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Suppression of the development of hypertension by the inhibitor of inducible nitric oxide synthase

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1 Our previous study demonstrated that the aortic inducible nitric oxide synthase (iNOS) expression and the plasma nitrite level in spontaneously hypertensive rats (SHR) were greater than that in age-matched Wistar-Kyoto rats (WKY). We subsequently hypothesized that the over-expression of iNOS might play an important role in the pathogenesis of hypertension in SHR.

2 In the present study, pyrrolidinedithiocarbamate (PDTC, 10 mg kg^{-1} day^{-1}, p.o., antioxidant and nuclear factor-κ B inhibitor) and aminoguanidine (15 mg kg^{-1} day^{-1}, p.o., selective inhibitor of iNOS) was used to treat SHR and WKY from age of 5 weeks through 16 weeks.

3 We found that PDTC and aminoguanidine significantly suppressed the development of hypertension and improved the diminished vascular responses to acetylcholine in SHR but not in WKY. Likewise, the increase of iNOS expression, nitrotyrosine immunostaining, nitric oxide production and superoxide anion formation in adult SHR were also significantly suppressed by chronic treatment with PDTC and aminoguanidine.

4 In conclusion, this study demonstrated that both PDTC and aminoguanidine significantly attenuated the development of hypertension in SHR. The results suggest that PDTC suppresses iNOS expression due to its anti-oxidant and/or nuclear factor-κ B inhibitory properties. However, the effect of aminoguanidine was predominantly mediated by inhibition of iNOS activity, thereby reducing peroxynitrite formation. We propose that the development of a more specific and potent inhibitor of iNOS might be beneficial in preventing pathological conditions such as the essential hypertension.

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Abbreviations: iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-kappa B; PDTC, pyrrolidine dithiocarbamate; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats

Introduction

Nitric oxide (NO), an important mediator in cardiovascular system, is synthesized by nitric oxide synthase (NOS), comprising a family of at least three NOS isoforms: neuronal (nNOS), endothelial (eNOS) and inducible NOS (iNOS) (Forstermann *et al*., 1991). In normal cardiovascular function, L-arginine is converted into NO *via* catalysis by eNOS, existing in endothelial cell (Palmer *et al*., 1988). However, in pathological cardiovascular diseases, such as septic shock, iNOS, mostly existing in smooth muscle cells, produces 1000-fold more NO than that produced by eNOS (Kuo & Schroeder, 1995). The formation of massive amount of NO *via* iNOS has potentially a cytotoxic effect, while relative small amounts of NO formed *via* eNOS has a cytoprotective action in the cardiovascular system (Loscalzo & Welch, 1995).

L-arginine induces a greater fall of blood pressure in spontaneously hypertensive rats (SHR) compared with Wistar-Kyoto rats (WKY) (Schleiffer *et al*., 1991). The NO synthesis is also remarkably increased in SHR (Singh *et al*., 1996, Wu *et al*., 1996). In a previous study, we demonstrated that the expression of iNOS in the aorta was significantly greater in SHR than in WKY (Chou *et al*., 1998, Hong *et al*., 1998). Thus, we hypothesized that the amount of NO could significantly change cardiovascular function and potentially induce cardiovascular diseases, including hypertension.

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Massive and sustained generation of NO may contribute to the oxidant-mediated endothelial barrier dysfunction (Marin & Rodriguez-Martinez, 1997). Superoxide anion may enhance iNOS expression by activating nuclear factor-kappa B (NF-κB) (Blackwell & Christman, 1997). The parallel time course of the generation of superoxide anion and iNOS indicates an efficient simultaneous reaction: $NO + O_2{}^{*-} \rightarrow ONOO^-$ (peroxynitrite) (Herce-Pagliai *et al*., 1998). Peroxynitrite is a short-lived and potently damaging oxidant that contributes significantly to pathological oxidative stress in living tissues (Beckman *et al*., 1990). Since peroxynitrite formation was previously identified through immunostaining of nitrotyrosine at the local site of infected organs (Herce-Pagliai *et al*., 1998), in the present study we used Western blotting to detect nitrotyrosine expression in the thoracic aorta as evidence of changes in peroxynitrite levels. The unique chemical reactions of peroxynitrite, such as protein nitration, DNA-single-strand breakage and guanidine nitration, are not only cytotoxic but are also mutagenic (Ducrocq *et al*., 1999). Virag *et al*. (1998) demonstrated that the cytotoxic effect of peroxynitrite is mediated by a nuclear enzyme, poly [ADP-ribose] synthase (PARS), which can be inhibited by compounds like 3-amniobenzamide. Blockade of NF-κB activation and iNOS activity could theoretically protect against the pathogenesis of hypertension. In the present study, we used SHR as a model to examine whether pyrrolidinedithiocarbamate (PDTC), an antioxidant and a NF-κB inhibitor (Schreck *et al*. 1992,

Ziegler-Heitbrock *et al*. 1993) and aminoguanidine, a selective iNOS inhibitor (Wolff & Lubeskie, 1995), can prevent hypertension development.

Methods

Animals

Four-week and sixteen-week-old aged-matched male SHR and WKY rats, whose stock originated from the Charles River Breeding Laboratories (Tokyo, Japan), were purchased from the National Laboratory Animal Breeding and Research Center of the National Science Council, Taiwan. Animals were caged individually in clear plastic cages and kept in an environmentally controlled room maintained at a room temperature of $23 \pm 1^{\circ}C$, relative humidity of $55 \pm 5\%$ and a light–dark cycle of 12 h/12 h. Aminoguanidine (15 mg kg^{-1} day^{-1}), pyrrolidine dithiocarbamate (PDTC, 10 mg kg^{-1} day^{-1}) and 3-aminobenzamide (2 mg $kg^{-1}day^{-1}$), were administered in drinking water from 5 weeks of age through 16 weeks of age.

Mean arterial blood pressure measurement

Mean arterial blood pressure was measured weekly in conscious rats by the tail-cuff method using an automatic blood pressure monitoring system (UR-5000, UETA, Tokyo, Japan). In brief, a tail cuff was used to constrict caudal artery flow and photoelectric sensors detected the tail pulses, as cuff pressure was reduced (Dilley & Nataatmadja, 1998).

Superoxide anion detection by chemiluminescence

On the day of study, rats were anaesthetized by an intraperitoneal injection of urethane (1.2 g kg^{-1}). The descending thoracic aorta was isolated and removed, taking care not to damage the endothelium, 5 mm ring segments of thoracic aorta (Hong *et al*., 1998). After dissecting the connective tissue, ring segments were incubated in Krebs-HEPES buffer solution containing (mM): NaCl 99.01, KCl 4.69, $CaCl_2$ 1.87, $MgSO_4$ 1.2, K_2HPO_4 1.03, $NaHCO_3$ 25.0, N-[2-hydroxyethyl] piperazine-N′ [2-ethanesulphonic acid] (HEPES) 20.0 and glucose 11.1; initially gassed with 95% O_2 and 5% CO_2, pH 7.4) and maintained at 37°C for 30 min. Subsequently, the ring segments were gently transferred to a polystyrene 96-well plate containing 0.25 mM lucigenin. Counts were obtained at 15 min intervals at 37°C using a luminescence measurement system (microLumate plus LB96V, EG & G Berthold, Bad Wildbad, Germany) (Ohara *et al*., 1993).

Plasma nitrate determination

After the rats were anaesthetized, 1 ml blood was withdrawn from the abdominal artery and immediately centrifuged ($3000 \times g$ for 10 min). Plasma was stored at -70 °C until use. A sample of thawed plasma was deproteined with two volumes of 4°C 99% ethanol and centrifuged ($3000 \times g$ for 10 min) (Hong *et al*., 1998). These plasma samples (100 μl) were injected into a collection chamber containing 5% VCl_3. This strong reducing environment converts both nitrate and nitrite to NO. A constant stream of helium gas carried NO into a NO analyser (Seivers 270B NOA; Seivers Instruments Inc., Boulder, CO, U.S.A.), where the NO reacted with ozone, resulting in the emission of light. Light emission is proportional to the NO formed; standard amounts of nitrate were used for calibration.

iNOS and nitrotyrosine detection by Western blotting

Rats were anaesthetized, the thoracic aortas were excised and placed in cold phosphate buffer saline (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.4) 4°C. The aortic vessels were homogenated and then centrifuged at $3000 \times g$ for 20 min at 4°C. Twenty μg of each sample was diluted in sodium dodecylsulfate (SDS)-treated buffer and heated to 95°C for 5 min. Gels were run at 200 V for 40 min and then transferred to nitrocellulose at 15 V for 16 h with the transfer buffer (1.2 g Tris[hydroxymethyl]aminomethane, 57.6 glycine, 3200 ml H_2O and 800 ml methanol). Membranes were treated with 5% non-fat-milk for 1 h then probed with mouse anti-iNOS (Transduction Laboratories, Lexington, Kentucky, U.S.A.) and rabbit anti-nitrotyrosine (Upstate Biotechnology, Saranac Lake, NY, U.S.A.) 1 μg ml^{-1} overnight at 4°C. The blot was washed three times with TTBS (0.01 M Tris, 0.15 M NaCl, 0.1% Tween 20) and then incubated for 1.5 h with secondary antibody, goat anti-mouse and anti-rabbit-horseradish peroxidase, conjugated (1 : 3000). The blot was washed three times with TTBS, then 1.5 ml mixed ECL chemiluminescence was added for 1 min. The blot was then exposed to X-ray film for 5 min (Chou *et al*., 1998).

Vascular reactivity determination

Rats were anaesthetized, the thoracic aortas were excised and placed in cold PSS (4°C). Fat and connective tissue were trimmed from the aortas. The aortas were cut into 3–4 mm rings. Some rings from each vessel were rubbed gently with a finger to remove the endothelium in order to assess the relaxation responses to L-arginine (Hong *et al*., 1998). Later, the lack of a relaxation response to acetylcholine (ACh, 1 μM) following precontraction of rings with phenylephrine (0.3 μM) was considered as evidence that the endothelium had been denuded. Care was taken to preserve the endothelium of other rings (Hong *et al*., 1998). Rings were mounted in organ baths containing 20 ml PSS bubbled with a mixture of O_2 (95%) and CO_2 (5%). The pH of the PSS solution was 7.4 and the composition was as follows (in mM): NaCl 118; KCl 4.7; $NaHCO_3$ 25; KH_2PO_4 1.2; $MgCl_2$ 1.25; $CaCl_2$ 2.5; glucose 11. Indomethacin (5.6 μM) was added to the PSS to prevent the production of prostanoids. Rings were connected to Grass ET03C force transducers (Grass Instrument Co., Duincy, MS, U.S.A.) and changes of vascular tension were recorded isometrically on a 7D Grass polygraph.

Preparations were left to equilibrate for 2 h under an optimal resting tension of 2 g. Before the commencement of experiments, rings were challenged twice with phenylephrine (0.3 μM) to ensure that the same contractile response was obtained both times. Drugs were removed from the organ bath by several washes with PSS and the tension was allowed to return to baseline. Rings were contracted with noradrenaline (NA, 1 μM); when a maximum stable contractile response was reached, ACh (10 nM to 10 μM) was added to the organ bath in a cumulative manner. In endothelium-denuded preparations, rings were challenged with ACh to ensure that the endothelium had been removed. Again, rings were contracted with NA (1 μM). When a maximum stable contractile response was reached, L-arginine (10 nM to 10 μM) was added to the bath in a cumulative manner (Hong *et al*., 1998).

Chemicals

Phynylephrine HCl, Acetylcholine chloride, L-arginine HCl, Noradrenalin bitartrate, pyrrolidinedithiocarbamate (PDTC) HCl, indomethacin, 3-aminobenzamide, HEPES, Tween 20 Tris[hydroxymethyl]aminomethane, glycine and aminoguanidine HCl were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Glucose, potassium chloride, potassium dihydrogenphosphate, sodium hydrogen carbonate and calcium chloride were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Vanadium chloride was purchased from Merck Chemical Co. (Darmstadt, Germany).

Statistical analyses

Mean values are quoted ± standard error of mean (s.e. mean), with sample size (n). A two-way ANOVA was performed in the statistical analysis of data. When group comparisons showed a significant difference, the Newman–Keul test was used. A P-value of 0.05 or less was accepted to indicate statistical significance.

Results

Effects of chronic treatment with aminoguanidine and PDTC on mean arterial blood pressure

As shown in Figure 1, the mean arterial blood pressure was not significantly different between SHR and WKY (98 ± 9 vs 95 ± 7 mmHg, $n=8$; $P<0.05$) at the age of 5 weeks. However at 11 weeks the mean arterial blood pressure of SHR was markedly increased compared with age-matched WKY (173 ± 5 vs 123 ± 4 mmHg, $n=8$; $P<0.05$), reaching a maximum differential (195 ± 5 mmHg, $n=8$) at 16 weeks. The increase of mean arterial blood pressure during the development of SHR from age 5 to 16 weeks was significantly reduced by chronic treatment with aminoguanidine (15 mg kg^{-1} day^{-1}) and PDTC (10 mg kg^{-1} day^{-1}). The same treatment had no effect in WKY.

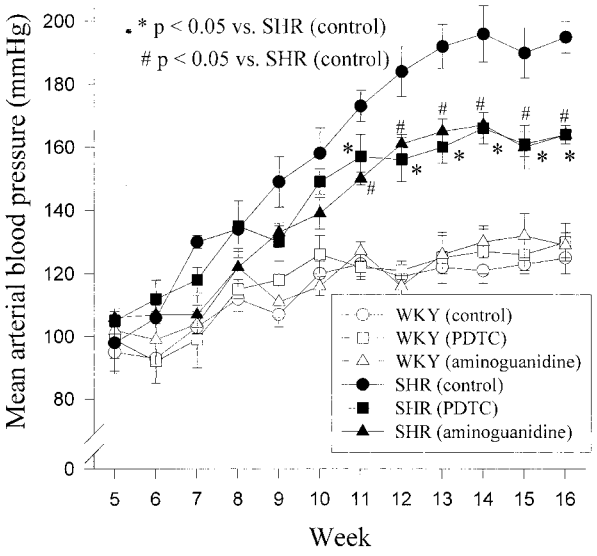

Figure 1 Effects of chronic treatment with pyrrolidine dithiocarbamate (PDTC) and aminoguanidine on mean blood pressure in conscious age-matched SHR and WKY. Each value represents mean ± s.e.mean, $n=8$. *$P<0.05$ PDTC-treated SHR vs untreated SHR. #$P<0.05$ aminoguanidine-treated SHR vs untreated SHR.

Effects of chronic treatment with aminoguanidine and PDTC on plasma nitrate and iNOS expression in aortic tissues

To investigate the possible mechanism of action of aminoguanidine and PDTC on blood pressure *in vivo*, the plasma nitrite/nitrate levels and the aortic iNOS expression were measured before and after chronic treatment with aminoguanidine and PDTC. Figure 2 shows that the plasma nitrite/nitrate levels in young SHR were not significantly different from age-matched WKY (11.75 ± 0.26 vs 10.89 ± 1.40 μM, $n=8$; $P>0.05$). However, the plasma nitrite/nitrate levels in adult SHR were significantly greater than that in age-matched WKY (21.90 ± 0.22 vs 15.54 ± 0.52 μM, $n=8$; $P<0.05$). The plasma nitrite/nitrate levels and iNOS expression were also reduced after chronic treatment with aminoguanidine and PDTC from the age of 5–16 weeks in SHR but not in WKY. These results showed that the dosage of aminoguanidine and PDTC used in this study was high enough to obtain inhibition iNOS and NF-κB (i.e. 15 mg kg^{-1} and 10 mg kg^{-1}, respectively). Thus, unless otherwise stated, these dosages of the two drugs were used throughout the present study.

As shown in Figure 3, there was little expression of iNOS in both young SHR and WKY and in old WKY. The iNOS expression in aorta of adult SHR was significantly higher than that of age-matched WKY. This increased expression of iNOS in SHR was significantly suppressed by chronic treatment with either aminoguanidine or PDTC.

Effects of chronic treatment with aminoguanidine and PDTC on superoxide anion formation in aortic tissues

It has been suggested that PDTC has a significant anti-oxidant action (Schreck *et al*., 1992). A small antioxidant effect has also been reported with aminoguanidine (Holstad *et al*., 1997; Yildiz *et al*., 1998). Thus, we have investigated the effect of aminoguanidine and PDTC on superoxide anion formation in aortic tissues from SHR and WKY. As shown in Figure 4, superoxide anion formation in aortic tissues from young SHR was not significantly different from that in age-matched WKY (114.8 ± 27.1 vs 86.0 ± 15.1 pmol 15 min^{-1} mg^{-1}, $n=8$; $P>0.05$). However, the superoxide anion level in adult SHR

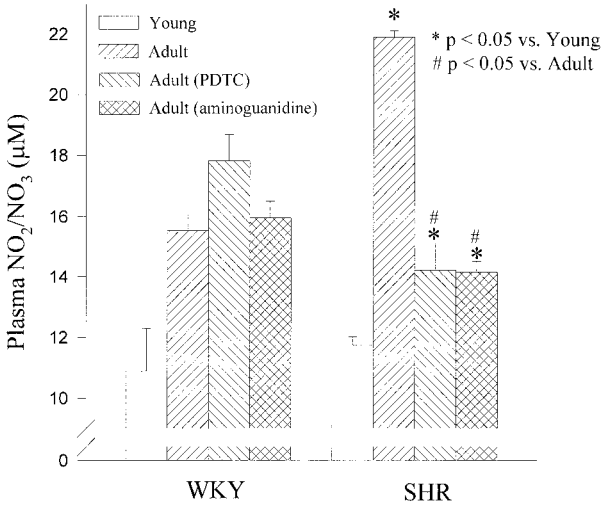

Figure 2 Effects of chronic treatment with PDTC and aminoguanidine on plasma nitrite level in age-matched SHR and WKY. Each value represents mean ± s.e.mean, $n=8$. *$P<0.05$ vs young untreated SHR. #$P<0.05$ vs adult untreated SHR.

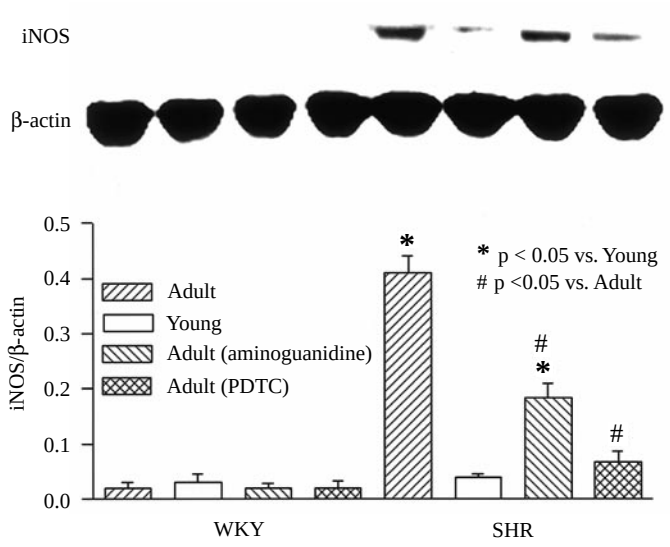

Figure 3 Inducible NOS expression in aortic tissues from age-matched SHR and WKY after chronic treatment with PDTC and aminoguanidine. Each value represents mean±s.e.mean, $n=8$. *$P<0.05$ vs young untreated SHR. #$P<0.05$ vs adult untreated SHR.

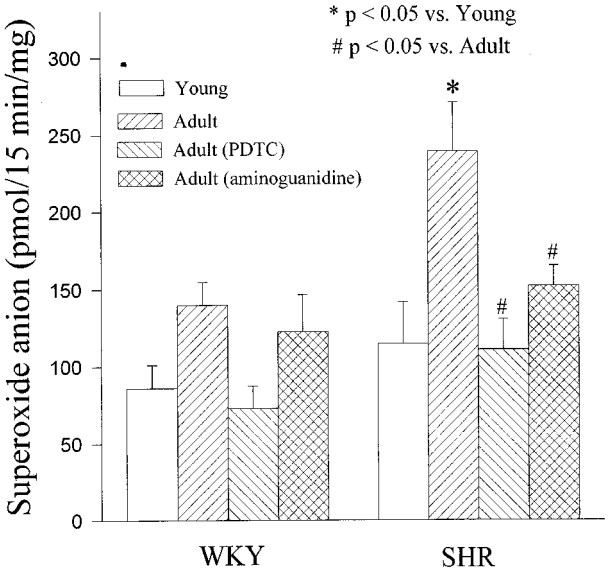

Figure 4 Effects of chronic treatment with PDTC and aminoguanidine on superoxide anion formation in age-matched SHR and WKY. Each value represents mean±s.e.mean, $n=8$. *$P<0.05$ vs young untreated SHR. #$P<0.05$ vs adult untreated SHR.

was greater than that in age-matched WKY (239.0±31.9 vs 140.0±14.8 pmol 15 min^{-1} mg^{-1}, $n=8$; $P<0.05$). The superoxide anion formation in adult SHR was significantly reduced by chronic treatment with aminoguanidine and PDTC from the age of 5 to 16 weeks (239.0±31.9 vs 152±13.1 and 111.0±19.9 pmol 15 min^{-1} mg^{-1}, $n=8$; $P<0.05$). However, the superoxide anion formation in aortic tissues from WKY was only reduced by chronic treatment with PDTC (140.0±14.8 vs 73.0±14.6 pmol 15 min^{-1} mg^{-1}, $n=8$; $P<0.05$), but was not affected by treatment of aminoguanidine (140.0±14.8 vs 122.7±23.9 pmol 15 min^{-1} mg^{-1}, $n=8$; $P>0.05$). These results suggest that superoxide anion is indeed higher in adult SHR than in age-matched WKY. Interestingly, not only PDTC, but also aminoguanidine significantly reduced the superoxide anion formation in SHR. This result indicates that aminoguanidine possesses an iNOS inhibitory effect as well as an anti-oxidant effect that might further affect iNOS expression (see Discussion for details).

Effects of chronic treatment with aminoguanidine and PDTC on vascular reactivity

As shown in Figure 5, ACh (10 nM to 10 μM)-induced relaxations were significantly reduced in intact aortic rings from adult SHR as compared with age-matched WKY ($n=8$; $P<0.05$). The change of ACh-induced relaxation was significantly reversed by chronic treatment with either aminoguanidine or PDTC. These findings suggest that the endothelium function in SHR is less efficient than that in WKY. Furthermore, aminoguanidine and PDTC improved the vascular reactivity to ACh. In contrast, as shown in Figure 6, relaxations induced by L-arginine (10 nM to 10 μM) in endothelium-denuded aortic preparations from adult SHR was significantly greater than in preparations from age-matched WKY ($n=8$; $P<0.05$). These results further confirm that iNOS expression in SHR is higher than in WKY. The alteration of L-arginine-induced relaxation was also significantly reversed by chronic treatment with either aminoguanidine or PDTC.

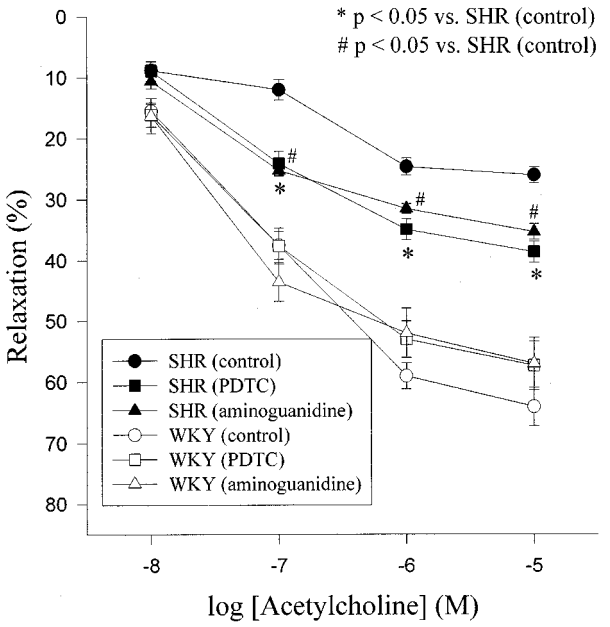

Figure 5 Acetylcholine-induced relaxation in aortic tissues from age-matched SHR and WKY after chronic treatment with PDTC and aminoguanidine. Each value represents mean±s.e.mean, $n=8$. *$P<0.05$ PDTC-treated SHR vs untreated SHR. #$P<0.05$ aminoguanidine-treated SHR vs untreated SHR.

Effects of chronic treatment with aminoguanidine and PDTC on nitrotyrosine immunostaining in aortic tissues

NO is known to react with superoxide anion to form an even more toxic material, peroxynitrite. Nitrotyrosine is a major product from spontaneous reaction of peroxynitrite with proteins, and can be used as a marker of peroxynitrite formation in various tissues. To investigate the effect of PDTC

and aminoguanidine on peroxynitrite formation, the aortic nitrotyrosine immunostaining was measured before and after treatment with PDTC and aminoguanidine. As shown in Figure 7, the nitrotyrosine immunostain in aortic tissues from young SHR was not different from that from the age-matched WKY. However, the nitrotyrosine immunostain in adult SHR was significantly greater than that in age-matched WKY. In aortic tissues from aminoguanidine and PDTC-treated adult SHR, the nitrotyrosine immunostain was almost completely abolished. This phenomenon was not observed in WKY. Furthermore, as shown in Figure 8, chronic treatment with 3-aminobenzamide (a PARS inhibitor, 2 mg kg^{-1} day^{-1}) also significantly attenuated the development of hypertension in SHR. These results imply that peroxynitrite is involved in the deterioration of cardiovascular functions.

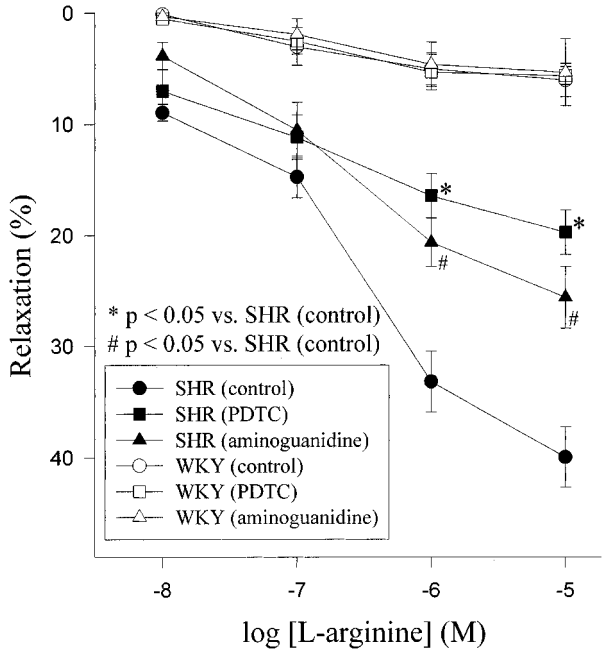

Figure 6 L-arginine-induced relaxation in aortic rings from age-matched SHR and WKY after chronic treatment with PDTC and aminoguanidine. Each value represents mean ± s.e.mean, $n = 8$. $^*P < 0.05$ PDTC-treated SHR vs untreated SHR. $\#P < 0.05$ aminoguanidine-treated SHR vs untreated SHR.

Discussion

The present study has evaluated the salutary effects of PDTC (antioxidant and NF-κB inhibitor) and aminoguanidine (iNOS inhibitor) on the development of hypertension by monitoring iNOS expression, nitrotyrosine immunostain, superoxide anion formation and plasma nitrate in aortic tissues among young SHR, adult SHR, and age-matched WKY. The *ex vivo* vascular hyporeactivity in aortic tissues among young and adult SHR and age-matched WKY were also checked. Results

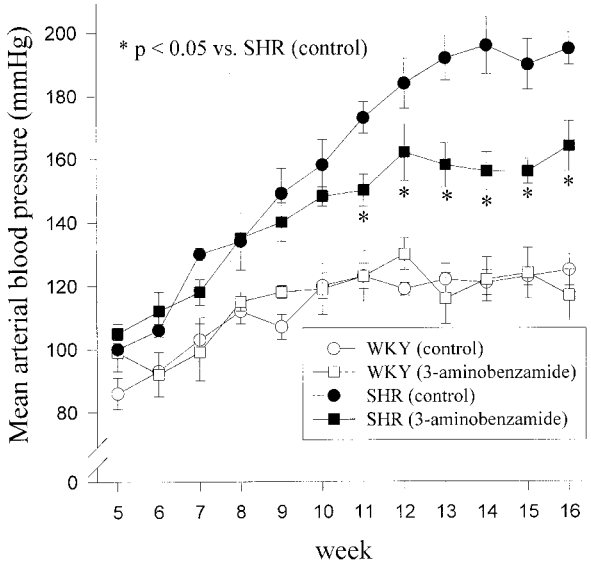

Figure 8 Effects of chronic treatment with 3-aminobenzamide on mean blood pressure in conscious age-matched SHR and WKY. Each value represents mean ± s.e.mean, $n = 8$. $^*P < 0.05$ vs untreated SHR.

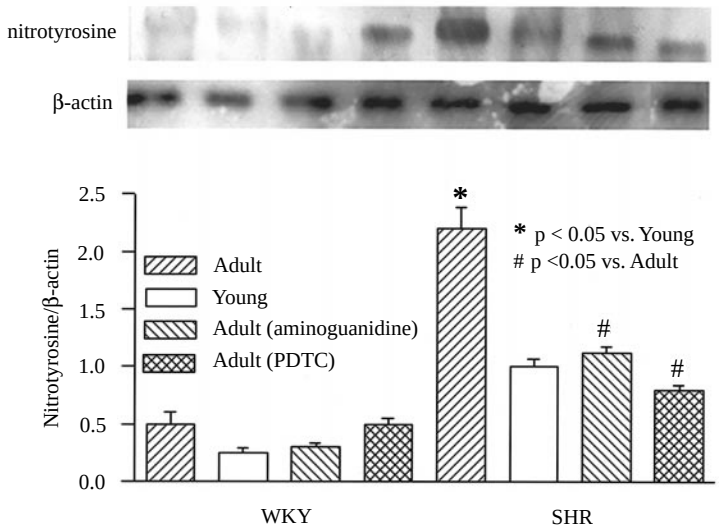

Figure 7 Nitrotyrosine immunostain in aortic tissues from age-matched SHR and WKY after chronical treatment with PDTC and aminoguanidine. Each value represents mean ± s.e.mean, $n = 8$. $^*P < 0.05$ vs young untreated SHR. $\#P < 0.05$ vs adult untreated SHR.

demonstrated that chronic treatment with aminoguanidine significantly reduced the development of hypertension and improved vascular hyporeactivity in SHR but not in WKY.

Changes of iNOS expression, superoxide anion and peroxynitrite in the development of hypertension

It has been suggested that alteration in NO metabolism is implicated in hypertension (Miyamoto *et al*., 1998). Indeed, genetic hypertension has often been found to be associated with an apparent endothelial dysfunction and impaired endothelium-dependent vasodilatation in response to increased flow and receptor-dependent agonists (Boulanger, 1999). Our present results, as shown in Figures 3 and 5, further confirm the involvement of iNOS expression and endothelial dysfunction in the hypertensive condition, i.e. in adult (16-week-old) SHR but not in young SHR (5-week-old) or age-matched WKY. These results are consistent with our previous studies (Hong *et al*., 1998; Wu *et al*., 1996). Furthermore, Liu *et al*. (1998) found that L-NAME, a non-selective NOS inhibitor, markedly elevated arterial blood pressure. However, results from the present study show that aminoguanidine, a selective iNOS inhibitor, not only does not increase blood pressure but also attenuates the development of hypertension in SHR. The reason for this discrepancy may be due to the cytotoxic action of peroxynitrite, a short-lived and reactive oxidant produced from the reaction of nitric oxide with superoxide anion (Beckman *et al*., 1990).

It has been well documented that superoxide anion plays an important role in many cardiovascular diseases, including endothelial dysfunction, atherosclerosis and hypertension (Sagar *et al*., 1992). Moreover, treatment with anti-oxidants, like vitamin C, has a protective effect in these diseases (Solzbach *et al*., 1997). Indeed, as shown in Figures 4 and 5, we also found the increase of superoxide anion formation in aortic tissues from SHR was significantly higher than that from WKY and PDTC, a well known anti-oxidant, significantly improved the endothelium lesions and dysfunction in SHR. These indicate that increase of superoxide anion formation, diminish anti-oxidant capacity and/or reduce of superoxide anion-inactivating enzymes. However, PDTC, which suppresses NF-κB activity both directly (Ziegler-Heitbrock *et al*., 1993) and indirectly *via* its anti-oxidant effect (Schreck *et al*. 1992), is a useful pharmacological tool for the analysis of NF-κB-regulated gene expression, including iNOS (Hong *et al*., 1998, Liu *et al*., 1997). Our results confirmed that PDTC inhibits iNOS expression in aortic tissues and the plasma NO level (Figures 2 and 3). Together, our results support that PDTC suppress the development of hypertension in SHR *via* inhibiting NF-κB activity (finally reducing iNOS formation) as well as its anti-oxidant activity.

Potential protective cardiovascular effect of iNOS inhibitor

To determine whether the iNOS inhibitory effect alone was important in the development of hypertension, aminoguanidine, an acknowledged iNOS inhibitor (Wolff & Lubeskie, 1995), was tested subsequently. A recent study reported that aminoguanidine has a salutary effect in septic shock and diabetes *via* inhibition of iNOS activity (Wu *et al*., 1995, Holstad *et al*., 1997). However, some reports showed that aminoguanidine has some anti-oxidant activity, which subsequently inhibits iNOS expression (Yildiz *et al*., 1998, Giardino *et al*., 1998). This was demonstrated in the present study as chronic treatment of aminoguanidine not only decreased the NO (Figure 2) but also reduced the superoxide anion formation (Figure 4) and iNOS expression (Figure 3) in aortic tissues from SHR. To compare the anti-oxidant effect of aminoguanidine and PDTC, the superoxide anion was determined from the vessels *in vitro*. Superoxide anion generated by aortic vessel rings obtained from SHR was significantly reduced by PDTC (10^{-2} M) (252 ± 31 vs 107 ± 19 pmol 15 min^{-1} mg^{-1}, $n=4$, $P<0.05$), but not by aminoguanidine (10^{-2} M) (252 ± 31 vs 228 ± 29 pmol 15 min^{-1} mg^{-1}, $n=4$, $P>0.05$); unpublished observations. These results suggested that the antioxidant effect of aminoguanidine is less potent than that of PDTC on the basis of the same condition.

To distinguish whether the anti-oxidant or the iNOS inhibitory effects of aminoguanidine play the predominant role in suppressing the development of hypertension in SHR, additional *in vitro* experiments were performed. In endothelium-denuded aortic rings from SHR, L-arginine (10^{-4} M)-induced relaxation from SHR with and without aminoguanidine (10^{-2} M) was $3 \pm 1\%$ and $26 \pm 3\%$, respectively (unpublished observations). These results further indicated that iNOS activity could be almost completely blocked by aminoguanidine at the concentration of 10^{-2} M. Moreover, in intact aortic rings from SHR, ACh-induced relaxation was significantly improved by ascorbic acid (as a positive anti-oxidant control, 10^{-4} M, from $28 \pm 3\%$ to $48 \pm 5\%$), but not by aminoguanidine (10^{-2} M, from $28 \pm 3\%$ to $33 \pm 4\%$); unpublished observations. This ascorbic acid data confirms the report of Akpaffiong & Taylor (1998). In addition, the non-significant change of aminoguanidine indicated that aminoguanidine has only a lithe antioxidant effect in the present study. These results also suggest that any contribution of *in vitro* anti-oxidant activity due to the remaining aminoguanidine in vessels treated with this drug seems to be unlikely. We therefore propose that the suppression of the development of hypertension by aminoguanidine was mainly due to inhibition of iNOS activity.

In addition, aminoguanidine also significantly inhibited nitrotyrosine immunostain in aortic tissues from adult SHR (Figure 7). These data indicate that peroxynitrite may be a pivotal endogenous mediator responsible for the cytotoxic effect on vascular function in SHR. The cytotoxic effect of peroxynitrite might offset the compensatory effect of NO on vascular reactivity during the development of hypertension in SHR. Indeed, as shown in Figures 1 and 7, aminoguanidine not only prevented progressive blood pressure increase in SHR but also suppressed nitrotyrosine immunostain in aortic tissue from SHR as compared with untreated SHR. These results suggest that the protective effect of chronic inhibition of iNOS may be mediated by decreasing peroxynitrite formation. Therefore, it is likely that a small amount of NO produced by eNOS has a beneficial effect on the cardiovascular system, but a large amount of NO produced by iNOS and encountered in pathophysiological conditions, is potentially cytotoxic. Moreover, it has been suggested that 3-aminobenzamide, a PARS inhibitor, can attenuate the damage caused by peroxynitrite (Bowes & Thiemermann, 1998). Our results also demonstrated that chronic treatment with 3-aminobezamide significantly attenuated the development of hypertension in SHR (Figure 8). This further supports that peroxynitrite is a major cytotoxic mediator in the modulation of hypertension in SHR.

In conclusion, our results suggest that: (1) overexpression of iNOS and superoxide anion formation may play an important role in the development of hypertension in SHR; (2) peroxynitrite might be a pivotal endogenous mediator responsible for the cytotoxic effect on vascular function in SHR; and (3) chronic use of aminoguanidine attenuates the

development of hypertension in SHR mainly due to the inhibition of iNOS. The underlying protective mechanism of this iNOS inhibitor might be mediated *via* a reduction in peroxynitrite formation, following inhibition of NO concentration in the SHR. Thus, the discovery of a more specific and potent inhibitor of iNOS may be therapeutically useful in pathological conditions, such as essential hypertension and atherosclerosis.

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