Original Article

Increased Expression of gp91phox Homologues of NAD(P)H Oxidase in the Aortic Media during Chronic Hypertension: Involvement of the Renin-Angiotensin System

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Although vascular cells express multiple members of the Nox family of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, including gp91phox, Nox1, and Nox4, the reasons for the different expressions and specific roles of these members in vascular injury in chronic hypertension have remained unclear. Thus, we quantified the mRNA expressions of these NAD(P)H oxidase components by real-time polymerase chain reaction and evaluated superoxide production and morphological changes in the aortas of 32-week-old stroke-prone spontaneously hypertensive rats (SHRSP) and age-matched Wistar Kyoto rats (WKY). The aortic media of SHRSP had an approximately 2.5-fold greater level of Nox4 mRNA and an approximately 10-fold greater level of Nox1 mRNA than WKY. The mRNA expressions of gp91phox and p22phox in SHRSP and WKY were comparable. SHRSP were treated from 24 weeks of age for 8 weeks with either high or low doses of candesartan (4 mg/kg/day or 0.2 mg/kg/day), or a combination of hydralazine (30 mg/kg/day) and hydrochlorothiazide (4.5 mg/kg/day). The high-dose candesartan or the hydralazine plus hydrochlorothiazide decreased the blood pressure of SHRSP to that of WKY, whereas the low-dose candesartan exerted no significant antihypertensive action. Media thickening and fibrosis, as well as the increased production of superoxide in SHRSP, were nearly normalized with high-dose candesartan and partially corrected with low-dose candesartan or hydralazine plus hydrochlorothiazide. These changes by antihypertensive treatment paralleled the decrease in mRNA expression of Nox4 and Nox1. These results suggest that blood pressure and angiotensin II type 1 receptor activation are involved in the up-regulation of Nox1 and Nox4 expression, which could contribute to vascular injury during chronic hypertension. (*Hypertens Res* 2006; 29: 813–820)

***Key Words*: stroke-prone spontaneously hypertensive rats, NAD(P)H oxidase, oxidative stress, angiotensin II, aorta**

Introduction

Accumulating evidence indicates that reactive oxygen species play a pivotal role in the development of vascular dysfunction caused by various disease states, including hypertension (*1–3*). An exaggerated production of superoxide anion (O_2^-) by the vascular wall has been observed in different animal mod-

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Table 1. Primers of NAD(P)H Oxidase Components and Their mRNA Expressions in the Aortic Media

Target mRNA	mRNA expression#		Primer sequence 5′ to 3′
	WKY	SHRSP	
Rat Nox1	3±1	33±6*	Fwd: TGGACGAATTAGGCAAACCG Rev: TTGGGGTGGGCAGTAGCTAT
Rat gp91phox	34±3	66±18	Fwd: TGGTGATGTTAGTGGGAGC Rev: CTTTCTTGCATCTGGGTCT
Rat Nox4	617±32	1,534±114**	Fwd: ATCTGGGTCTGCAGAGACAT Rev: CTGAGGTACAGCTGGATGTT
Rat p22phox	836±34	805±58	Fwd: TCCACTTACTGCTGTCCGT Rev: TCAATGGGAGTCCACTGCT
Rat GAPDH	—	—	Fwd: TGAACGGGAAGCTCACTGG Rev: TCCACCACCCTGTTGCTGTA

#Relative expression normalized by 10,000 copies of GAPDH expression. $p<0.0005$, **$p<0.0001$. WKY, Wistar Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats. $n=6$ in each group.

els of hypertension. Among the multiple enzymatic systems that produce superoxide in vascular cells, the major enzymatic source of intracellular superoxide is NAD(P)H oxidase, which generates O_2^- by a one-electron reduction of molecular oxygen (*2*, *3*). NAD(P)H oxidase consists of multiple subunits, electron transfer moieties such as gp91phox, Nox1, and Nox4, and another membrane component, p22phox. It also includes regulatory subunits such as p47phox, p67phox, and rac1. The enhanced expression and/or enhanced activation of NAD(P)H oxidase have been suggested as a mechanism underlying the enhancement of superoxide anion production in hypertension (*2*, *3*).

Continuous infusion of angiotensin II to various animal models has been shown to cause sustained hypertension and vascular injury. In the aortas from angiotensin II–infused rabbits, adventitial fibroblasts and smooth muscle were found to have important roles in the superoxide production, and the expression of p67phox in adventitia was increased (*4*). In aortas from angiotensin II–infused rats, the expressions of Nox1, gp91phox, Nox4, and p22phox were increased (*5*). In spontaneously hypertensive rats (SHR), in which the renin-angiotensin system has a pivotal role in the development and maintenance of hypertension as well as in vascular injury, a previous study examined only p22phox and found that its expression in SHR aortas was up-regulated or comparable to that in Wistar Kyoto Rats (WKY) (*6*, *7*). Another study showed that expressions of Nox1 mRNA, gp91phox mRNA, and p22phox mRNA were comparable between SHR and WKY (3–4 months old and 9–12 months old, respectively). In contrast, a study of cerebral arteries from stroke-prone SHR (SHRSP) showed increased expression of Nox4 in SHRSP, whereas the expressions of Nox1, gp91phox, p22phox, and p47phox did not differ between SHRSP and WKY (*8*, *9*). Thus, the alteration in the expression of NAD(P)H oxidase components in vascular tissue from the chronic hypertension model appears to differ from that in the angiotensin II–infusion model, and the alteration in the expression of NAD(P)H oxidase components in the former model is still controversial. In addition, it is not known whether antihypertensive treatment could normalize the altered expression of NAD(P)H oxidase, and particularly the expression of Nox family members, in hypertension. Thus, in the present study we used aortas from 32-week-old SHRSP and WKY, as well as those from chronically treated SHRSP beginning at 24 weeks of age and continuing for 8 weeks, to evaluate the expression of NAD(P)H oxidase, superoxide production, and the remodeling of the aorta after administration of high- or low-dose angiotensin II type 1 receptor blocker or hydralazine plus hydrochrolothiazide.

Methods

Animals and Tissue Preparation

The present study was approved by the Committee on the Ethics of Animal Experimentation of Kyushu University. Male SHRSP/IZM and WKY/IZM (Disease Model Cooperative Research Association, Kyoto, Japan) were fed a standard rat chow and had free access to tap water. At 24 weeks of age, SHRSP were assigned to either a control group (SP-C) or one of three treatment groups: candesartan (Takeda Pharmaceutical Co., Osaka, Japan) 0.2 mg/kg/day (SP-Cand(low)), candesartan 4 mg/kg/day (SP-Cand(high)), or a combination of hydralazine 30 mg/kg/day and hydrochlorothiazide 4.5 mg/kg/day (SP-HH) for 8 weeks. All drugs were given in the drinking water. Systolic blood pressure (SBP) was measured by the tail-cuff method.

RNA Preparation and Real-Time Quantitative Polymerase Chain Reaction

At 32 weeks of age, the rats were anesthetized with ether and killed by decapitation. The aortas were quickly excised and washed in ice-cold phosphate-buffered saline. Adventitial tis-

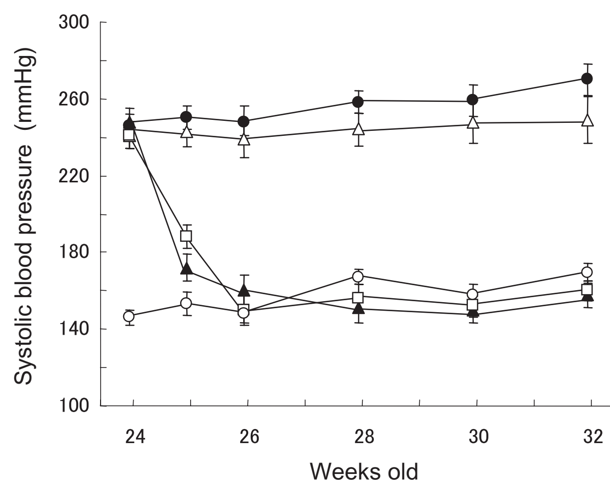

Fig. 1. *Changes in systolic blood pressure (SBP) level in Wistar Kyoto rats (WKY; open circles) and stroke-prone spontaneously hypertensive rats (SHRSP) untreated (closed circles) or treated with high-dose candesartan (closed triangles), low-dose candesartan (open triangles), or hydralazine plus hydrochlorothiazide (open squares). Drugs were administered from 24 weeks of age for 8 weeks. The SBP of untreated SHRSP was different from that of WKY, and from SHRSP treated with high-dose candesartan, and hydralazine plus hydrochlorthiazide* ($p<0.001$)*. Low-dose candesartan did not significantly decrease SBP.*

sue was cleaned from the vessels and the endothelium was removed by rubbing. The tissues were quickly frozen in liquid nitrogen and stored at −80°C until further use. Total RNA was isolated using the Isogen total RNA isolation system (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol, and was reverse-transcribed using *Bca*PLUS RTase (Takara, Osaka, Japan). Aliquots of the reverse-transcription products were used for quantitative polymerase chain reaction (PCR) in the LightCycler™ PCR and Detection System using the Fast Start DNA Master SYBER Green I kit (both from Roche Molecular Biochemicals) as described by the manufacturer.

Optimized amplification conditions were 0.3 μmol/l primers for gp91phox and p22phox (Table 1), 3 nmol/l $MgCl_2$, and annealing at 63°C; and, for Nox1, Nox4, and GAPDH, 0.5 μmol/l primers (Table 1), 3 nmol/l $MgCl_2$, and annealing at 60°C. The number of copies was calculated by the software included with the instrument and normalized by that of the GAPDH housekeeping gene.

Morphometry of the Aorta

The rats were killed with a sodium pentobarbital overdose and were perfusion-fixed for 5 min at 120 mmHg in WKY, SP-Cand(high), and SP-HH, and at 200 mmHg in SP-C and SP-Cand(low), with 4% paraformaldehyde buffered with 0.1 mol/l NaH_2PO_4. The aortas were excised, fixed in the same buffer for 24 h, then dehydrated and embedded in paraffin. The paraffin slices (4 μm thick) were stained with hematoxylin-eosin and Azan staining solutions. The wall thickness/lumen ratio (the medial thickness to the internal diameter) and fractional Azan staining (area of fibrosis) were determined. These measurements were performed from five randomly selected fields in one cross section of aorta (magnification: ×200) with NIH Image (version 1.59) software.

Oxidative Fluorescence Microtopography

A portion of the aorta was excised and snap-frozen with OCT compound (Sakura Finetek, Inc., Tokyo, Japan) in isopentane prechilled with liquid nitrogen to produce fresh-frozen sections for oxidative fluorescence microtopography. Dihydroethidium (DHE) oxidative fluorescence dye was used to evaluate the *in situ* production of superoxide. Unfixed frozen samples were cut into 30-μm–thick sections and placed on glass slides. Dihydroethidium (10 μmol/l) was applied to each section, and then the section was incubated in a light-protected humidified chamber at 37°C for 30 min. A fluorescence image of DHE was obtained by a laser scanning confocal imaging system (LSM5 PASCAL; Carl Zeiss, Tokyo, Japan) with a 560-nm–long pass filter.

The image was selectively and quantitatively analyzed using NIH Image software to evaluate the cellular site of superoxide production in the media areas, which were predetermined with hematoxylin-eosin–stained serial sections. These data were expressed as fold increases against the corresponding data of the control group.

Statistical Analysis

Data are presented as the means±SEM. Comparisons between groups were made by one-way ANOVA, followed by Scheffe's least significant difference post hoc test. A value of $p<0.05$ was considered statistically significant.

Results

Expression of NAD(P)H Oxidase mRNA in the Aortic Media of SHRSP and WKY

The mRNA expressions of NAD(P)H oxidase components in the aortic media of SHRSP and WKY were assessed by quantitative PCR (Table 1). In both SHRSP and WKY, the expression of Nox4 mRNA was highest in homologues of gp91phox. Between SHRSP and WKY, the expressions of Nox4 mRNA and Nox1 mRNA were significantly higher in the former (Table 1). No significant differences were found between WKY and SP-C in the expression of gp91phox or in that of p22phox.

Table 2. Blood Pressure, Heart Rate, and Body Weight in 32-Week-Old Rats

	Blood pressure (mmHg)	Heart rate (/min)	Body weight (g)
WKY	169±3	413±24	451±5
SP-C	270±8*	372±19	336±5*
SP-Cand(low)	249±12*	347±11	347±5*
SP-Cand(high)	$158±7^{††,\#}$	403±12	348±9*
SP-HH	$160±4^{††,\#}$	$475±9^{†,§,\#}$	339±8*

n=7–11 in each group. SP-C, untreated stroke-prone spontaneously hypertensive rats (SHRSP); SP-Cand(low), SHRSP treated with low-dose candesartan; SP-Cand(high), SHRSP treated with high-dose candesartan; SP-HH, SHRSP treated with hydralazine plus hydrochlorothiazide. *$p<0.0001$ *vs*. WKY, $^{†}p<0.01$ *vs*. SP-C, $^{††}p<0.001$ *vs*. SP-C, $^{§}p<0.0001$ *vs*. SP-Cand(low), $^{\#}p<0.005$ *vs*. SP-Cand(high).

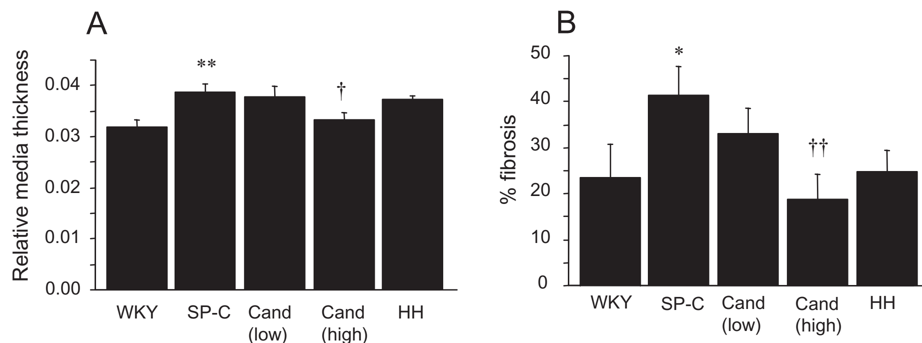

Fig. 2. *Morphological evaluation of the aorta from WKY, untreated SHRSP (SP-C), and SHRSP treated with a low dose of candesartan (SP-Cand(low)), with a high dose of candesartan (SP-Cand(high)), or with hydralazine plus hydrochlorothiazide (SP-HH). A: Relative media thickness (ratio of medial thickness to lumen diameter) evaluated by hematoxylin-eosin staining. B: Percent fibrosis in the media evaluated by Azan staining.* n = 7 *in each group, in both A and B.* *p < *0.05* vs. *WKY,* **p < *0.001* vs. *WKY,* †p < *0.05* vs. *SP-C,* ††p < *0.01* vs. *SP-C.*

Systolic Blood Pressure, Heart Rate, and Body Weight

We next tested whether or not antihypertensive treatment would affect the expressions of Nox1 and Nox4, superoxide production in the media, and remodeling and fibrosis of the aorta. The changes in SBP during drug administration are shown in Fig. 1. Chronic treatment with high-dose candesartan or hydralazine plus hydrochlorothiazide decreased blood pressure to a level comparable to that of WKY. On the other hand, low-dose candesartan did not significantly decrease blood pressure. Table 2 shows the SBP, heart rate, and body weight of WKY, SP-C, SP-Cand(low), SP-Cand(high), and SP-HH at 32 weeks of age. SBP was significantly higher in SP-C than in WKY. Body weight was significantly smaller in the three SHRSP groups than in WKY. Heart rate was significantly greater in SP-HH than in the other groups.

Morphology of the Aorta

The wall thickness-to-lumen ratio in the SP-C was significantly higher than that in WKY. In SP-Cand(high), that ratio was nearly the same as in WKY (Fig. 2A). Similarly, total fractional fibrosis in the media was significantly higher in SP-C than in WKY, and was nearly normalized in SP-Cand(high) (Fig. 2B). The wall-to-lumen ratio and fractional fibrosis in the media in SP-Cand(low) and SP-HH tended to decrease, but the changes were not statistically significant.

Oxidative Fluorescence Microtopography

Figure 3 shows superoxide content in the vessel wall assessed by DHE fluorescence. The SP-C group showed an increase in DHE fluorescence, reflecting the increased superoxide content throughout the vessel wall (Fig. 3A). The WKY group and the SP-Cand(high) group showed only a minimal fluorescence in the media. In another set of experiments in which the sections were additionally exposed to diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, the fluorescence was minimal from the aortic media in all groups and the increased fluorescence in SHRSP was not evident (data not shown).

Figure 3B shows the results of semi-quantitative analysis. The level of fluorescence in aortic media from the SP-C

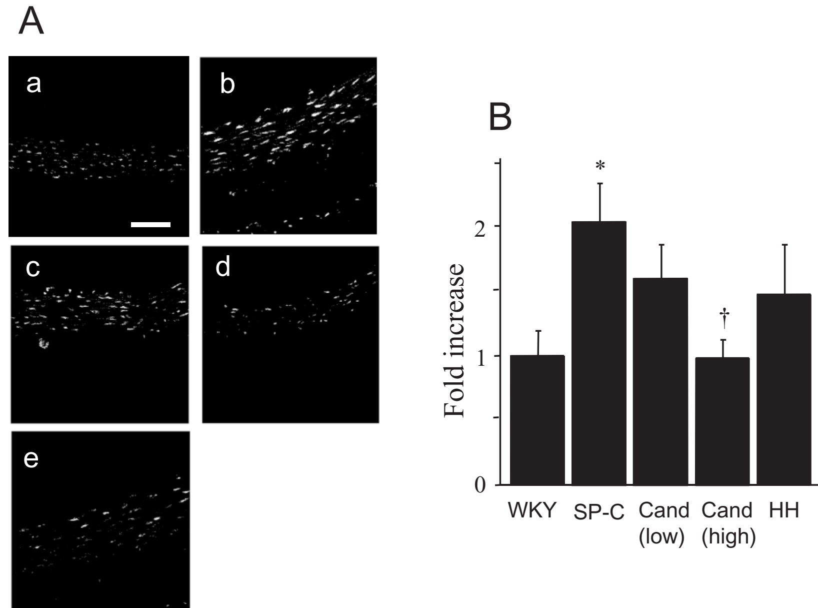

Fig. 3. *Fluorescence intensities by dihydroethidium, reflecting superoxide production in the aorta. A: Fluorescence signals in the aorta from WKY (a), SP-C (b), SP-Cand(low) (c), SP-Cand(high) (d), and SP-HH (e). Bar indicates 100 μm. The upper side of the figure corresponds to the lumen. B: Level of fluorescence intensity from the aortic media in each group.* n = 7 *in each group.* *$p < 0.05$ vs. *WKY,* †$p < 0.05$ vs. *SP-C.*

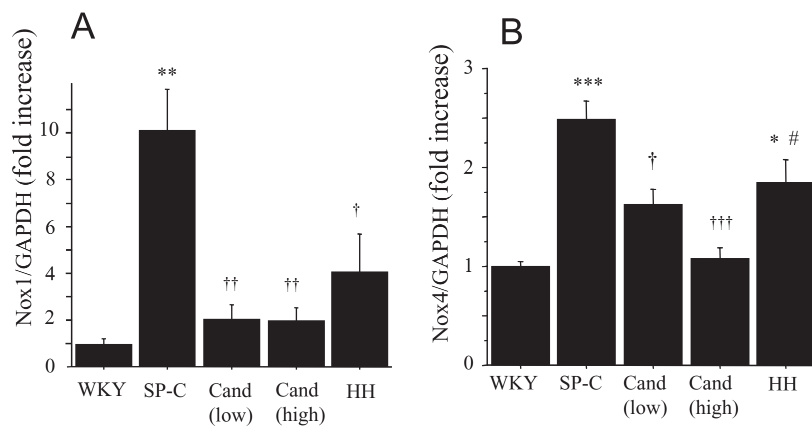

Fig. 4. *Expressions of Nox1 mRNA (A) and Nox4 mRNA (B) in the aortic media. Relative expressions are shown, with those in WKY normalized to 1.0.* n = 6 *in each group, in both A and B.* *$p < 0.05$ vs. *WKY,* **$p < 0.0005$ vs. *WKY,* ***$p < 0.0001$ vs. *WKY,* †$p < 0.05$ vs. *SP-C,* ††$p < 0.0005$ vs. *SP-C,* †††$p < 0.0001$ vs. *SP-C,* #$p < 0.05$ vs. *SP-Cand(high).*

group was significantly higher than that from the WKY group. The level of fluorescence from the SP-Cand(high) group was reduced significantly, to the level of the WKY group. Fluorescence levels in the SP-Cand(low) and SP-HH groups decreased, but were in between those of SP-C and WKY.

Expression of Nox4 mRNA and Nox1 mRNA

The Nox4 mRNA level was significantly lower than that of SP-C in all treatment groups (Fig. 4). The Nox4 mRNA level of SP-Cand(high) was comparable to that of WKY. In contrast, the levels of SP-Cand(low) and SP-HH were in between the levels of WKY and SP-C. Unlike the increase in the mRNA level of Nox1 in the SP-C group, the level was significantly decreased in the SP-Cand(low), SP-Cand(high), and

SP-HH groups. The Nox1 mRNA levels of SP-Cand(low), SP-Cand(high), and WKY were comparable.

Discussion

In the present study, we have shown that the mRNA expressions of Nox1 and Nox4 were higher in the aortic media from SHRSP than in those from WKY, whereas the mRNA expressions of p22phox and gp91phox were comparable from strain to strain. Furthermore, antihypertensive treatment for 8 weeks corrected the increased expressions of Nox1 and Nox4 in SHRSP, in parallel with the decrease in superoxide production and with the correction of the wall remodeling. High-dose candesartan nearly normalized these abnormalities, which developed during chronic hypertension. Collectively, these results indicate that both angiotensin II and chronic hypertension up-regulated vascular NAD(P)H oxidases, especially Nox1 and Nox4, and could contribute to the development of hypertensive vascular injury.

Expression of Nox Family Members in the Aortic Media

In vascular tissues, the expressions of Nox1 and Nox4 are high, but that of gp91phox is relatively low (*2*, *3*). Nox4 mRNA expression is about 50 times higher in the rat aorta and about 150 times higher in the rat carotid artery than Nox1 mRNA expression (*5*, *10*). In the present study, the Nox4 mRNA level in the aortic media was 300- to 500-fold higher than that of Nox1. In cultured vascular muscle cells from the rat aorta, the expressions of Nox1 and Nox4 are relatively high and comparable to each other, but that of gp91phox is low (*11*). In contrast, a significant expression level of gp91phox was reported in cultured vascular muscle cells from human resistance arteries (*12*). The different expression patterns among these studies might be attributable to differences in vascular tissues (species and regional differences) and/or those in experimental conditions (*in vitro vs*. *in vivo*).

Increased mRNA Expression of Nox1 and Nox4 in the Aortic Media from SHRSP

We have shown that the expression of Nox1 mRNA and Nox4 mRNA in the aortic media of SHRSP were 10-fold and 2.5-fold those of WKY, respectively. Only three studies have examined the altered expression of Nox family members in vascular tissues from a chronic hypertensive model. One study showed that in cerebral arteries from SHR, up-regulation of Nox4 mRNA was 4-fold that of WKY, whereas Nox1 mRNA and gp91phox mRNA were comparable between the strains (*8*). Another study showed that mRNA expressions of Nox1 and gp91phox in the aorta were comparable between SHR and WKY (3–4 months old and 9–12 months old, respectively), although that of Nox4 was not evaluated (*9*). The third study examined transgenic hypertensive rats overexpressing the mouse Ren 2 gene, TGR(mREN2)27; such mice have an overactive tissue renin-angiotensin system and develop severe hypertension at an early age (*13*). In aortas from 10-week-old TGR(mREN2)27, mRNA and protein of Nox4 were increased by about 60% and 100%, respectively, compared to control rats, while the expression of gp91phox mRNA was not evaluated and Nox1 mRNA was detected only in TGR(mREN2)27, not in control rats. Although the alteration varies among different disease models, the increased expression of NAD(P)H oxidase components appears to be a common mechanism for the increased superoxide production.

In the present study, the level of p22phox mRNA did not differ between SHRSP and WKY. Other reports have also shown that expression of p22phox mRNA did not differ between SHRSP and WKY rats aged 9–12 months (*9*), nor did they differ in cerebral vessels from 26-week-old SHR and WKY (*8*). This is in contrast to previous reports that p22phox immunostaining was enhanced in aortas from female SHRSP aged 9–12 months compared to age-matched WKY (*6*), and that p22phox mRNA was higher in aortas from 30-week-old SHRSP than in age-matched WKY (*7*). The present study did not reveal the reasons for the discrepant results among these studies.

Infusion of angiotensin II has been shown to cause acute hypertension and inflammation in cardiovascular tissues (*4*, *5*). In the aortas of angiotensin II–infused rats, mRNA expressions of Nox1, gp91phox, and p22phox were up-regulated several fold, whereas the increase in Nox4 mRNA was small (*5*, *14*). This result is in contrast to a finding of the present study, that Nox1 and Nox4 in the aortic media from SHRSP were up-regulated while p22phox was not. The mechanism underlying the increased superoxide production in the aorta from angiotensin II–infusion model rats might differ from that in SHR. Since we removed the adventitia from the samples, the changes of p22phox and gp91phox, which are known to localize and play important roles for superoxide production in the adventitia (*4*), might have been less evident in the present study compared to other studies.

Antihypertensive Treatment

Treatment with high-dose candesartan decreased the blood pressure level of SHRSP to that of WKY. In addition, the expressions of Nox1 mRNA and Nox4 mRNA were decreased, and superoxide production was nearly normalized. Low-dose candesartan, which did not decrease blood pressure, also decreased the expressions of Nox1 and Nox4 in SHRSP. It is thus likely that activation of the angiotensin II type 1 receptor contributes to the elevation of Nox1 and Nox4 mRNA expressions independently from blood pressure. The Nox1 mRNA level in the low-dose candesartan group was fully normalized, whereas the Nox4 mRNA level was in between the levels of SHRSP and WKY. Thus, the expression of Nox1 is more dependent on the renin-angiotensin system

than is that of Nox4. This result is in good accord with a previous report in which angiotensin II increased Nox1 mRNA but not Nox4 mRNA in rat cultured vascular muscle cells (*11*). Our finding is also supported by the results that in the aortas of angiotensin II–infused rats, the mRNA expression of Nox1 was up-regulated, but the increase in Nox4 mRNA was small (*5*, *14*). Although angiotensin II is known to stimulate superoxide production by modulating signal transduction, including Src, protein kinase C, phospholipase D, small G protein (rac), phosphoinositol 3 kinase, and epidermal growth factor receptor activation (*15*, *16*), the angiotensin II–dependent increase in the expression of the NAD(P)H component could also be an important mechanism for the increased superoxide production of vascular tissues during chronic hypertension.

Antihypertensive treatment of SHRSP with diuretics and a vasodilator decreased their level of blood pressure to that of WKY. This treatment partially reduced the mRNA expressions of Nox1 and Nox4 and the superoxide production. Chronic pressure overload to the aorta *per se* appears to increase the expressions of Nox1 and Nox4, but it could not explain all of the changes observed in SHRSP. Our findings might be supported by *in vitro* evidence that a mechanical stretch enhanced Nox1 mRNA in cultured aortic vascular cells from mice (*17*). It has been reported that mechanical stress activated the angiotensin II type 1 receptor without the involvement of angiotensin II (*18*). The correction of mechanical stress and pressure overload to the aorta by a vasodilator and diuretics might suppress angiotensin II type 1 activation. Considering the data concerning antihypertensive treatment, it can be inferred that both strict control of blood pressure and inhibition of the angiotensin II type 1 receptor are required in order to repair the hypertensive vascular damage. Inversely, chronic overload and angiotensin II type 1 receptor activation play important roles in increasing superoxide production during chronic hypertension.

Both low-dose candesartan and hydralazine plus hydrochlorothiazide significantly decreased Nox1 and Nox4, but the inhibition of superoxide contents in aortic media by these treatments was mild. Although the reason for this discrepancy in the present study remains unknown, it is possible that changes in other components of NAD(P)H oxidase, such as p47phox and p67phox, may play a role in the level of superoxide. In addition, various enzymes or systems which generate or scavenge superoxide, including NAD(P)H oxidase, xanthin oxidase, electron transport chain in mitochondria, superoxide dismutase, glutathione peroxidase, *etc*., may also affect the level of superoxide content. In hypertensive rats, the expression and activity of superoxide dismutase, which scavenges superoxide, was decreased compared to that in normotensive controls (*19*). Administration of angiotensin II receptor blocker or angiotensin converting enzyme inhibitor further enhanced the superoxide dismutase expression (*20*).

A recent study showed that a lipophilic angiotensin II receptor blocker had more pronounced inhibitory action on p22phox expression than did a hydrophilic one in the aorta from SHRSP when drugs were administered for 2 weeks (*21*). We did not compare types of angiotensin II receptor blockers in the present study, although candesartan is lipophilic. It remains uncertain whether our findings would be common to all angiotensin II receptor blockers.

The limitations of the present study are as follows. First, although we prepared aortic media by rubbing the endothelium and adventitia, a minute contamination might have been present, and could have affected the results. Second, we quantified the expression levels of mRNA but not those of protein. This was because no antibodies specific to rat Nox family members were available. The relationship between mRNA expressions of Nox 1 and Nox 4 and superoxide production were in good accordance. Thus, the evaluation of the mRNA expression of Nox family members might be useful at the present. Third, the mechanism underlying the association between Nox homologue expression and angiotensin II or hypertension remains unknown in the present study. It has been reported that angiotensin II may increase the Nox1 expression, but not the Nox4 expression, in cultured cells (*5*, *14*), whereas tumor necrosis factor-α and pholbor ester increase the latter but not the former (*22*, *23*). Fourth, although we have shown that the mRNA expressions of Nox1 and Nox4 were up-regulated in the aortic media from SHRSP, it remains unknown what the specific roles of Nox1 and Nox4 are in smooth muscle during the development of hypertensive vascular injury *via* superoxide production. It has been suggested that Nox1 may be associated with cell proliferation or cell growth, whereas Nox4 may be associated with cell differentiation or senescence (*24–27*). Future studies are needed to clarify this question.

References

1. Taniyama Y, Griendling KK: Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension* 2003; **42**: 1075–1081.
2. Van Heerebeek L, Meischl C, Stooker W, Meijer CJ, Niessen HW, Roos D: NADPH oxidase(s): new source(s) of reactive oxygen species in the vascular system? *J Clin Pathol* 2002; **55**: 561–568.
3. Lassegue B, Clempus RE: Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol* 2003; **285**: R277–R297.
4. Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM, Quinn MT: Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. *Proc Natl Acad Sci U S A* 1997; **94**: 14483–14488.
5. Mollnau H, Wendt M, Szocs K, *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.
6. Hamilton CA, Brosnan MJ, McIntyre M, Graham D, Dominiczak AF: Superoxide excess in hypertension and aging: a common cause of endothelial dysfunction. *Hyper-*

tension 2001; **37**: 529–534.

7. Zalba G, Beaumont FJ, San Jose G, *et al*: Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. *Hypertension* 2000; **35**: 1055–1061.
8. Paravicini TM, Chrissobolis S, Drummond GR, Sobey CG: Increased NADPH-oxidase activity and Nox4 expression during chronic hypertension is associated with enhanced cerebral vasodilatation to NADPH *in vivo*. *Stroke* 2004; **35**: 584–589.
9. Hamilton CA, Brosnan MJ, Al-Benna S, Berg G, Dominiczak AF: NAD(P)H oxidase inhibition improves endothelial function in rat and human blood vessels. *Hypertension* 2002; **40**: 755–762.
10. Szocs K, Lassegue B, Sorescu D, *et al*: Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury. *Arterioscler Thromb Vasc Biol* 2002; **22**: 21–27.
11. Lassegue B, Sorescu D, Szocs K, *et al*: Novel gp91(phox) homologues in vascular smooth muscle cells: Nox1 mediates angiotensin II–induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* 2001; **88**: 888–894.
12. Touyz RM, Chen X, Tabet F, *et al*: Expression of a functionally active gp91phox-containing neutrophil-type NAD(P)H oxidase in smooth muscle cells from human resistance arteries: regulation by angiotensin II. *Circ Res* 2002; **90**: 1205–1213.
13. Wingler K, Wunsch S, Kreutz R, Rothermund L, Paul M, Schmidt HH: Upregulation of the vascular NAD(P)H-oxidase isoforms Nox1 and Nox4 by the renin-angiotensin system *in vitro* and *in vivo*. *Free Radic Biol Med* 2001; **31**: 1456–1464.
14. Fukui T, Ishizaka N, Rajagopalan S, *et al*: p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. *Circ Res* 1997; **80**: 45–51.
15. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK: Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* 2002; **91**: 406–413.
16. Touyz RM, Schiffrin EL: Increased generation of superoxide by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients: role of phospholipase D–dependent NAD(P)H oxidase-sensitive pathways. *J Hypertens* 2001; **19**: 1245–1254.
17. Grote K, Flach I, Luchtefeld M, Akin E, *et al*: Mechanical stretch enhances mRNA expression and proenzyme release of matrix metalloproteinase-2 (MMP-2) *via* NAD(P)H oxidase–derived reactive oxygen species. *Circ Res* 2003 **13**; 92: e80–e86.
18. Zou Y, Akazawa H, Qin Y, *et al*: Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II. *Nat Cell Biol* 2004; **6**: 499–506.
19. Umemoto S, Tanaka M, Kawahara s, *et al*: Cralcium antagonist reduces oxidative stress by upregulating Cu/Zn superoxide dimutase in stroke-prone spontaneously hypertensive rats. *Hypertens Res* 2004; **27**: 877–885.
20. Tanaka M, Umemoto S, Kawahara S, *et al*: Angiotensin II type 1 receptor antagonist and angiotensin-converting enzyme inhibitor altered the activation of Cu/Zn-containing superoxide dismutase in the heart of stroke-prone spontaneously hypertensive rats. *Hypertens Res* 2005; **28**: 67–77.
21. Takai S, Kirimura K, Jin D, *et al*: Significance of angiotensin II receptor blocker lipophilicities and their protective effect against vascular remodeling. *Hypertens Res* 2005; **28**: 593–600.
22. Moe KT, Aulia S, Jiang F, *et al*: Differential upregulation of Nox homologues of NADPH oxidase by tumor necrosis factor-alpha in human aortic smooth muscle and embryonic kidney cells. *J Cell Mol Med* 2006; **10**: 231–239.
23. Kuroda J, Nakagawa K, Yamasaki T, *et al*: The superoxide-producing NAD(P)H oxidase Nox4 in the nucleus of human vascular endothelial cells. *Genes Cells* 2005; **10**: 1139–1151.
24. Suh YA, Arnold RS, Lassegue B, *et al*: Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 1999; **401**: 79–82.
25. Geiszt M, Kopp JB, Varnai P, Leto TL: Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U S A* 2000; **97**: 8010–8014.
26. Su B, Mitra S, Gregg H, *et al*: Redox regulation of vascular smooth muscle cell differentiation. *Circ Res* 2001; **8**: 39–46.
27. Shiose A, Kuroda J, Tsuruya K, *et al*: A novel superoxide-producing NAD(P)H oxidase in kidney. *J Biol Chem* 2001; **276**: 1417–1423.