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ORIGINAL ARTICLE

Telmisartan improves endothelial dysfunction and renal autoregulation in Dahl salt-sensitive rats

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Hypertensive vascular disorders are characterized by endothelial dysfunction. Loss of renal autoregulation causes glomerular hypertension. However, the relationship between the autoregulatory response and glomerular damage has not been well examined. We examined the contributions of uncoupled endothelial nitric oxide synthase (eNOS) in hypertensive renal disease, and the relationship between the degree of autoregulation impairment and glomerular injury. We also investigated the effects of telmisartan on eNOS coupling and renal autoregulation. Male Dahl salt-sensitive hypertensive (DS) rats (14-week old) fed an 8% salt diet were used to examine endothelial dysfunction and impaired renal autoregulation caused by glomerular hypertension. Some DS rats were treated with telmisartan (3.0 mg kg^{-1} day^{-1}), an angiotensin receptor blocker, for 2 weeks. Increased superoxide production and decreased nitric oxide production, as detected by fluorescent indicator perfusion methods, were observed in the glomeruli and arterioles of hypertensive DS rats. Telmisartan improved the imbalance of superoxide and nitric oxide in the glomeruli and arterioles. Decreased serum tetrahydrobiopterin levels and coupled eNOS seen in the DS rat kidney were improved with telmisartan treatment. The endothelial relaxation reaction was impaired in DS rat aortic arteries. Autoregulatory capacity in response to step changes in perfusion pressure was also impaired in DS rat kidney. Treatment with telmisartan improved these abnormalities. Endothelial dysfunction in the glomeruli and impaired renal autoregulation, which may cause glomerular sclerosis, were observed in DS rat kidney. Telmisartan treatment improves these dysfunctions in hypertensive renal disease.
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Keywords: angiotensin receptor blocker; eNOS uncoupling; nitric oxide; superoxide

INTRODUCTION

Hypertension is a well-known complication in chronic kidney disease and, at the same time, is one of the important risk factors for the development of cardiovascular disease and for the progression of renal insufficiency.[1,2] Indeed, various studies have indicated that subjects with hypertension are at risk of developing end-stage renal disease.[3–5] Hypertensive vascular disorders are characterized by pathological changes in endothelial cells in the early stage of hypertension, and endothelial cell dysfunction causes an imbalance between reactive oxygen species (ROS) and nitric oxide (NO) production.[6] There are several overlapping mechanisms for endothelial dysfunction, including NO trapping by free radicals and reduced endothelial NO synthase (eNOS) activity. The eNOS is a homodimeric enzyme that generates NO and L-citrulline from L-arginine (L-Arg). However, in certain circumstances, eNOS may cause uncoupling that generates superoxide.[7] Recent studies report the involvement of uncoupled eNOS during increased oxidative stress and endothelial dysfunction in association with atherosclerosis[8] and diabetes.[9] Evidence also suggests that the absence of the substrate L-Arg or cofactor tetrahydrobiopterin (BH4) uncouples eNOS, thereby producing superoxide rather than NO,[10–12] which may contribute to the pathogenesis of endothelial dysfunction in arteries exposed to hypertension. However, little is known about the contributions of uncoupled eNOS in the context of hypertensive glomerular injury.

Pressure-mediated autoregulatory behavior operates by recognizing a change in renal perfusion pressure and initiating induction of autoregulatory resistance changes intended to stabilize renal blood flow.[13] The net result is that renal blood flow and glomerular filtration rates remain relatively stable over a wide range of renal perfusion pressures. Autoregulatory behavior is significantly less efficient in pathological settings, such as in diabetes mellitus and in some forms of hypertension. Loss of autoregulatory efficiency could be one contributing factor, which leads to glomerular stress, glomerular compensation and perhaps glomerular injury and failure. Evidence indicates that renal autoregulation is impaired in some experimental animal models of hypertension.[14,15] However, the relationship between the degree of autoregulation impairment and glomerular injury have not been well examined, and it is unclear whether blockade of renal autoregulation impairment would effectively inhibit hypertensive renal injury.

In this study, we examined whether uncoupled eNOS, which is a marker of endothelial dysfunction, and loss of renal autoregulation

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occur during hypertensive glomerular injury. We used Dahl salt-sensitive hypertensive (DS) rats to examine the NOS coupling statement in the kidney using the dihydroethidium (DHE) assay[16] and *in vivo* NO and ROS imaging,[17] and examined autoregulation capacity by measuring renal microvascular responses to step changes in renal perfusion pressure. Moreover, to elucidate whether angiotensin receptor blockade (ARB) has a beneficial effect against eNOS uncoupling and renal autoregulation, we examined DS rats treated with telmisartan, an angiotensin receptor blocker.

METHODS

Experimental protocol

Male DS rats (8-week old) were purchased from Clea Japan (Osaka, Japan). Animals were housed in a temperature- and humidity-controlled room with a 12:12 h light–dark cycle, and were fed standard laboratory animal chow with free access to tap water. In the first 6 weeks, rats were fed an 8% salt diet to induce hypertension. Systolic blood pressure (SBP) was also measured weekly in each rat using the tail-cuff method using an automatic sphygmomanometer (BP98A; Softron, Tokyo, Japan). After confirming the induction of hypertension, rats were randomly divided into three groups: the DS-H group (n=15) consisting of untreated DS rats; the DS-H-Hyd group (n=15) consisting of DS rats treated with hydralazine (5.0 mg per rat per day); and the DS-H-Tel group (n=15) consisting of DS rats treated with telmisartan (3.0 mg kg^{-1} day^{-1}). As a control, we used another group of DS normotensive rats fed a 0.9% salt diet (DS-L, n=8). The experimental protocol (No. 07–060) was approved in advance by the Ethics Review Committee for Animal Experimentation of Kawasaki Medical School, Kurashiki, Japan.

During the experimental period, body weight was measured weekly for each rat. All rats were killed after 2 weeks of treatment. Serum creatinine and blood urea nitrogen levels were measured after killing the rats. Before killing, 24-h urine samples were collected after placing the rats in metabolic cages and urine protein concentrations were measured spectrophotometrically using the Bradford method (Bio-Rad Protein Assay; Bio-Rad Laboratories, Tokyo, Japan).

Glomerular damage score

Half of the left kidneys were preserved in 4% paraformaldehyde for light microscopy. Paraffin-embedded tissue samples were cut into 4 μm-thick sections and stained with periodic acid-Schiff for histological studies. In each animal, 100 glomeruli were evaluated for the presence of sclerotic lesions. The severity of glomerular lesions was graded with a score of 0–3 based on the percentage of glomerular involvement, as described previously.[18]

Fluorescence spectrometric assay of O_2- production in the renal cortex

To assess the source of ROS production, we performed fluorescence spectrometric assays. Fluorescence spectrometry of tissue O_2- production was carried out using the fluorogenic oxidation of DHE to ethidium as a measure of O_2-, as previously described.[16] The renal cortex of the right kidney obtained from each rat was cut into small pieces, and the glomeruli were isolated by the mechanical graded sieving technique. After isolation, the purity of the final suspension was determined by light microscopic examination. On average, tubular contamination was $<$5%. Isolated glomeruli were homogenized in ice-cold 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing 25 mM HEPES, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation of the homogenate, the supernatant was used for this DHE assay. A part of the supernatant was used for western blotting. Enzyme activities of different pathways are expressed relative to the control. Following substrates or inhibitors were used in this study: nicotinamide adenine dinucleotide phosphate (NADPH) (0.1 mM) was used as a substrate for NADPH oxidases; L-Arg (1 mM) was used as a substrate for NOS; NG-nitro-L-Arg methyl ester (1 mM) was used to block NOS activity; succinate (5 mM) was used as a substrate for intramitochondrial O_2- production; antimycin (0.05 mM) was used to block the normal reaction in the respiratory chain; and xanthine (0.1 mM) was used as a substrate for xanthine oxidase. The effect of BH4 (0.01 mM) on L-Arg-induced O_2- production was also examined.

In situ detection of NO and ROS

To visualize the endothelial production levels of NO and ROS resulting from NOS coupling, *in situ* NO and ROS were imaged by confocal laser microscopy after perfusion with fluorescence indicators, as previously described.[17] In brief, an 18-gauge needle connected to an infusion pump was inserted in the left ventricle. After the right atrium was cut, the whole body was perfused with PBS maintained at 37 °C. After exsanguination, the whole body was perfused with diaminorhodamine–4 M acetoxymethyl ester (DAR–4 M AM; Daiichi Pure Chemicals, Tokyo, Japan) and 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA; Invitrogen, Tokyo, Japan) with L-Arg and $CaCl_2$. All unreacted DAR–4 M AM and DCFH-DA were removed by postperfusion with PBS. After fixation with 4% paraformaldehyde perfusion, the tissues were cut into 1 mm-thick sections and placed on a slide glass. Fluorescent images of NO and ROS were obtained using confocal laser-scanning microscopy (TCS-NT, Leica-Microsystems Japan, Tokyo, Japan). The mean NO and ROS fluorescence intensity of glomeruli (total of 100 glomeruli from five rats in each group) was analyzed using Leica TCS-NT system software (Leica-Microsystems).

Western immunoblotting

Western blots were performed to quantify total eNOS and its active dimer. Portions of the glomeruli samples (50 μg per lane) were subjected to SDS-PAGE. For immunoblot analysis of total eNOS, the samples were heated at 95 °C for 5 min before electrophoresis. For immunoblot analysis of the dimeric form of eNOS, samples were not heated, and the temperature of the gel was maintained below 15 °C during electrophoresis (low-temperature SDS-PAGE).[17] Primary antibody 0.1 μg/ml rabbit anti-eNOS polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied for 180 min at room temperature. The antibody was visualized using an enhanced chemiluminescence method (ECL plus; GE Healthcare Japan, Tokyo, Japan). The integrated density (density 3 area) of the bands was quantified using Image-J software (http://rsbweb.nih.gov/ij/).

Determination of serum hydrobiopterin concentrations

BH4 and dihydrobiopterin (BH2) concentrations were determined by high-performance liquid chromatography, as described previously.[17] Serum samples were mixed at a ratio of 1:1 with a solution of 0.5 M perchloric acid containing 0.1 mM Na_2-ethylenediaminetetraacetic acid and 0.1 mM $Na_2S_2O_3$ for protein separation. After filtration, BH4 concentrations in the samples were measured by HPLC. Detection of BH4 was carried out fluorometrically at wavelengths of 350 nm for excitation and of 440 nm for emission by postcolumn $NaNO_2$ oxidation using a reversed-phase ion-pair LC system (LC-10 series; Shimadzu, Kyoto, Japan).

Real-time quantitative PCR

The mRNA expression of guanosine-5′-triphosphate cyclohydrolase 1 (GTPCH1), which is the rate-limiting enzyme in BH4 synthesis, and purinergic receptor P2X, ligand-gated ion channel 1 (P2rx1) were determined by real-time quantitative PCR. RNA isolation and real-time quantitative PCR for GTPCH1 and P2rx1 were performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA), as described previously.[19] Primers and probes for P2rx1 (NM_012997) were as follows: rat P2rx1 forward primer: 5′-CGTCATTGGGTGGGTGTT-3′; reverse primer: 5′-AGCTGGGTCA CAGCCAAG-3′; TaqMan probe: 5′-FAM-TCAGCAGTGTGTCCGTGAAGCT CA-TAMRA-3′. Fold-change analysis was based on standardizing RNA levels by normalizing to glyceraldehyde-3-phosphate dehydrogenase levels in the sample.

Endothelium-dependent vascular responses

Endothelium-dependent vascular responses were measured after preparation of thoracic rat aortic rings, as reported previously.[20] Briefly, cylindrical 3.0 mm-long segments were dissected from the aorta and bathed in Krebs bicarbonate saline (120 mM NaCl, 5.2 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.03 mM Na_2-ethylenediaminetetraacetic acid and 11 mM dextrose (pH 7.4)) equilibrated with 95% O_2 and 5% CO_2, and maintained at 37 °C. The rings were suspended under 1 g of tension and preconstricted by adding 3×10^{-7} M norepinephrine. After the contraction force had reached a plateau,

acetylcholine (10^{-9}–10^{-5} M; endothelium-dependent vasodilator) or sodium nitroprusside (10^{-9}–10^{-5} M; endothelium-independent vasodilator) was added incrementally to the bath. The force of isometric contraction was measured using a force-displacement transducer (Model MTOB-1Z; Labo Support, Osaka, Japan). Responses to acetylcholine were expressed as the percentage of the preconstricted tension induced by norepinephrine. Nonlinear regression (GraphPad Prism5.0; GraphPad Software, La Jolla, CA, USA) was used to determine pEC_{50} and maximum responses (R_{max}) to acetylcholine and nitroprusside in each ring.

Renal microvascular responses to step changes in renal perfusion pressure

The effects of perfusion pressure on renal microvascular tone were examined by slight modification of the isolated perfusion technique described by van Dokkum *et al*.[21] For perfusion of the kidneys, animals were anesthetized with sevoflurane, the left kidney was denervated by stripping all visible nerves from the renal artery, the artery was coated with a 5% solution of phenol in ethanol, and the left renal artery was then cannulated by introducing a perfusion cannula through the mesenteric artery and across the aorta. The perfusion medium consisted of Krebs–Ringer bicarbonate buffer containing 6.5 g/100 ml bovine serum albumin, 5 mM D-glucose and a complement of amino acids. The perfusion pressure, monitored at the level of the renal artery, was altered by controlling the backpressure regulator. The renal artery was supplied with a 1-mm Transonic flow probe connected to a Transonic T206 flowmeter (Transonic Systems, Ithaca, NY, USA) to monitor renal flow. Renal perfusion pressure was maintained at 100 mm Hg. The pressure was then raised in a stepwise manner by 20-mm Hg increments to 160 mm Hg. Autoregulatory index (AI) between 100–160 mm Hg of pressure changes was calculated by the method of Semple and de Wardener[22] as follows: $AI=[(\text{renal flow } (RF)_2-RF_1)/RF_1]/[(\text{renal perfusion pressure } (RPP)_2-RPP_1)/RPP_1]$.

Statistical analysis

Values are expressed as mean ± s.e.m. Statistical comparisons except the glomerular damage score were made using the one-factor analysis of variance with a Tukey–Kramer test for multiple comparisons. The glomerular damage score was evaluated by the Kruskal–Wallis test with the Steel–Dwass test for multiple comparisons. A *P*-value <0.05 denoted a statistically significant difference.

RESULTS

Pathophysiological data

Systolic blood pressure was significantly higher in DS-H rats than in DS-L rats (Table 1). A two-week treatment with telmisartan decreased systolic blood pressure in a manner similar to hydralazine. There were no differences in serum creatinine and blood urea nitrogen levels among DS-H, DS-H-Hyd and DH-H-Tel rats. Urinary protein excretion in DS-H rats was greater than in DS-L rats ($P<0.05$). Urinary protein excretion was significantly decreased in both DS-H-Hyd and DH-H-Tel rats compared with DS-H rats ($P<0.05$ each), and was much lower in DH-H-Tel rats than in DS-H-Hyd rats ($P<0.05$). Glomerular damage, which was worse in DS-H rats than in DS-L rats, also did not worsen after treatment with telmisartan compared with hydralazine (data not shown). Glomerular damage score was significantly improved in DH-H-Tel rats compared with DS-H and DS-H-Hyd rats (Table 1).

Source of ROS production in glomeruli of salt-sensitive hypertensive rats

The production of ROS was measured by dihydroethidium assay. The production of ROS was higher in the glomeruli of DS-H rats than DS-L rats (Figure 1a; $P<0.05$) and increased in the former group after the addition of NADPH (3.2 ± 0.4-fold; $P<0.05$ *vs.* DS-H (−)). Moreover, ROS production was accelerated by the addition of L-Arg (2.5 ± 0.4-fold, $P<0.05$ *vs.* DS-H (−)) and decreased by the addition of NG-nitro-L-Arg methyl ester (1.2 ± 0.1-fold, $P<0.05$ *vs.* DS-H (−)). There were no changes observed with the addition of succinate plus antimycin or xanthine. Furthermore, in DS-H-Hyd rats, ROS production was accelerated by the addition of L-Arg (Figure1b; 2.2 ± 0.3-fold, $P<0.05$ *vs.* DS-H-Hyd (−)) and diminished by incubation with BH4 (1.2 ± 0.2-fold, $P<0.05$ *vs.* DS-H-Hyd+L-Arg). However, L-Arg did not increase ROS production in DS-H-Tel rats.

In situ detection of NOS coupling in glomeruli and small arteries of salt-sensitive hypertensive rats

Figure 2 shows *in situ* endothelial NO and ROS images for the four groups of rats. The NO detected by DAR–4 M AM appears red (Figures 2a–d), and ROS detected by DCFH-DA appears green (Figures 2e–h). NO, but not ROS, was detected in the glomeruli and arterioles of DS-L rats. However, ROS production was increased and NO production was decreased in the glomeruli and arterioles of DS-H rats compared with DS-L rats. The production of ROS was increased and NO production was reduced in the glomeruli and arterioles of the Hyd group. Treatment with telmisartan normalized the production of NO and ROS in the glomeruli and arterioles.

Western blot analysis of eNOS in glomeruli of salt-sensitive hypertensive rats

Total eNOS was examined by standard SDS–PAGE. Dimer/monomer eNOSs were examined by low-temperature SDS–PAGE. Total eNOS

Table 1 Pathophysiological data

	DS-L	*DS-H*	*DS-H-Hyd*	*DS-H-Tel*
Physiological data (n=15)				
Body weight (g)	433 ± 10	386 ± 7*	402 ± 9	402 ± 8
Systolic blood pressure (mm Hg)	114 ± 2	190 ± 3*	136 ± 3**	134 ± 3**
Serum creatinine (mg 100 ml^{-1})	0.27 ± 0.01	0.37 ± 0.02*	0.35 ± 0.02	0.38 ± 0.02
Blood urea nitrogen (mg 100 ml^{-1})	18.9 ± 1.0	24.2 ± 1.3*	22.9 ± 1.8	22.3 ± 1.1
Urinary protein excretion (mg per day)	27.0 ± 2.9	106.4 ± 6.9*	70.7 ± 5.7**	52.1 ± 3.3**,***
Histology (n=5)				
Glomerular damage (score 0–4)	0.18 ± 0.02	1.35 ± 0.14*	1.10 ± 0.06**	0.79 ± 0.08**,***

Abbreviations: DS-H, untreated DS rats; DS-H-Hyd, DS rats treated with hydralazine; DS-L, DS normotensive rats fed a 0.9% salt diet; DS-H-Tel, DS rats treated with telmisartan.
Data are expressed as mean ± s.e.m.
*$P<0.05$ *vs.* DS-L.
**$P<0.05$ *vs.* DS-H.
***$P<0.05$ *vs.* DS-H-Hyd.

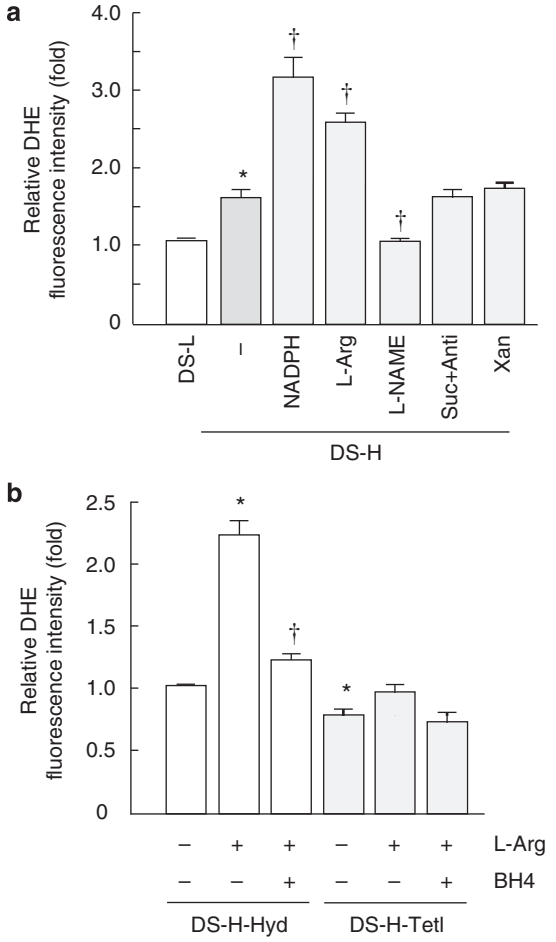

Figure 1 Source of reactive oxygen species (ROS) production. ROS production was measured by dihydroethidium (DHE) assay. (**a**) L-Arg, L-arginine; Suc+Anti, succinate+antimycin; Xan, xanthine. $n=5$ in each group. $^{*}P<0.05$ *vs.* DS-L (DS normotensive rats fed a 0.9% salt diet). $^{\dagger}P<0.05$ *vs.* DS-H (untreated DS rats) (−). (**b**) $n=5$ in each group. $^{*}P<0.05$ *vs.* DS-H-Hyd (DS rats treated with hydralazine) (−). $^{\dagger}P<0.05$ *vs.* DS-H-Hyd L-Arg (+). DS, Dahl salt-sensitive hypertensive.

production was similar in the four groups (Figure 3a). However, the ratio of dimer to monomer eNOS was lower in DS-H rats than DS-L rats (Figure 3b, $P<0.05$). Treatment with telmisartan ($P<0.05$ *vs.* DS-H-Hyd), but not hydralazine, ameliorated the decrease of the eNOS dimer/monomer ratio.

Biopterin production and concentration in salt-sensitive hypertensive rats

Tetrahydrobiopterin (BH4) is an essential cofactor required for the production of NO by each of the NOS isoforms. Synthesis of BH4 involves a multistep process in which GTPCH1 is the rate-limiting enzyme required for the initial step in conversion of guanosine-5′-triphosphate to BH4. The mRNA expression of GTPCH1, as measured by real-time PCR (Figure 3c), was increased in DS-H, DS-H-Hyd and DS-H-Tel rats compared with DS-L rats, but there were no differences between the three groups. Serum BH4 levels were decreased in DS-H and DS-H-Hyd rats, but preserved in DS-H-Tel rats (Figure 3d). However, serum levels of BH2, which is the oxidized form of BH4, were increased in DS-H and DS-H-Hyd rats, but preserved in DS-H-Tel rats (Figure 3e).

Aortic artery endothelial function in salt-sensitive hypertensive rats

Endothelium-dependent relaxation of aortic artery rings in response to acetylcholine was significantly depressed in DS-H rats compared with DS-L rats (Figure 4a). Acetylcholine caused concentration-dependent vasorelaxations in preconstricted control rings from DS-L rats ($pEC_{50}=7.2\pm0.1$; $R_{max}=82\pm4\%$; $n=5$). In contrast, both the sensitivity and maximum relaxations to acetylcholine enhanced in collared rings were depressed in rings from DS-L rats ($pEC_{50}=6.5\pm0.2$; $R_{max}=27\pm3\%$; $n=5$; $P<0.05$). Telmisartan and hydralazine significantly ($P<0.05$) improved both sensitivity ($pEC_{50}=6.9\pm0.1$ and 6.9 ± 0.1, respectively; $n=6$ each) and maximum relaxation ($R_{max}=60\pm1$ and $37\pm2\%$, respectively; $n=5$ each) to acetylcholine in aortic artery ring segments compared with the rings from DS-H rats. Regarding maximum relaxation, telmisartan treatment afforded more improvement ($P<0.05$) than hydralazine treatment. However, endothelium-independent relaxation of the aortic artery rings in response to sodium nitroprusside did not differ in any group of rats (Figure 4b). To determine whether endothelial cells were histologically damaged, expression of rat endothelial cell antigen 1 in the isolated aortic artery was examined. Endothelial cells were preserved in DS-H rats (data not shown).

Renal flow autoregulation in salt-sensitive hypertensive rats

Alterations in renal flow during step changes in renal perfusion pressure are shown in Figure 5. The capacity to autoregulate the steady-state renal flow in response to step changes was decreased in DS-H rats (Figure 5b) compared with DS-L rats (Figure 5a), as indicated by the difference in the calculated AI between 100–160 mm Hg of pressure changes ($AI=0.56\pm0.07$ and 0.22 ± 0.03, respectively, $P<0.05$). The renal flow autoregulation in response to step changes in perfusion pressure was well improved in DS-H-Tel rats (Figure 5d; $AI=0.34\pm0.05$) but not in DS-H-Hyd rats (Figure 5c; $AI=0.64\pm0.08$, $P<0.05$ *vs.* DS-H-Tel).

Purinergic receptor expression in salt-sensitive hypertensive rats

The mRNA expression levels of P2rx1 in renal cortex were examined by quantitative real-time PCR. The P2rx1 mRNA expression could be detected in the renal cortex, and the expression was decreased in hypertensive rat glomeruli (Figure 6; 0.75 ± 0.06-fold in DS-H compared with DS-L, $P<0.05$). Telmisartan treatment restored the P2rx1 expression to control levels (0.96 ± 0.12-fold).

DISCUSSION

In this study, we explored whether uncoupled eNOS parallel with endothelial dysfunction and deterioration of renal autoregulation were involved in the progression of hypertensive renal disease. Our results demonstrated that NO/ROS imbalance, which is indicative of endothelial dysfunction, exists in DS rat glomerular and pre-glomerular arterioles, and that telmisartan has beneficial effects against eNOS uncoupling, endothelial dysfunction and loss of renal autoregulation in a Dahl rat model for salt-sensitive hypertensive renal disease.

We reported previously that NADPH oxidase and uncoupled NOS are major sources of glomerular superoxide in streptozotocin-induced rats with diabetic nephropathy,[17] and NOS uncoupling has been reported to occur in the renal medulla of DS rats.[23] In this study, we demonstrated that uncoupled eNOS also occurs in the glomeruli of DS rats. The eNOS uncoupling was found to participate not only in kidney disease, but also in arteriosclerosis and endothelial dysfunction in large blood vessels of diabetics.[8] Thus, eNOS uncoupling seems to be a common mechanism of endothelial dysfunction, leading to reduced NO production and increased ROS production in the blood vessel wall.

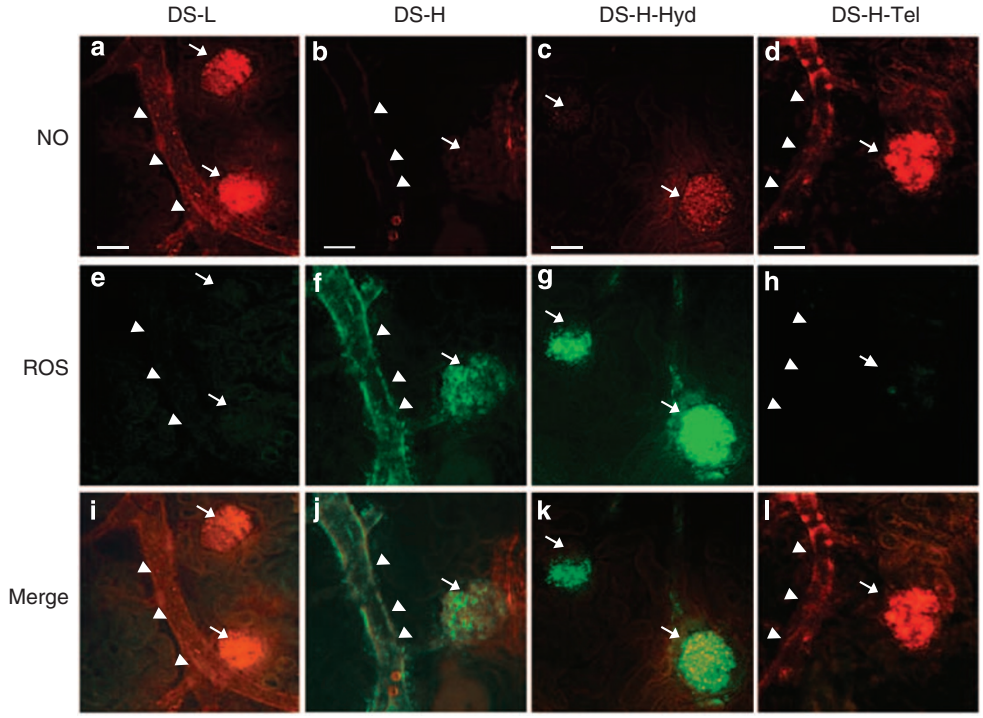

Figure 2 *In situ* detection of nitric oxide (NO) and reactive oxygen species (ROS). (**a**–**d**) Upper panel: NO detected by diaminorhodamine–4 M acetoxymethyl ester (DAR–4 M AM). (**e**–**h**) Middle panel: ROS detected by dichlorodihydrofluorescein (DCFH). (**i**–**l**) Lower panel: merged images of NO with ROS. Arrow: glomeruli; arrowheads: small lobar artery. Data are representative of five independent experiments. Bar=100 μm.

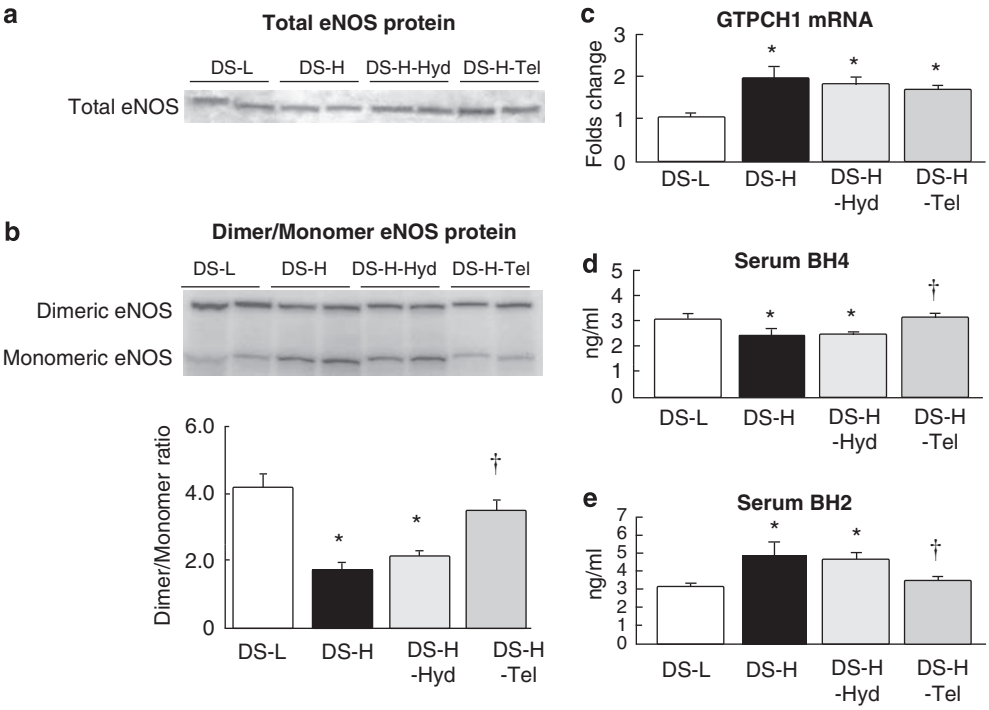

Figure 3 Western blot analysis of endothelial nitric oxide synthase (eNOS) and biopterin production. (**a**) Total eNOS was examined by normal SDS–PAGE. (**b**) Dimer/monomer eNOS were examined by low temperature SDS–PAGE. n=6 in each group. *P<0.05 *vs.* DS-L (DS normotensive rats fed a 0.9% salt diet), †P<0.05 *vs.* DS-H-Hyd (DS rats treated with hydralazine). (**c**) Real-time PCR for *guanosine-5′-triphosphate cyclohydrolase 1* (*GTPCH1*) mRNA expression. n=5 in each group. *P<0.05 *vs.* DS-L. (**d**) Serum tetrahydrobiopterin (BH4) concentration. (**e**) Dihydrobiopterin (BH2) concentration. n=5 in each group. *P<0.05 *vs.* DS-L, †P<0.05 *vs.* DS-H-Hyd. DS, Dahl salt-sensitive hypertensive.

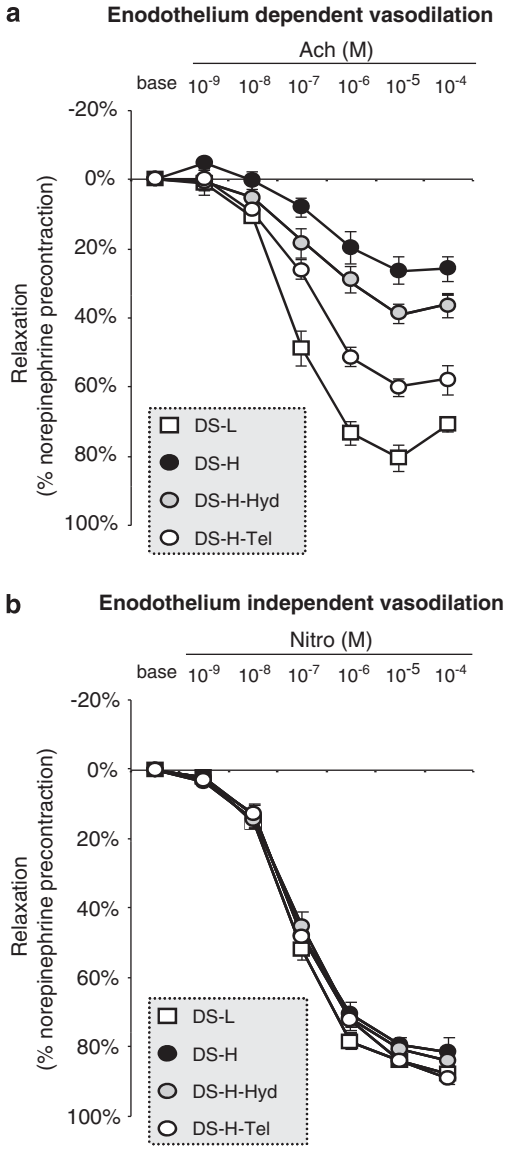

Figure 4 Endothelial function of aortic artery. (**a**) Endothelium-dependent vasodilation examined by acetylcholine. (**b**) Endothelium-independent vasodilation examined by nitroprusside. n=5 in each group.

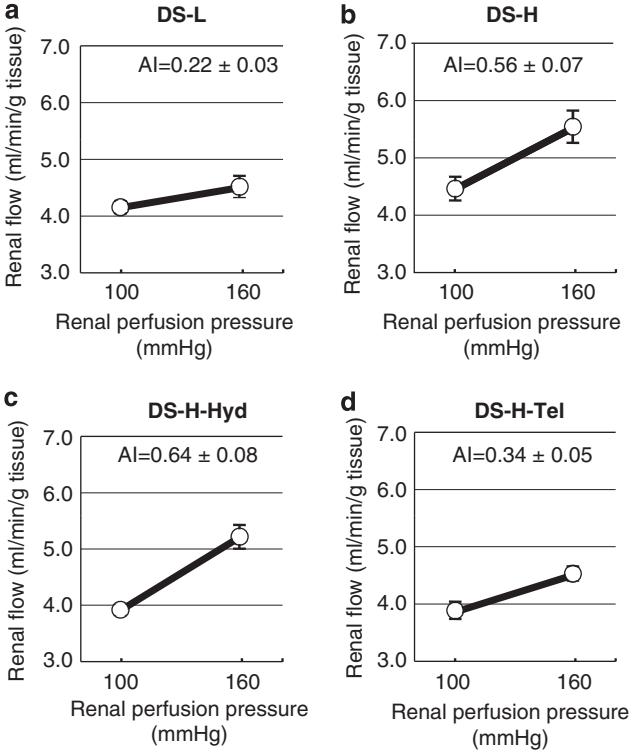

Figure 5 Renal flow autoregulation in DS-L (DS normotensive rats fed a 0.9% salt diet) (**a**), DS-H (untreated DS rats) (**b**), DS-H-Hyd (DS rats treated with hydralazine) (**c**) and DS-H-Tel (DS rats treated with telmisartan) (**d**). n=6 in each group. Autoregulatory index (AI)=[(RF_2–RF_1)/RF_1]/[(RPP_2–RPP_1)/RPP_1]. DS, Dahl salt-sensitive hypertensive; RF, renal flow; RPP, renal perfusion pressure.

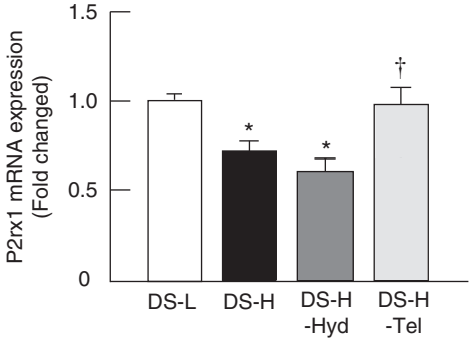

Figure 6 Effects of telmisartan on renal *P2rx1* mRNA expression in Dahl salt-sensitive hypertensive (DS) rats. Real-time PCR for *P2rx1* mRNA expression. n=6 in each group. *$P<0.05$ *vs.* DS-L (DS normotensive rats fed a 0.9% salt diet), †$P<0.05$ *vs.* DS-H-Hyd (DS rats treated with hydralazine).

In DS rats, high salt intake induced the activation of NADPH oxidase ,[24] and in another study, such activation increased ROS production, with subsequent endothelial dysfunction.[25] The production of ROS by NADPH oxidase oxidized BH4 to BH2 and caused eNOS uncoupling. In addition, ROS produced by NADPH oxidase reacted with NO immediately and produced peroxynitrite.[26] Reaction of this peroxynitrite with the protein promotes nitrotyrosine formation of the protein. Nitrotyrosine formation of eNOS may obstruct eNOS dimers. In addition, the resolution of GTPCH1, which is an enzyme involved in the production of BH4, is promoted by proteasome-dependent degradation under the presence of peroxynitrite.[27] Consequently, reduced production of BH4 may induce eNOS uncoupling.

Treatment with ARB clearly improved eNOS uncoupling. The mechanism of this action of ARB is thought to be mainly a reduction of ROS production by attenuation of glomerular hypertension and inhibition of NADPH oxidase. There is a close link between NADPH oxidase activation and NOS uncoupling,[28] and it has been reported that restraint of NADPH oxidase improved NOS uncoupling.[29] In DS rats, NADPH oxidase-induced superoxide production may trigger eNOS uncoupling, leading to impaired NO signaling and to endothelial dysfunction. Angiotensin receptor blockade (ARB) was demonstrated to decrease NADPH oxidase activity.[18] Therefore, reduction of NADPH oxidase activity by ARB seems the most efficient method to improve endothelial function through NOS recoupling. However, the

abnormal feature in DS rats is characterized by the dysfunction of NO system. Barton *et al*.[30] reported that DS rats failed to increase renal NOS activity in response to salt loading. The ARB treatment may improve the NOS activity in response to salt loading.

The inability of eNOS to produce NO on a regular basis leads to an imbalance between ROS and NO, and endothelial dysfunction. In this study, we measured endothelium-dependent relaxation of the aortic artery rings in response to acetylcholine. The relaxation response significantly fell in DS rats with disproportionate NO/ROS production. In addition, we measured the ability of autoregulatory responses, which was significantly lower in DS rat kidneys. The autoregulatory response is reported to be significantly decreased in DS rats[31] and remnant kidney rats, a model of progressive renal damage.[32] The renal autoregulatory response is coordinated by two mechanisms: myogenic response and tubulo-glomerular feedback.[33] On the basis of the results of our experiments in DS rats, ARB seems to improve the renal autoregulatory response in parallel with endothelial function. The endothelium has a central role in early functional adaptations to hypertension. Pressure-induced myogenic constriction is enhanced due to the augmented release of endothelium-derived constrictor factors that modulate arteriolar smooth muscle sensitivity to Ca^{2+}.[34] In contrast, flow/shear stress-induced dilation of arterioles is reduced in hypertension, due to the impaired mediation of the response by NO.[35] It has also been proposed that high salt intake reduces endothelium-dependent dilation of mouse arterioles by enhancing the release of ROS from NOS, which then interferes with the synthesis and/or action of endothelium-derived mediators.[36] Treatment with ARB might maintain and improve endothelial function by reducing superoxide production, which could lead to inhibition of vascular constrictive changes due to the improved mediation of the response by NO and consequently may maintain a normal pressure-induced myogenic constriction.

In general, angiotensin II promotes the autoregulatory response reaction in normal rats by increasing tubulo-glomerular feedback.[37,38] So, ARB may dampen the strength of tubulo-glomerular feedback helping to restore the normal GFR response.[39] Enhanced tubulo-glomerular feedback responses may be the result of an angiotensin II-induced reduction in local levels of nitric oxide.[40] Tubulo-glomerular feedback responses have been reported to be enhanced in spontaneously hypertensive rats,[41] but there are few reports regarding to tubulo-glomerular feedback in DS rats. It is well accepted that DS rats on high-salt diet exhibit a suppressed systemic RAS (rennin–angiotensin system) accompanied with inappropriately activated renal RAS.[42] Indeed, there is a possibility that the tubuloglomerular feedback response in the DS rats are reduced similar to deoxycorticosterone acetate (DOCA)– salt hypertensive rat or Goldblatt hypertensive rats.[43] So, ARB might have little effects on the renal autoregulatory mechanism via tubulo-glomerular feedback in DS rats.

We have shown that the renal autoregulation in the DS rat was impaired. Conclusive identification of the signaling molecules that mediate autoregulation remains controversial, but it does seem clear that extracellular ATP and P2 purine receptor activation is required for autoregulatory control of renal hemodynamics.[44,45] Indeed, ATP is released from many different cell types in response to stretch, osmotic stimuli[46] and activation of P2rx1 receptors, which is an essential step in pressure-mediated afferent arteriolar vasoconstriction. Blockade of P2rx1 receptors or deletion of P2rx1 receptors in knockout mice eliminates pressure-mediated afferent arteriolar contraction.[47] In hypertensive renal disease, pathological changes arise in small arteries in the early stage.[48] So, this ATP–P2X axis might be damaged, which could subsequently lead to down regulation of pressure sensory ability and cause glomerular hypertensive injury. Actually, mRNA expression levels of P2rx1 in the renal cortex of DS rats were decreased, which seem to be parallel with impairment of renal autoregulation. Telmisartan effectively prevented the deterioration of renal autoregulation, which might be due to the preservation of P2rx1.

The relationship between endothelial function and renal autoregulational ability is not clear from the results of this study. Decreased endothelial function might induce the failure of autoregulation. We demonstrated that treatment with telmisartan improved both functions in DS rat kidney, but there are no data to support the hypothesis that the decreased endothelial function lies behind the decreased autoregulation. Similarly, whether a causal relation exists between improved autoregulation and a decrease in renal damage cannot be determined from this study. It is very difficult to design experiments that directly address these important questions, but this is what is needed if we are to gain new insight into these questions in the future.

In summary, we detected glomerular ROS/NO imbalance and impairment of renal autoregulatory response in rats with salt-induced hypertension. We also showed that correction of the ROS/NO disproportion and endothelial dysfunction were improved by ARB treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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