). Interestingly, PPAR-γ has a protective role in vascular function and structure via anti-inflammatory, anti-fibrotic, and antioxidant actions (35, 36). Recent studies have shown that telmisartan has an ameliorative effect on endothelial dysfunction by improving the PPAR-γ–eNOS system in Dahl salt-sensitive hypertensive rats (37) or NO bioavailability in Watanabe heritable hyperlipidemic rabbits (38). Thus, in addition to effects on the response to NO in vascular smooth muscle cells, improvement of endothelial dysfunction might, in part, participate in the beneficial effects of telmisartan on NO-mediated vasorelaxation dysfunction in SHR-cp.

In summary, the present study demonstrated that in SHR-cp, a model of metabolic syndrome, chronic peroxynitrite formation occurs via the angiotensin II /NADPH oxidase pathway in vascular walls. Long-term actions of peroxynitrite may cause gradual denaturation of proteins, especially sGC, leading to impairment of NO-dependent vasorelaxation. Our results suggest that angiotensin type 1–receptor antagonism can effectively

prevent dysfunction of vasodilation in the metabolic syndrome. SHR-cp may be a suitable animal model for studying the influence of peroxynitrite on vascular function in the metabolic syndrome.

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References

1 Reaven GM. Role of insulin resistance in human disease (syndrome X): an expanded definition. Annu Rev Med. 1993;44:121–131.
2 Maxwell AJ, Tsao PS, Cooke JP. Modulation of the nitric oxide synthase pathway in atherosclerosis. Exp Physiol. 1998;83:573–584.
3 Schulz E, Anter E, Keaney JF. Oxidative stress, antioxidants, and endothelial function. Curr Med Chem. 2004;11:1093–1104.
4 Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, et al. Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. Circ Res. 2000;86:E85–E90.
5 Patel RP, Moellering D, Murphy-Ullrich J, Jo H, Beckman JS, Darley-Usmar VM. Cell signaling by reactive nitrogen and oxygen species in atherosclerosis. Free Radic Biol Med. 2000;28:1780–1794.
6 Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension. What is the clinical significance? Hypertension. 2004;44:248–252.
7 Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res. 2000;86:494–501.
8 Bengtsson SH, Gulluyan LM, Dusting GJ, Drummond GR. Novel isoforms of NADPH oxidase in vascular physiology and pathophysiology. Clin Exp Pharmacol Physiol. 2003;30:849–854.
9 Warnholtz A, Nickenig G, Schulz E, Macharzina R, Bräsen JH, Skatchkov M, et al. Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. Circulation. 1999;99:2027–2033.
10 Shinozaki K, Ayajiki K, Kashiwagi A, Masada M, Okamura T. Malfunction of vascular control in lifestyle-related diseases: mechanisms underlying endothelial dysfunction in the insulin-resistant state. J Pharmacol Sci. 2004;96:401–405.
11 Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87:315–424.
12 Escobales N, Crespo MJ. Oxidative-nitrosative stress in hypertension. Curr Vasc Pharmacol. 2005;3:231–246.
13 Pacher P, Obrosava IG, Mabley JG, Szabo C. Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. Curr Med Chem. 2005;12:267–275.
14 Pacher P, Schulz R, Liaudet L, Szabo C. Nitrosative stress and pharmacological modulation of heart failure. Trends Pharmacol Sci. 2005;26:302–310.
15 Zaki MH, Akuta T, Akaike T. Nitric oxide-induced nitrative stress involved in microbial pathogenesis. J Pharmacol Sci. 2005;98:117–129.
16 Yamaguchi Y, Yoshikawa N, Kagota S, Nakamura K, Haginaka J, Kunitomo M. Elevated circulating levels of markers of oxidative-nitrative stress and inflammation in a genetic rat model of metabolic syndrome. Nitric Oxide. 2006;15:380–386.
17 Kagota S, Yamaguchi Y, Tanaka N, Kubota Y, Kobayashi K, Nejime N, et al. Disturbances in nitric oxide/cyclic guanosine monophosphate system in SHR/NDmcr-cp rats, a model of metabolic syndrome. Life Sci. 2006;78:1187–1196.
18 Kagota S, Tada Y, Kubota Y, Nejime N, Yamaguchi Y, Nakamura K, et al. Peroxynitrite is involved in the dysfunction of vasorelaxation in SHR/NDmcr-cp rats, spontaneously hypertensive obese rats. J Cardiovasc Pharmacol. 2007;50:677–685.
19 Setsukinai K, Urano Y, Kakinuma K, Majima HJ, Nagano T. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. J Biol Chem. 2003;278:3170–3175.
20 Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res. 1994;74:1141–1148.
21 Fukui T, Ishizaka N, Rajagopalan S, Laursen JB, Capers Q 4th, Taylor WR, et al. p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. Circ Res. 1997;80:45–51.
22 Rajagopalan S, Kurz S, Münzel T, Tarpey M, Freeman BA, Griendling KK, et al. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. J Clin Invest. 1996;97:1916–1923.
23 Zhen J, Ku H, Wang ZQ, Vaziri ND, Zhou Z. Upregulation of endothelial and inducible nitric oxide synthase expression by reactive oxygen species. Am J Hypertens. 2008;21:28–34.
24 Mollnau H, Wendt M, Szöcs K, Lassègue B, Schulz E, Oelze M, et al. Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric oxide/cGMP signaling. Circ Res. 2002;90:E58–E65.
25 Chalupsky K, Cai H. Endothelial dihydrofolate reductase: critical for nitric oxide bioavailability and role in angiotensin II uncoupling of endothelial nitric oxide synthase. Proc Natl Acad Sci U S A. 2005;102:9056–9061.
26 Vásquez-Vivar J, Kalyanaraman B, Martásek P, Hogg N, Masters BS, Karoui H, et al. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc Natl Acad Sci U S A. 1998;95:9220–9225.
27 Xu J, Xie Z, Reece R, Pimental D, Zou MH. Uncoupling of endothelial nitric oxidase synthase by hypochlorous acid. Role of NAD(P)H oxidase-derived superoxide and peroxynitrite. Arterioscler Thromb Vasc Biol. 2006;26:2688–2695.
28 Simons JM, Hart BA, Ip Vai Ching TR, Van Dijk H, Labadie RP. Metabolic activation of natural phenols into selective oxidative burst agonists by activated human neutrophils. Free Radic Biol Med. 1990;8:251–258.

29 Heumüller S, Wind S, Barbosa-Sicard E, Schmidt HH, Busse R, Schröder K, et al. Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. Hypertension. 2008;51: 211–217.
30 Touyz RM. Apocynin, NADPH oxidase, and vascular cells: a complex matter. Hypertension. 2008;51:172–174.
31 Itoh T, Kajikuri J, Tada T, Suzuki Y, Mabuchi Y. Angiotensin II-induced modulation of endothelium-dependent relaxation in rabbit mesenteric resistance arteries. J Physiol. 2003;584:893–906.
32 Weber M, Lauer N, Mülsch A, Kojda G. The effect of peroxynitrite on the catalytic activity of soluble guanylyl cyclase. Free Radic Biol Med. 2001;31:1360–1367.
33 Stasch JP, Schmidt PM, Nedvetsky PI, Nedvetskaya TY, H S AK, Meurer S, et al. Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. J Clin Invest. 2006;116:2552–2561.
34 Kunitomo M, Yamaguchi Y, Kagota S, Otsubo K. Beneficial effect of coenzyme Q10 on increased oxidative and nitrative stress and inflammation and individual metabolic components developing in a rat model of metabolic syndrome. J Pharmacol Sci. 2008;107:128–137.
35 Schiffrin EL, Amiri F, Benkirane K, Iglarz M, Diep QN. Peroxisome proliferator-activated receptors: vascular and cardiac effects in hypertension. Hypertension. 2003;42:664–648.
36 Beyer AM, Baumbach GL, Halabi CM, Modrick ML, Lynch CM, Gerhold TD, et al. Interference with PPAR-gamma signaling causes cerebral vascular dysfunction, hypertrophy, and remodeling. Hypertension. 2008;51:867–871.
37 Kobayashi N, Ohno T, Yoshida K, Fukushima H, Mamada Y, Nomura M, et al. Cardioprotective mechanism of telmisartan via PPAR-gamma-eNOS pathway in dahl salt-sensitive hypertensive rats. Am J Hypertens. 2008;21:576–581.
38 Ikejima H, Imanishi T, Tsujioka H, Kuroi A, Kobayashi K, Shiomi M, et al. Effects of telmisartan, a unique angiotensin receptor blocker with selective peroxisome proliferator-activated receptor-gamma-modulating activity, on nitric oxide bioavailability and atherosclerotic change. J Hypertens. 2008;26:964–972.