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RAPID REPORT

Paradoxical effects of streptozotocin-induced diabetes on endothelial dysfunction in stroke-prone spontaneously hypertensive rats

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Non-technical summary Elevated blood glucose is generally regarded as one of the risk factors that lead to coronary heart disease in patients with type 2 diabetes. However, our studies show that after inducing short-term damage, high blood glucose subsequently provides paradoxical protection for vessel function of animals with high blood pressure. Vessels can adapt to sustained high blood glucose and produce different stress proteins to counteract, to some extent, the damage brought about by hypertension. The results help us understand part of the basis for vessel adaptation in diabetes. The implication for treatment of diabetes is that if the patients have long-standing diabetes and established cardiovascular disease, the target of blood glucose lowering should be less stringent and reached gradually to avoid abrupt cancellation of the pre-existing adaptations.

Abstract Although both diabetes and hypertension are risk factors for cardiovascular disease, the role of hyperglycaemia *per se* in endothelial dysfunction is controversial. This study was designed to examine whether hyperglycaemia, or streptozotocin-induced diabetes, could aggravate endothelial dysfunction in stroke-prone spontaneously hypertensive rats (SHRSP). Hyperglycaemia was induced by streptozotocin in 2-month-old SHRSP and age-matched normotensive Wistar–Kyoto (WKY) rats. The aorta was isolated 8 weeks after induction of hyperglycaemia to record its function and to examine its morphology with transmission electron microscopy. Endothelial/inducible nitric oxide synthase (eNOS/iNOS) and inducible/constitutive haem oxygenase (HO-1/HO-2) levels were determined with Western blotting. Aortic endothelial function and production of reactive oxygen species and nitric oxide were assayed after incubation *in vitro* in hyperglycaemic, hyperosmolar solution. Streptozotocin-induced diabetes of 8 weeks duration did not result in endothelial dysfunction in normotensive WKY rats. In contrast, hyperglycaemic WKY rats showed significantly enhanced endothelium-dependent vasodilatation, which was abrogated by simultaneous blocking of NOS and HO. The enhanced vasodilatation was associated with elevation of vascular eNOS and HO-1. Significant endothelial dysfunction and massive macrophage–monocyte infiltration were found in SHRSP aorta (the ratio of the number of macrophages to endothelial cells in the intima, expressed as a percentage, was $20.9 \pm 2.8\%$ in SHRSP *versus* $1.9 \pm 0.5\%$ in WKY rats, $P < 0.01$), which was attenuated significantly in hyperglycaemic SHRSP ($11.3 \pm 1.6\%$, $P < 0.01$ *versus* SHRSP). Acute hyperglycaemia (10 min) aggravated endothelial dysfunction in SHRSP, with a marked

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increase in intracellular reactive oxygen species and NO production. Sustained *in vitro* incubation in hyperglycaemic/hyperosmolar conditions (addition of an extra 50 mmol L^{-1} of glucose or mannitol to the usual buffer, to produce a final osmolarity of 350 mosmol L^{-1}) for 5 h enhanced endothelium-dependent vasodilatation, with elevated vessel NO production and upregulation of eNOS/HO-1 proteins. Sustained hyperglycaemia does not aggravate endothelial dysfunction and macrophage infiltration in SHRSP. Hyperglycaemia/hyperosmolarity-induced upregulation of eNOS and HO-1 may play a role in this paradoxical adaptation of endothelial function.

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Abbreviations ACCORD, Action to Control Cardiovascular Risk in Diabetes; ADVANCE, Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation; eNOS, endothelial nitric oxide synthase; HO-1, inducible haem oxygenase; HO-2, constitutive haem oxygenase; iNOS, inducible nitric oxide synthase; KHB, Krebs–Henseleit buffer; Nrf2, nuclear factor-erythroid 2-related factor; ROS, reactive oxygen species; SHRSP, stroke-prone spontaneously hypertensive rats; SNP, sodium nitroprusside; STZ, streptozotocin; WKY, Wistar–Kyoto; ZnPPIX, protoporphyrin IX zinc (II).

Introduction

Both hypertension and type 2 diabetes are risk factors for cardiovascular disease. While hypertension is recognized as the single most important contributing factor to cardiovascular disease, hyperglycaemia is sometimes considered a ‘bystander’, and its role in large vessel lesions is controversial. Therapies aiming at lowering blood pressure are effective in reducing cardiovascular mortality in diabetes (Reaven, 1988; Bakris *et al.* 2000; Jackson *et al.* 2005), confirming the causative role of hypertension in cardiovascular disease. In contrast, studies on the relationship of hyperglycaemia with the severity of coronary lesions and attempts to lower blood glucose have shown considerable discrepancies, and the role of hyperglycaemia in macrovascular complications remains elusive (Waller *et al.* 1980; Stratton *et al.* 2000; Alexander *et al.* 2003; Wiernsperger, 2003; Gerstein *et al.* 2005; Ray *et al.* 2009; Molitch *et al.* 2010).

The results from large-scale clinical trials, such as the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications studies, have shown that in patients with type 1 diabetes, tight glucose control reduced the incidence of both micro- and macrovascular complications during long-term follow up. The benefit remained even after the glucose returned to the control level. Thus, prior glucose control exerted a benefit by way of ‘glucose memory’ (Nathan *et al.* 2005). The United Kingdom Prospective Diabetes Study also found that despite a loss of glycaemic differences after the study had finished, emergent risk reductions for myocardial infarction and death from any cause were observed during 10 years of post-trial follow up in patients with type 2 diabetes (Holman *et al.* 2008), conforming the long-term benefits of a ‘memory’ of intensive glucose control in macrovascular outcomes. However, some other large-scale clinical studies, such as the Action to Control Cardiovascular Risk in Diabetes (ACCORD), Action in Diabetes and Vascular Disease (ADVANCE) and Veterans Affairs Diabetes Trial, failed to show a significant benefit of intensive glucose control on overall cardiovascular disease in patients with long-term type 2 diabetes, apart from a favourable effect on microvascular complications, and the ACCORD trial indicated that an aggressive lowering of haemoglobin A_{1c} was associated with an increase risk of all-cause mortality (Action to Control Cardiovascular Risk in Diabetes Study Group *et al.* 2008; ADVANCE Collaborative Group *et al.* 2008; Duckworth *et al.* 2009).

Likewise, animal studies also present controversies over the role of hyperglycaemia in cardiovascular outcomes. Some experiments have shown that severe hyperglycaemia decreases myocardial sensitivity to ischaemic injury and/or enhances endothelium-dependent vasodilatation (Tani & Neely, 1988; Liu *et al.* 1993; Schaffer *et al.* 2000; Ravingerova *et al.* 2001; Chen *et al.* 2003*a*, 2006; Wang & Chatham, 2004; Shen *et al.* 2008; Chu *et al.* 2010); however, hyperglycaemia was also reported to exert differential effects on relaxations in various arteries. For example, incubation with high glucose concetrations for 2 h impaired bradykinin-induced relaxation in human subcutaneous vessels, but augmented acetylcholine-induced relaxation in mesenteric and subcutaneous vessels (MacKenzie1 *et al.* 2008). In addition, other risk factors, such as obesity and insulin resistance, could lower the threshold for high-glucose-induced depression of vessel function (Bohlen, 2004). It was also found that in the absence of insulin, the natural protection by *in vitro* high glucose for cardiomyocytes was unmasked (Ricci *et al.* 2008). Therefore, the effect of hyperglycaemia on vessel function is complex when the confounding factors are considered.

Hypertension, frequently encountered in type 2 diabetes as in metabolic syndrome, is the major cause of endothelial dysfunction, an early stage

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of the atherosclerotic cascade that culminates in coronary occlusion and life-threatening myocardial infarction. As type 2 diabetes and hypertension-related co-morbidities strongly predispose people to poor cardiovascular outcomes, and most of the animal studies have not included the influence of co-morbid hypertension in diabetes, we designed the present study to investigate the effects of hyperglycaemia on large vessel function in established hypertension, using a type 1 diabetes model to avoid confounding factors, such as obesity and hyperinsulinaemia. Hyperglycaemia was induced by streptozotocin in 2-month-old stroke-prone spontaneously hypertensive rats (SHRSP) and age-matched normotensive control Wistar–Kyoto (WKY) rats. Aortic endothelial function, protein expressions of endothelial and inducible forms of nitric oxide synthase (eNOS and iNOS), inducible and constitutive haem oxygenase (HO-1 and HO-2; also called heat shock protein 32, Hsp32), together with vessel morphology were examined 8 weeks after induction of hyperglycaemia. The effects of *in vitro* hyperglycaemia/hyperosmolarity on endothelial function of SHRSP, protein expressions of eNOS and hyperosmolarity-related heat shock proteins (Hsp32, Hsp90 and Hsp110) were also examined.

Methods

Animals and induction of hyperglycaemia

All experimental procedures were performed under protocols approved by the Animal Care Committee of the Animal Centre at the Chinese Academy of Sciences in Shanghai, and the experiments comply with the policies and regulations of *The Journal of Physiology* given by Drummond (2009). Two-month-old male WKY rats and SHRSP were obtained from the Shanghai Laboratory Animal Centre, Chinese Academy of Science. After blood pressure measurement and 12 h fasting with free access to water, the rats received an intraperitoneal injection of streptozotocin (STZ; 75 mg kg^{-1} in citrate buffer with an injection volume of 1 ml (kg body weight)$^{-1}$, pH 6.0). Control rats were injected with the same volume of citrate buffer. All the rats were kept for a further 8 weeks before assessment of vessel function. All animals were housed, two per cage, in a temperature-controlled room with a 12 h–12 h light–dark cycle and received water and chow *ad libitum.*

Blood pressure measurement

Blood pressure and heart rate were measured in the conscious state by the tail-cuff method using an animal blood pressure analyser (BP-98A; Softron, Beijing, China) at the beginning of the experiment and before killing.

Biochemical determinations

Blood samples were collected at the time of killing. Serum insulin was determined with a rat insulin radioimmunoassay kit (Linco Research, St Charles, MI, USA) and blood glucose with a biochemistry automatic analyser (Hitachi, Tokyo, Japan).

Assessment of vessel function *in vitro*

After induction of deep general anaesthesia by intraperitoneal injection of sodium pentobarbital (60 mg (kg body weight)$^{-1}$), the rat was killed quickly by open-chest heart excision. Then the thoracic aorta was carefully dissected, cleaned of fat and adherent connective tissues, cut into segments of 2–3 mm in length and mounted on two stainless-steel stirrups in a 10 ml organ chamber containing Krebs–Henseleit buffer (KHB) of the following composition (in mmol L^{-1}): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.0; and Na_2-EDTA, 0.5. The KHB was kept at 37°C and bubbled continuously with 95% O_2 and 5% CO_2. One stirrup was connected to an isometric force transducer for tension measurement in the organ bath (ALC-M tissue organ system; Alcott Biotech, Shanghai, China) and recorded with a data-acquisition system (MPA 2000; Alcott Biotech; Shen *et al.* 2008).

The vessel rings were stretched to a resting tension of 2.0 g and allowed to equilibrate for 40–50 min, with the buffer changed every 10–15 min. After equilibration, the rings were challenged twice with 60 mmol L^{-1} of KCl by substituting an equimolar amount of KCl for NaCl in the KHB. Endothelium-dependent vasodilatation was assessed by application of 1 μmol L^{-1} of ACh when a contractile plateau was reached after 1 μmol L^{-1} of phenylephrine. Then, dose–response contractions to cumulative phenylephrine and dilatations to cumulative ACh/sodium nitroprusside (SNP) were examined. Doses were added every 3 min.

To investigate the possible roles of NOS and HO responsible for relaxation changes, the rings were further incubated with the NOS blocker L-NAME (10 μmol L^{-1}; 30 min) and the HO blocker protoporphyrin IX zinc (II) (ZnPPIX; 2 μmol L^{-1}; 30 min). Acetylcholine-induced vasodilatation was re-examined after NOS and NOS/HO blockades.

Hyperglycaemic/hyperosmolar incubation *in vitro*

In separate experiments, aortic rings from SHRSP were incubated in hyperglycaemic/hyperosmolar solution (addition of an extra 50 mmol L^{-1} of glucose or mannitol to the usual KHB, to give a final osmolarity of 350 mosmol L^{-1}). Mannitol-supplemented KHB served as a hyperosmotic control. Acetylcholine-induced

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vasodilatation was examined after 10 min, 2 and 5 h of incubation in hyperglycaemic/hyperosmolar solution. The hyperosmotic solution was changed to the usual KHB 5 min before the addition of phenylephrine and ACh. Additional aortic rings of SHRSP without tension recording were incubated with oxygenated high-glucose or high-mannitol buffer at 37°C continuously for 5 h and then examined with transmission electron microscopy.

Measurement of reactive oxygen species and nitric oxide

After hyperglycaemic/hyperosmolar incubation for 10 min, 2 and 5 h, changes in intracellular levels of reactive oxygen species (ROS) in aorta from normal WKY rats were determined by measuring oxidative conversion of cell-permeable 2′,7′-dichlorofluorescein diacetate into fluorescent dichlorofluorescein, which was detected by fluorospectrophotometer at an excitation wavelength of 488 nm and an emission wavelength of 535 nm (Jia *et al.* 2006).

To measure intracellular NO production, the aortic rings were loaded with 5 μmol L^{-1} of the membrane-permeable fluorescent indicator 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate for 30 min at 37°C, and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm every 1 min for 30 min (Burger *et al.* 2006).

Western blotting

The upper parts of the thoracic aorta were homogenized in lysis buffer (50 mmol L^{-1} β-glycerophosphate, 100 mmol L^{-1} Na_3VO_4, 2 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} EGTA, 0.5% Triton X-100 and 1 mmol L^{-1} DTT) containing protease inhibitors (20 mmol L^{-1} pepstatin, 20 mmol L^{-1} leupeptin, 1000 U ml^{-1} aprotinin and 1 mmol L^{-1} phenylmethylsulfonyl fluoride). The soluble lysates were subjected to 10% (w/v) SDS-PAGE, and proteins were then transferred to nitrocellulose membranes and blocked with 5% (w/v) non-fat milk in Tris-buffered saline Tween 20 for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies against tubulin (1:5000 dilution; Sigma-Aldrich, St Louis, MO, USA), catalase (1:1000 dilution; Calbiochem, San Jose, CA, USA), eNOS (1:1000 dilution; Sigma, St Louis, MO, USA), iNOS (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), HO-1 (1:1000 dilution; Assay Designs Stressgen, Ann Arbor, MI, USA), and HO-2 (1:1000 dilution; Calbiochem, San Jose, CA, USA), Hsp90 (1:1000 dilution; Stressgen, Victoria, BC, Canada), or Hsp110 (1:1000 dilution; Sigma-Aldrich, St Louis, MO, USA) in 5% (w/v) milk in Tris-buffered saline Tween 20. The membranes were then incubated with corresponding horseradish peroxidase-conjugated secondary antibody and immunoreactive bands visualized using electrochemoluminescence. The relative protein levels were semi-quantified with scanning densitometry and normalized against tubulin (Shen *et al.* 2008).

Transmission electron microscopy

Segments of aortic rings that had not been used for the tension experiment were directly fixed in 2% glutaraldehyde for examination by transmission electron microscopy. Within the semi-thin transverse sections of aortic rings, random areas, including the endothelial layer, were identified and taken for transmission electron microscopy (Philips CM-120). All the endothelial cells and mononuclear–macrophage cells found under the electron microscope were counted carefully along the intima layer. The ratio of the number of macrophages to endothelial cells in the intima was expressed as a percentage. The intima thickness was measured and recorded under transmission electron microscopy, with at least 10 randomized areas from each specimen (Chen *et al.* 2011).

Statistical analyses

Data are expressed as means ± SEM and analysed with ANOVA followed by Student–Newman–Keuls *post hoc* analysis. Repeated-measures ANOVA was used for concentration–response relationships or time-dependent vasodilatation data. Significance was defined as $P < 0.05$.

Results

General data

The mean values of systolic blood pressure at the beginning of the experiments were 126 ± 1 mmHg ($n = 21$) in WKY rats and 242 ± 2 mmHg ($n = 26$) in SHRSP. All of the WKY and SHRSP injected with STZ (WKY+STZ and SHRSP+STZ) developed different degrees of hyperglycaemia within 3 days of injection. Three of 12 SHRSP and 4 of 14 SHRSP+STZ died of stroke before the vessel experiments. Three SHRSP+STZ died of hypoglycaemia within 2 days of STZ injection (STZ induced significant necrosis of β cells, which leads to short-term massive release of insulin).

As shown in Table 1, injection of STZ induced severe hyperglycaemia and a marked reduction of insulin levels in both WKY rats and SHRSP, with no significant impact on blood pressure (data of rats that of early hypoglycaemia after STZ injection or stroke before and after blood pressure measurement are not included).

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Table 1. General data of normal Wistar–Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSP) with and without streptozotocin (STZ)-induced diabetes

	WKY	WKY+STZ	SHRSP	SHRSP+STZ
n	10	9	9	7
Body weight (g)	391 ± 17	266 ± 24*	281 ± 6*	203 ± 10*†
Systolic blood pressure (mmHg)	127 ± 3	133 ± 5	285 ± 8*	280 ± 10*
Heart rate (beats min^{-1})	366 ± 8	325 ± 9*	402 ± 5*	394 ± 8*
Blood glucose (mmol L^{-1})	5.8 ± 0.2	26.2 ± 0.6*	7.4 ± 0.4*	25.5 ± 0.8*†
Serum insulin (ng ml^{-1})	1.70 ± 0.11	0.34 ± 0.06*	3.28 ± 0.42*	0.24 ± 0.10*†

*$P < 0.01$ *versus* WKY rats; †$P < 0.01$ *versus* SHRSP.

Vessel function

As shown in Fig. 1*A* and *B*, SHRSP showed significantly reduced vasodilatation in response to either ACh or SNP. In contrast, hyperglycaemic WKY rats (WKY+STZ) showed significantly enhanced vasodilatation in response to ACh. It was surprising to find that hyperglycaemic SHRSP (SHRSP+STZ) also exhibited significantly enhanced vasodilatation compared with the non-hyperglycaemic SHRSP. The aorta of SHRSP exhibited a significantly depressed contraction response to phenylephrine, which was completely reversed in hyperglycaemic SHRSP (data not shown).

The aortic dilatation in response to ACh was largely abrogated in WKY rats by NOS inhibition with L-NAME (10 μmol L^{-1}), and completely abrogated in SHRSP. After NOS inhibition in WKY+STZ and SHRSP+STZ, there was a significant remaining dilatation in response to ACh, which could be further abrogated by the HO-1 blocker ZnPPIX (2 μmol L^{-1}) in the presence of L-NAME (10 μmol L^{-1}; Fig. 1*C* and *D*).

Aortic NOS and HO protein expressions

There were significant increases in catalase, eNOS and HO-1 in aorta of hyperglycaemic WKY rats compared with non-hyperglycaemic WKY rats (Fig. 2*A*). Although aorta from SHRSP had higher expression of eNOS than normal WKY rats, its endothelium-dependent vasodilatation was reduced, indicating an uncoupling of eNOS or other factors that counteract vasodilatation. The SHRSP+STZ group showed a greater increase in aortic eNOS compared with SHRSP (Fig. 2*B*). The elevation of iNOS in WKY+STZ and SHRSP+STZ indicated increased oxidative stress in hyperglycaemic conditions, which could be a strong factor inducing vascular antioxidants.

Morphology of aortic intima

In comparison with normal WKY rats, SHRSP intima showed significant thickening of the subendothelial layer, oedema and macrophage infiltration (the ratio of the number of macrophages to endothelial cells in the intima, expressed as a percentage, was 1.9 ± 0.5% in WKY rats and 20.9 ± 2.8% in SHRSP; $P < 0.01$; Fig. 3). Surprisingly, hyperglycaemia did not add to endothelial damage, but instead reduced the thickness of the intima and macrophage infiltration in SHRSP (Fig. 3).

Effects of acute or sustained hyperglycaemia and hyperosmolarity

The endothelium-dependent vasodilatation in SHRSP was further impaired by 10 min acute stimulation with hyperglycaemia/hyperosmolarity, but was enhanced after sustained (2–5 h) incubation in hyperglycaemic/hyperosmolar conditions (Fig. 4*A*). Vascular ROS production increased markedly after 10 min hyperglycaemia and returned to the control level after sustained hyperglycaemia (Fig. 4*B*). Hyperosmolarity induced by mannitol increased NO but not ROS production. After 5 h incubation in hyperglycaemic/hyperosmolar conditions, NO production was still higher than control values (Fig. 4*B*). Protein expressions of eNOS and three kinds of hyperosmolarity-related antioxidative heat shock proteins, i.e. Hsp32 (HO-1), Hsp90 and Hsp110, were all upregulated (Fig. 4*C*).

The main morphological characteristic of SHRSP intima after 5 h *in vitro* incubation with the usual buffer was aggravated subendothelial oedema with sporadic large puffs, which were attenuated by high-glucose incubation. The postincubation values of intima thickness for the SHRSP control, SHRSP hyperglycaemia and SHRSP mannitol groups were 3.06 ± 0.21 ($n = 6$), 2.25 ± 0.11 ($P < 0.05$ *versus* SHRSP control, $n = 4$) and 2.70 ± 0.20 μm, respectively ($n = 4$).

Discussion

The main findings of the present study are as follows: (1) endothelium-dependent and -independent vasodilatation were impaired in SHRSP but remained normal in hyperglycaemic WKY rats, which was associated with elevation of eNOS and HO-1

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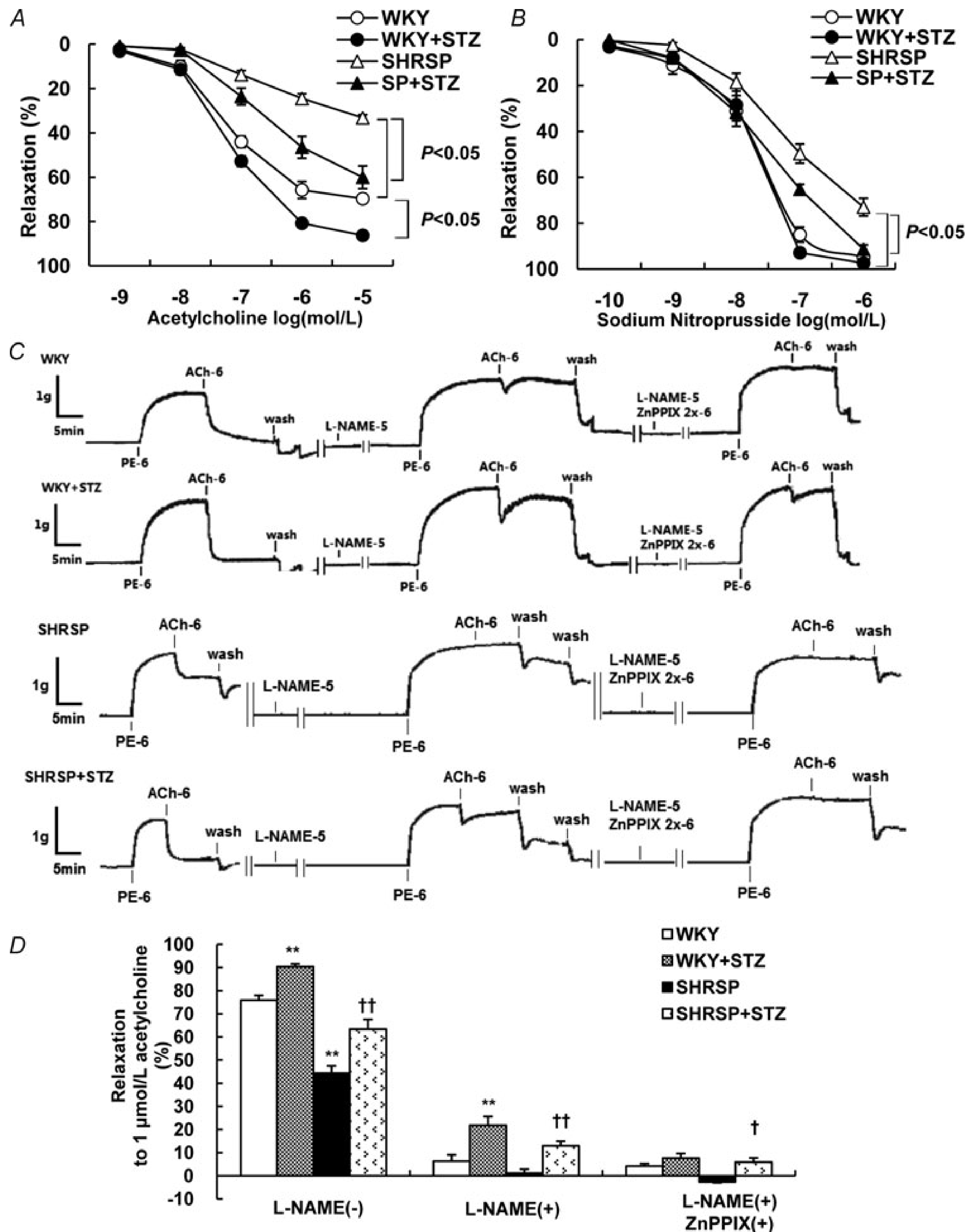

Figure 1. Chronic hyperglycaemia enhanced aortic vasodilatation
A and *B*, streptozotocin (STZ)-induced hyperglycaemia for 8 weeks enhanced endothelium-dependent and -independent vasodilatation in normotensive and hypertensive rats. *C* and *D*, the enhanced endothelium-dependent vasodilatation was abrogated after simultaneous blockade of nitric oxide synthase and haem oxygenase. Abbreviations: ACh, acetylcholine; HO, haem oxygenase; L-NAME, NOS blocker; NOS, nitric oxide synthase; PE, phenylephrine; SHRSP, stroke-prone spontaneously hypertensive rats; SHRSP+STZ, STZ-induced

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proteins; (2) hyperglycaemic SHRSP showed better endothelial function and less macrophage infiltration compared with non-hyperglycaemic SHRSP; and (3) sustained hyperglycaemia/hyperosmolarity enhanced endothelium-dependent vasodilatation and induced upregulation of eNOS and HO-1.

Although type 2 diabetes is associated with increased cardiovascular morbidity and mortality, there are also reports demonstrating that diabetic patients without metabolic syndrome do not have greater prevalence of coronary arterial disease (Alexander *et al.* 2003; Kasai *et al.* 2008). In addition, meta-analysis of major large clinical trials did not show a clear benefit of intensive glucose control on all-cause mortality, except for the reduction in cardiovascular risk (Ray *et al.* 2009). Therefore, the cause–effect relationship of hyperglycaemia and cardiovascular disease is yet to be established. As type 2 diabetes and hypertension-related co-morbidities strongly predispose people to cardiovascular injury, we checked the hypothesis that hyperglycaemia might act in combination with hypertension to worsen endothelial dysfunction, using a type 1 diabetes model to avoid confounding factors, such as obesity and hyperinsulinaemia.

It is surprising and very interesting to find that, instead of accelerating vessel damage, severe hyperglycaemia for 8 weeks significantly ameliorated endothelial function in SHRSP, with no effect on blood pressure. Morphological findings also showed that hyperglycaemia attenuated macrophage infiltration in SHRSP intima. We have previously found similar results in SHRSP with a slightly longer duration of hyperglycaemia (12 weeks; Chen *et al.* 2009). Therefore, our results support the idea that hyperglycaemia is unlikely to be the cause of endothelial dysfunction in diabetes with hypertension. On the contrary, after the initial damage to endothelium, severe hyperglycaemia may subsequently induce endogenous adaptation that attenuates endothelial dysfunction in SHRSP. Our data also support the suggestion that for patients with long-term type 2 diabetes, established cardiovascular disease and an increased baseline haemoglobin A_{1c} concentration, glucose control targets should be less stringent and be reached gradually (Ray *et al.* 2009; Mazzone, 2010), thus avoiding abrupt abrogation of the existing adaption that may be protective for the cardiovascular system.

The mechanism of enhanced vasodilatation in response to ACh was possibly related to NOS and HO, as simultaneous blockade of eNOS and HO was necessary to abrogate the vasodilatation. Haem oxygenase (also called Hsp32) is a member of the heat shock protein family that has antioxidative and vasodilator properties. When breaking down haem, HO creates carbon monoxide, an endogenous vasodilator that regulates vessel tension in a manner similar to NO (Abraham & Kappas, 2008). In addition to eNOS and HO, another protective protein, Hsp90, was also significantly upregulated, and this protein plays an important role in the balance of eNOS-generated NO and superoxide anion ($\cdot O_2^-$). Hsp90 can associate with eNOS and shifts NO and $\cdot O_2^-$ generation by eNOS from $\cdot O_2^-$ towards NO, therefore governing vasodilatation and radical generation (Pritchard *et al.* 2001). It was found that hyperglycaemia could induce eNOS expression but uncouple eNOS and promote $\cdot O_2^-$ production (Cosentino *et al.* 1997). In hyperglycaemic aorta, eNOS and Hsp90 were upregulated simultaneously, suggesting that hyperglycaemia-induced early ROS elevation may serve as a stimulus for subsequent eNOS/Hsp90 upregulation and association, thus favouring NO generation during sustained hyperglycaemia.

Bohlen and colleagues found that hyperosmolarity induced by NaCl caused a dose-dependent increase in NO production and vasodilatation, and the involvement of sodium channels, especially Na^+–Ca^{2+} exchanger, was suggested to be one of the mechanisms of the hyperosmolarity-induced NO production (Bohlen 1998; Zani & Bohlen, 2005). We also found that hyperosmotic NaCl could reduce myocardial ischaemia–reperfusion injury in SHRSP with elevated coronary flow (Chen *et al.* 2003*b*; Wang *et al.* 2006). The present results showed that hyperosmolarity induced eNOS, HO-1 and Hsp90 as hyperglycaemia did, supporting the hypothesis that hyperosmolarity may contribute partly to the hyperglycaemia-induced vasodilatation.

Zooming in on the intima layer of SHRSP, we found significant subendothelial thickening, oedema and massive macrophage infiltration. Although SHRSP had higher eNOS protein than normal WKY rats, functional examination showed reduced endothelium-dependent vasodilatation, indicating uncoupling of eNOS in SHRSP, or other factors that counteract the action of NO, such as the thickened intima being be an obstacle to NO diffusion. We have found that insulin administration to lean type 2 diabetic Goto–Kakizaki rats attenuated endothelium-dependent vasodilatation with increased intima thickness (Chen *et al.* 2011; Zhong *et al.* 2012). The higher insulin level of SHRSP could also contribute to the thickening of the aortic intima in the present study. In addition, our study of the

hyperglycaemic SHRSP; STZ, streptozotocin; WKY, Wistar–Kyoto rats; WKY+STZ, STZ-induced hyperglycaemic WKY rats; and ZnPPIX, HO blocker. $^{**}P < 0.01$ *versus* WKY rats; †$P < 0.05$, ††$P < 0.01$ *versus* SHRSP ($n = 10$–12 aortic segments per group).

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immunofluorescence co-localization of eNOS/iNOS and HO-1/HO-2 suggested that NOS and HO in SHRSP intima were mainly of macrophage origin (data not shown).

It was interesting to find that there were fewer macrophages in the aortic intima of hyperglycaemic SHRSP than non-hyperglycaemic SHRSP. The mechanisms for the hyperglycaemia-induced attenuation of hypertensive damage are not clear. As we found that severe hyperglycaemia and hyperosmolarity could exert myocardial protection with upregulation of antioxidants (Chen *et al.* 2006; Shen *et al.* 2008), we also checked the effect of *in vitro* hyperglycaemia and/or hyperosmolarity on endothelial function and endogenous antioxidants. It was found that hyperglycaemia (10 min) transiently aggravated endothelial dysfunction in SHRSP, with marked increases of both ROS and NO production. Given enough time (2–5 h), however, hyperglycaemia tended to enhance endothelium-dependent vasodilatation, with diminished ROS production and milder NO elevation, indicating rapid adaptation of the endothelium to hyperglycaemia. The hyperglycaemic/hyperosmolar conditions induced simultaneous increases of a spectrum of antioxidants, including eNOS, HO-1, Hsp90, Hsp110 and catalase, as well as pro-oxidative iNOS, which indicates that hyperglycaemia and/or hyperosmolarity can present as a redox stress for vascular tissue, which in turn acts to induce endogenous adaptation and protection.

The underlying mechanisms for antioxidant regulation in the present study are not clear. It has recently been reported that high glucose-induced advanced glycation end-products elicited increased ROS generation and nuclear factor-erythroid 2-related factor (Nrf2)-dependent HO-1 expression through activation of NAD(P)H oxidase (He *et al.* 2011). The NAD(P)H oxidases are a family of enzymes that generate ROS and are also the major sources of ROS in hypertension and diabetes, which contribute to oxidative stress in arterial walls in such conditions. However, NAD(P)H oxidase is also considered to be a double-edged sword, with transient activation providing a feedback antioxidant response to ROS via different pathways, including Nrf2 (Gao & Mann, 2009). When activated, Nrf2 leads to reduction of ROS and initiates transcription of the antioxidant HO-1, while keeping eNOS in the coupled state (Iori *et al.* 2008; Heiss *et al.* 2009). The detailed mechanisms for hyperglycaemia-mediated pro- and antioxidative

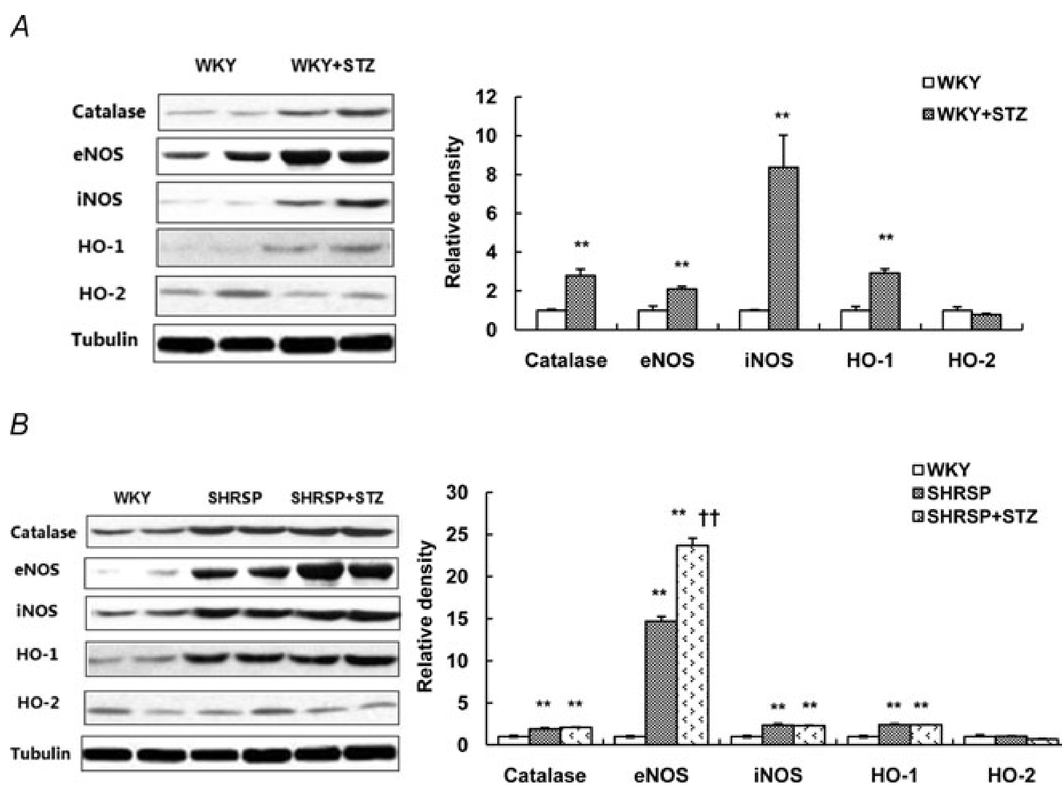

Figure 2. Chronic hyperglycaemia upregulated aortic antioxidants
Western blots show that STZ-induced hyperglycaemia of 8 weeks duration increased catalase and vasodilatation-related NOS and HO in WKY+STZ (*A*) and SHRSP+STZ (*B*). Abbreviations: eNOS, endothelial NOS; iNOS, inducible NOS; HO-1, inducible HO; and HO-2, constitutive HO. $^{**}P < 0.01$ *versus* WKY rats; $^{††}P < 0.01$ *versus* SHRSP ($n = 6$ per group).

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homeostasis in the hypertensive state require further investigation.

Our study has several potential limitations. First, the duration of diabetes was relatively short, because the high rate of stroke in SHRSP that frequently occurred after 4 months of age prevented us from longer observation of severe hyperglycaemia in this SHRSP model. Therefore, it is not clear whether the hyperglycaemia-induced adaptation would be lost when the disease progresses with time. However, our previous work in non-obese type 2

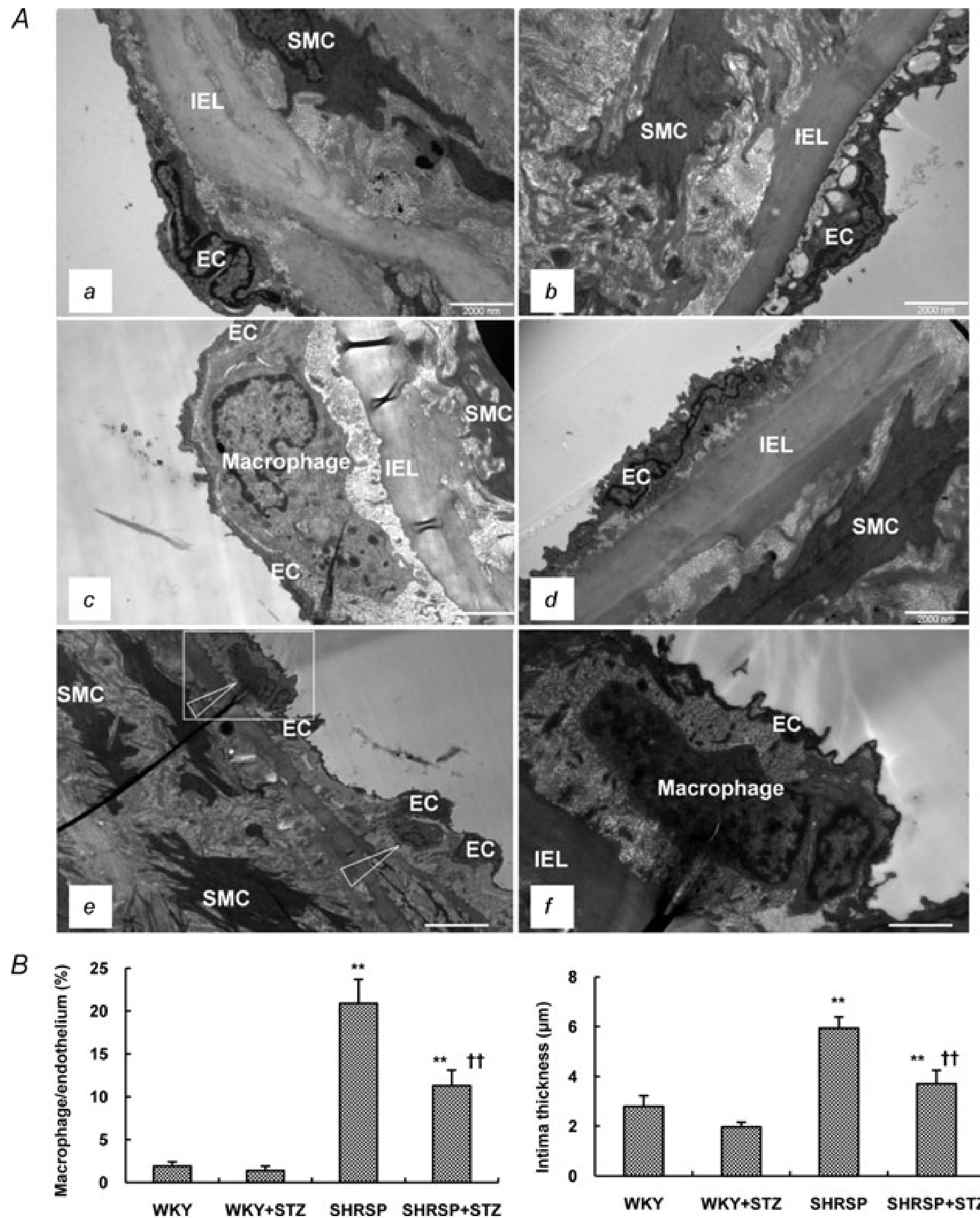

Figure 3. Morphology of the aortic intima

A, Compared with WKY rats (*a*), there were few changes in WKY+STZ (*b*). SHRSP (*c*) showed significant thickening of the intima, oedema and macrophage infiltration, which were attenuated in SHRSP+STZ (*d*). Scale bar in *Aa–d* represents 2000 nm. Two macrophages (indicated by white open arrowheads) were seen within one field in SHRSP (*Ae*; original magnification ×3400; scale bar represents 10 000 nm), with one macrophage from the boxed area shown in an enlarged view (*Af*; ×13 500). *B*, Intima thickness and the ratio of the number of macrophagesto endothelial cells in the aortic intima of SHRSP were significantly increased as compared to WKY and WKY + STZ, while STZ-induced diabetes attenuated the above changes in SHRSP. Abbreviations: EC, endothelial cell; IEL, internal elastic lamina; and SMC, smooth muscle cell. $^{**}P < 0.01$ *versus* WKY, $^{††}P < 0.01$ *versus* SHRSP ($n = 7–10$ per group).

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diabetic Goto–Kakizaki rats showed that the enhancement in endothelium-dependent vasodilatation existed at the age of 6 weeks, and persisted to 3, 6 and 12 months of age despite progressive elevation of blood glucose (Qiao *et al.* 2010; Chen *et al.* 2011). In contrast, the adaptive phenomenon was lost when hyperglycaemia was brought under control with insulin administration, showing attenuated endothelium-dependent vasodilatation and hyperplasia of the intima (Chen *et al.* 2011). Nonetheless, further studies with a longer duration of hyperglycaemia in another kind of hypertensive rats are warranted, and glucose control with different agents should provide valuable information for understanding the mechanisms of long-term cardiovascular adaptation to diabetes.

Second, our results should be interpreted with caution because they are inconsistent with many published results of animal experiments and epidemiological evidence. We tried to ensure the reliability of our findings by comparing results from different durations of diabetic animals and different arteries (aorta and carotid artery), checking the vessel function in spontaneous diabetes as well as STZ-induced diabetes. Carotid artery from SHRSP+STZ also showed ameliorated vessel function and intima morphology compared with SHRSP (data not shown). The lean spontaneously diabetic Goto–Kakizaki rats with severe hyperglycaemia and normal insulin levels demonstrated similar enhanced vasodilatation of both aorta and carotid artery, while the obese spontaneously diabetic Otsuka Long–Evans Tokushima

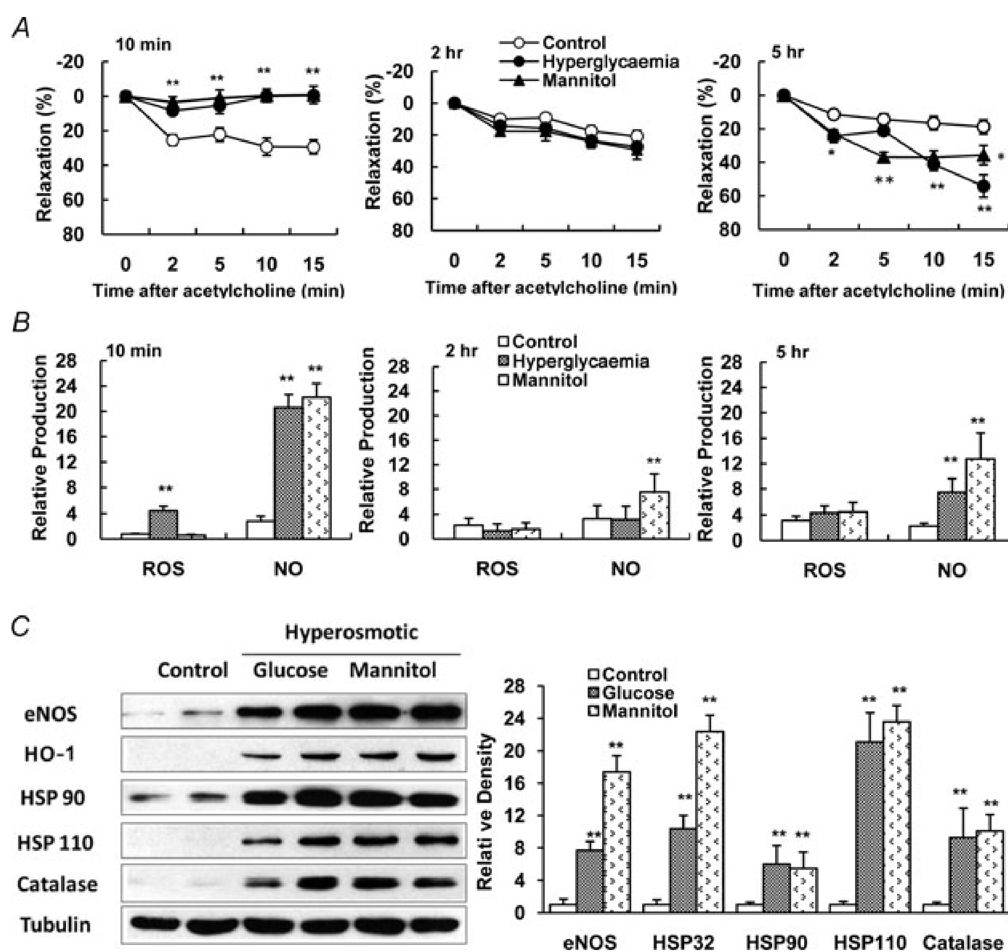

Figure 4. Effects of hyperglycaemia on vessel function and hyperosmolarity-related proteins
A, hyperglycaemic and hyperosmolar incubation *in vitro* for 10 min attenuated, but sustained hyperglycaemia for 5 h enhanced, acetylcholine-induced vasodilatation of SHRSP aorta. *B*, time-dependent changes in aortic intracellular reactive oxygen species (ROS) and nitric oxide (NO) production after stimulation with hyperglycaemia/hyperosmolarity. Vascular ROS production increased markedly after 10 min hyperglycaemia but returned to control level after sustained hyperglycaemic incubation. Hyperosmolarity induced by mannitol increased NO production but not ROS production. *C*, hyperglycaemia/hyperosmolarity for 5 h significantly increased protein expressions of aortic eNOS, catalase and hyperosmolarity-related heat shock proteins (Hsp32, Hsp90 and Hsp110). Abbreviation: HO-1, inducible haem oxygenase, also called Hsp32. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* respective control preparation ($n = 6$–10 segments per group).

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fatty rats showed significant early hyperinsulinaemia and endothelial dysfunction (Matsumoto *et al.* 2007; Chen *et al.* 2011; Zhong *et al.* 2012). Therefore, the evidence from animals with either type 1 or type 2 diabetes, with or without hypertension, supports a more important role of hyperinsulinaemia and hypertension in diabetes-related angiopathy.

In summary, although both hypertension and type 2 diabetes are well-established risk factors for cardiovascular disease, our findings suggest that in the SHRSP model of hypertension it is the high blood pressure that is responsible for endothelial dysfunction and infiltration of macrophages into the intima. Hyperglycaemia induced by STZ did not aggravate endothelial dysfunction in hypertensive rats. Endothelium may adapt to sustained hyperglycaemia which attenuated endothelial dysfunction in SHRSP aorta. The hyperglycaemia/hyperosmolarity-induced upregulation of the NOS/HO systems and other antioxidants may be one of the mechanisms for this paradoxical protection.

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Author contributions

H.C. and W.-L.S. designed, performed and co-ordinated the experiments, as well as preparing the manuscript. M.-F.Z., J.W. and J.H. carried out the functional experiments. J.Y. participated in the morphological examination. P.-P.W., Y.W. and L.Z. participated in revising the manuscript for important intellectual content. W.-J.Y. and H.H. contributed to the design and interpretation of the data. All authors have read and approved the final version. The experiments were done at the cardiovascular laboratory in the pharmacology department and the electron microscope laboratory at Shanghai Jiaotong University School of Medicine.

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