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Research Paper

NaHS ameliorates diabetic vascular injury by correcting depressed connexin 43 and 40 in the vasculature in streptozotocin-injected rats

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Abstract

Objectives Cardiovascular complication contributes an important role to morbidity and mortality in patients with diabetes. We hypothesized that these abnormalities are mainly mediated by oxidative stress, endothelial dysfunction and impaired intracellular communications. Thus, we examined vasoactivity and expression of connexin (Cx) 43 and 40, protein kinase C-ε (PKCε) and NADPH oxidase of the vasculature of thoracic aorta in streptozotocin (STZ)-injected rats, and whether NaHS could reverse these abnormalities compared with aminoguanidine.

Methods Male Sprague–Dawley rats were administered with STZ (60 mg/kg, i.p.) to induce diabetes. Diabetic rats were divided into untreated and treated groups in the 5th–8th week and intervention with either NaHS (5 mg/kg daily, s.c.) or aminoguanidine (100 mg/kg daily, p.o.) was made.

Key findings In rats with untreated diabetes, hyperglycaemia, increased activity of inducible nitric oxide (NO) synthase, increased NO, mild vascular spasm, reduced NO bioavailability and diminished vasorelaxation were found. These findings were accompanied by downregulated Cx43 and Cx40, and upregulated PKCε and NADPH oxidase subunits $p22^{phox}$/$p47^{phox}$/$p67^{phox}$ in the thoracic aorta. NaHS appears to be as effective as aminoguanidine in attenuating these abnormalities.

Conclusions NaHS shows promise in relieving diabetic vascular abnormality by upregulating junctional connexin Cx40 and Cx43, via normalizing NADPH oxidase and PKCε in the vasculature.

Keywords connexin; diabetes; NADPH oxidase; NaHS; PKCε

Introduction

An increasing incidence of diabetes mellitus raises considerable concerns in modern society. The majority of mortality in patients with diabetes mellitus is contributed to by cardiovascular complications, which mainly stem from endothelial dysfunction and oxidative stress.[1,2] Excess production of reactive oxygen species (ROS), including superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$), peroxide (H_2O_2) and peroxynitrite ($ONOO^{\bullet -}$), substantially contributes to deterioration of cardiovascular disease.[3] Subunits of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase ($p22^{phox}$ / $p47^{phox}$ / $p67^{phox}$), as a main source of ROS genesis, are stimulated by advanced glycation end products (AGEs) in diabetes. Indeed, ROS play an important role in intracellular signalling pathways in diabetes mellitus[4] in which an involvement of protein kinase C-ε (PKCε) has been found in ROS-induced insults to cardiomyocytes.[5] Evidence indicates that suppression of upregulated NADPH oxidase and PKCε hyperphosphorylation may provide a relief to vascular abnormality.[6]

Endothelial dysfunction characteristically can range from impaired vasodilatation to mild vascular spasm, involving pro-aggregation, procoagulation and vascular remodelling.[7] The pathogenesis of vascular complications is caused by increased AGEs[8] and ROS, in which nitric oxide (NO) bioavailability and endothelium-dependent vascular relaxation are impaired in diabetes mellitus, in association with activated endothelin type A (ET_A) receptors.[9,10]

Nitric oxide released from endothelial cells by endothelial NO synthase (eNOS) is a key signalling molecule in the regulation of vascular tone and permeability under physiological and pathological conditions,[11] and the function of physiological NO at the vasculature can

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be easily evaluated by bioavailable NO and vascular relaxation. Reduced NO bioavailability caused by oxidative stress may inversely result in potentiated vasoconstrictive substance.[12] An increase in $ONOO^{\bullet -}$ sourced from activated inducible nitric oxide synthase (iNOS) activity plays a major role in the tissue injury by ROS,[13,14] in which an increased activity of NADPH oxidases and iNOS account for the progression of diabetic vasculopathy.

Gap junction intercellular communication (GJIC) or cell–cell coupling in the vasculature is critical in the maintenance of vascular homoeostasis through intercellular channels that connect the cytoplasm of adjoining cells allowing the passage of small molecules between adjacent cells.[15] Gap junction channels possess two hexameric structures consisting of six connexin proteins.[16] It has been reported that hyperglycaemia impairs gap junction activity by altering connexin expression due to subcellular lesions caused by oxidative stress and abnormal PKC phosphorylation.[17–19] Connexin 43 and 40 (Cx43 and Cx40) in the absence of phosphorylation are essential for keeping GJIC in good order, which is necessary for keeping normal vascular function. Downregulated Cx43 and Cx40 caused by PKC hyperphosphorylation is responsible for impaired intracellular connection and abnormal cardiovascular activity.[20]

H_2S, an interesting gaseous signal, appears to be a vasodilator and interacts with ROS to exert an antioxidative activity in diabetic rats.[21,22] NaHS is a type of H_2S donor and can be converted rapidly to endogenous H_2S. Thus, NaHS may reverse vascular injury by attenuating oxidative stress in the presence of hyperglycaemia. Aminoguanidine is a special inhibitor of both iNOS and AGEs,[23,24] and exhibits potent antioxidation and anti-glycosylation properties to reverse detrimental processes in the cardiac and vascular tissue in diabetic rats.[25,26]

Hereby, we hypothesized that abnormal vascular activity in hyperglycaemia is likely to be related to downregulation of Cx43 and Cx40, which is related to upregulation of NADPH oxidase and PKCε in the vasculature. NaHS may ameliorate vascular activity by affecting the above abnormalities due to its antioxidant activity. We intended to explore whether NaHS, without a hyperglycaemia-lowering activity, could ameliorate vascular activity and normalize abnormal expression of Cx43 and Cx40, secondary to suppressing NADPH oxidase and phosphorylation of PKCε. The efficacy of NaHS in attenuating diabetic vascular alterations was compared with that of aminoguanidine in streptozotocin-injected rats.

Materials and Methods

Drugs and reagents

N^G-Nitro-L-arginine (L-NNA), phenylephrine, acetylcholine, noradrenaline (norepinephrine), NaHS and aminoguanidine were purchased from Sigma-Aldrich Co. (St Louis, USA). Krebs-Henseleit solution (K-H) was freshly prepared containing (mM): NaCl 119, KCl 4.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10. All solutions were freshly prepared with distilled water. Kits for biochemical detection, content of NO and activity of iNOS, were provided by Jiancheng Bio-engineering Company (Nanjing, China).

The reverse transcription–polymerase chain reaction (RT-PCR) reagents (oligo d (T)18, dNTP, Trizol, RNase inhibitor, avian myeloblastosis virus (AMV) reverse transcriptase, Taq DNA polymerase) were obtained from Promega (Madison, USA).

Animals

Male Sprague–Dawley rats, 70 in total, 220–270 g, were obtained from the Animal Center of Qinglongshan in Nanjing. All procedures were approved by the University Ethic Committee in accordance with the Guidelines for the Care and Use of Laboratory Animals in Jiangsu Province, China. Rats were kept in temperature-controlled (20–25°C) room with natural lighting and free access to water.

Experimental procedure

Rats were administered with streptozotocin (STZ; Lot B56981; Sigma) as a single intraperitoneal dose of 60 mg/kg in citrate buffer. Normal rats received an injection of the buffer only. Body weight and blood glucose levels were monitored weekly. Rats with a blood glucose level of > 16.7 mM on day 7 were recognized as being diabetic. On day 28 rats were randomly divided into five groups as follows and drug interventions lasted for another four weeks: normal group, STZ untreated group, STZ + NaHS (NaHS 5 mg/kg daily, s.c.), and STZ + AMG (aminoguanidine 100 mg/kg daily, p.o.), respectively. After treatment for four weeks rats were sacrificed and serum was collected and separated by centrifugation at 1500*g* and stored at −20°C before use. Vascular tissue of the thoracic aorta was harvested for vascular activity and the tissue intended for RT-PCR was kept in liquid nitrogen before use.

Vascular activity

On day 57, rats were exsanguinated under urethane anaesthesia and the chest was opened to dissect the thoracic aorta. Vascular activity was tested according to the previous practice.[6] Briefly, after peeling off connective tissue, the aorta was cut into rings, 2–3 mm in length, and each ring was mounted in a 3-ml organ bath filled with oxygenated K-H solution at 37°C with a preload tension of 1.0 g. After 60 min equilibration, pre-contraction by noradrenaline (10^{-6} M) was carried out twice, essential for stability of vascular activity. Thereafter, the cumulative concentration–constriction curve to phenylephrine (10^{-9} to 10^{-5} M) was established and vascular tension was compared among the five groups. Vascular relaxation of the thoracic aortic rings to acetylcholine (10^{-9} to 10^{-5} M) was conducted while the rings were pre-contracted with phenylephrine (10^{-7} M) so as to be at the plateau of vasoconstriction; acetylcholine was added to induce cumulative concentration–vasodilatation curves, and the reduced vascular tone was compared among groups.

NO bioavailability of the thoracic aorta was assessed by measuring the difference between the AUC (area under the vascular constrictive curve) of the phenylephrine-induced cumulative concentration–constriction curves in the absence and presence of L-NNA at 10^{-5} M, which was incubated for 20 min.

Table 1 Effect of NaHS and aminoguanidine in diabetic rats

	Normal	STZ	STZ + NaHS	STZ + aminoguanidine
Blood				
Blood glucose (mmol/l)	5.71 ± 1.10	25.48 ± 4.28**	22.30 ± 2.75	21.74 ± 4.47
NO (μmol/g protein)	34.77 ± 6.27	135.95 ± 19.04**	48.67 ± 11.66$^{\#\#}$	63.94 ± 11.12$^{\#\#}$
Activity of iNOS (U/ml)	0.63 ± 0.11	1.62 ± 0.39**	1.01 ± 0.20$^{\#\#}$	0.83 ± 0.15$^{\#\#}$
Vasculature				
NO bioavailability	1.48 ± 0.36	1.04 ± 0.22*	1.41 ± 0.28$^{\#}$	1.41 ± 0.18$^{\#}$
NADPH oxidase p47phox/β-actin	0.56 ± 0.07	0.85 ± 0.10**	0.66 ± 0.06$^{\#\#}$	0.69 ± 0.1$^{\#\#}$
NADPH oxidase p67phox/β-actin	0.38 ± 0.08	0.73 ± 0.09**	0.53 ± 0.07$^{\#\#}$	0.60 ± 0.07$^{\#\#}$

Abnormal blood glucose, the content of NO and activity of induced nitric oxide (iNOS) in serum, and NO bioavailability and mRNA expression of NAPDH oxidase $p47^{phox}$ and $p67^{phox}$ in the vasculature were determined in streptozotocin (STZ)-injected rats. These changes were attenuated significantly by NaHS and aminoguanidine, except the hyperglycaemia. Data are the means ± SD, $n = 6$. $^{**}P < 0.01$ vs normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs STZ group.

Biochemical assays

Serum samples were measured for glucose, NO and activity of iNOS. The measurements were conducted by using commercial kits (Nanjing Jiancheng biomedical engineering Co. Ltd, Nanjing, China).[10,27]

RT-PCR

Vascular tissue samples were processed following previous practice.[6] Briefly, the tissue was homogenized in Trizol (1 mL/100 mg tissue), containing 200 μl chloroform and then centrifuged at 12 000*g* at 4°C for 15 min. A sample of supernatant was added to cold 75% ethanol, centrifuged, dried and RNA was extracted. The RT-PCR of PKCε, NADPH oxidase $p22^{phox}$/$p47^{phox}$/$p67^{phox}$, Cx43 and Cx40 were conducted and the probes were used as below: PKCε: sense: 5′-CGAGGACGACTTGTTTGAATCC-3′ and antisense: 5′-CAGTTTCTCAGGGCATCAGGTC-3′; $p22^{phox}$: sense: 5′-GATCGAGTGGGCCATGT-3′ and antisense: 5′-TGCTTGATGGTGCCTCC-3′; $p67^{phox}$: sense: 5′-GAAAGCATGAAGGATGCCTGG-3′ and antisense: 5′-TGGACGGAAAGTAGCCTG-3′; $p67^{phox}$: sense: 5′-GAAAGCATGAAGGATGCCTGG-3′ and antisense: 5′-ATAGCACCAAGATCACATCTCC-3′; Cx43: sense: 5′-CTGGCTGCGAAAACGTCTGCTATG-3′ and antisense: 5′-CCACGGGAACGAAAATGAACACC-3′; Cx40: sense: 5′-TTCCCCATCTCCCACATTCGTTAC-3′ and antisense: 5′-TTCCGGGAGCCCAT GTTATTACTG-3′; β-actin: sense: 5′-GCCTCAAGATCATCAGCAAT-3′ and antisense: 5′-AGGTCCACCACTGACACGTT-3′. Following several steps, the amplified products were measured by imaging analysis. All the data were normalized to the expression of β-actin that served as the internal control.

Statistical analysis

Results were expressed as mean ± SD. Analysis of variance was used and the difference between two groups was calculated by the Student–Newman–Keuls test for the statistical significance and a level of $P < 0.05$ was considered as significant.

Results

Blood glucose, inducible nitric oxide synthase and nitric oxide

Following STZ injection in rats, the blood glucose escalated dramatically to reach a sustained level of 25 mM, which remained unchanged in the two intervention groups.

The activity of iNOS in serum was dramatically increased in STZ-injected rats ($P < 0.01$) relative to normal and, thus, the resultant serum NO was significantly increased. However, without suppressing hyperglycaemia, the respective NO content was significantly decreased following intervention with NaHS and aminoguanidine, and no significant difference could be found between them (Table 1).

Vascular activity

Hyperglycaemia harmed the vascular activity significantly if no intervention was conducted. Hyperglycaemia-induced vascular damage was evident as a mild spasm, which contributed to an increase in vascular tone to phenylephrine in the STZ-treated rats ($P < 0.01$), relative to the normal. Treatment with NaHS and aminoguanidine ameliorated the increment in vascular tension significantly ($P < 0.01$) compared with STZ alone (Figure 1a).

The maximal relaxant response to acetylcholine was significantly reduced in STZ rats, relative to normal group ($P < 0.01$), indicating that hyperglycaemia remarkably impaired endothelin-dependent relaxation, reflecting a reduced NO released from endothelium in the response to the agonist acetylcholine. NaHS was excellent in providing recovery of vasodilatation, as was the effect of aminoguanidine (Figure 1b).

Nitric oxide bioavailability

We intended to explore the basic release of NO in the resting state, which is likely to be changed in diabetes. An investigation of bioavailable NO was conducted indirectly by estimating the changes in the vasoconstrictive activity in STZ-injected rats compared with normal. After adding L-NNA, an inhibitor for eNOS activity, the reduction in NO bioavailability in STZ-injected rats was significant,

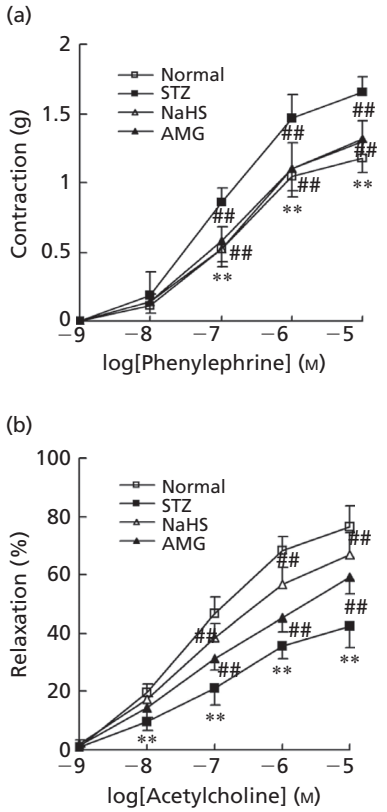

Figure 1 The cumulative concentration–response curves of rat thoracic aortic rings. (a) Vasoconstriction to phenylephrine and (b) vasorelaxation to acetylcholine are shown in normal, diabetic (streptozotocin; STZ), diabetic treated with NaHS and diabetic treated with aminoguanidine (AMG) rats. Data are the means ± SD, $n = 6$. $^{**}P < 0.01$ vs normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs STZ group

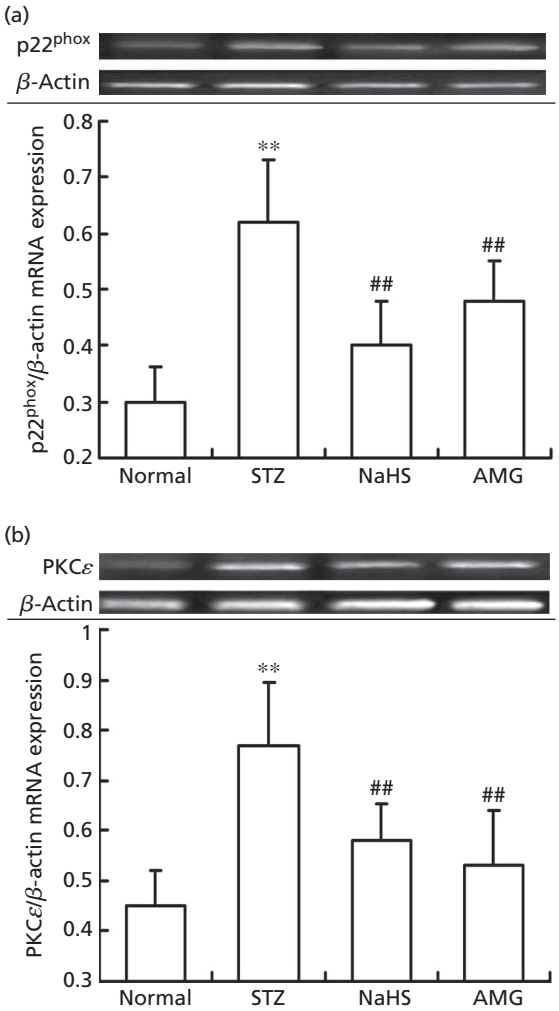

Figure 2 Downregulated expression of $p22^{phox}$ and upregulation of PKCε Cx43 and Cx40 were found in the vasculature of thoracic aorta from diabetic rats and were relieved by NaHS and aminoguanidine. Expression of (a) $p22^{phox}$ and (b) PKCε is shown in thoracic aorta vasculature from normal, diabetic (streptozotocin; STZ), diabetic treated with NaHS and diabetic treated with aminoguanidine (AMG) rats. Data are the means ± SD, $n = 6$. $^{**}P < 0.01$ vs normal group; $^{\#\#}P < 0.01$ vs STZ group

manifesting as an increase in the vascular tone in the presence of phenylephrine, relative to normal. An amelioration of the reduced NO bioavailability was achieved by NaHS and aminoguanidine (Table 1).

NADPH oxidase subunits and protein kinase C-ε

For the investigation into the factors accounting for the abnormal vascular activity, a portion of the vasculature mass was used for mRNA expression of bioactive molecules. Interestingly, upregulation of NADPH oxidase subunits ($p22^{phox}$/$p47^{phox}$/$p67^{phox}$) and PKCε was significant in the STZ-treated rats, compared with the normal group ($P < 0.01$) (Table 1, and Figure 2a, b). This indicated that a state of oxidative stress existed in the vascular wall, contributing a major role to endothelial dysfunction resulting in an exaggerated constriction and compromised relaxation. These changes were associated with an activated PKCε pathway in the vasculature. As expected, following interventions with NaHS and aminoguanidine, these changes were blunted significantly compared with the untreated group.

Connexin 43 and 40

Compared with the normal group, mRNA expression of gap junction protein Cx43 and Cx40 in the vasculature was downregulated markedly in diabetic rats ($P < 0.01$) suggesting an impairment of the signal passing route through intercellular connection (Figure 3a, b). NaHS and aminoguanidine significantly reversed the abnormality and the effect of NaHS was superior to that of aminoguanidine (Figure 3a). The intracellular communication damaged by hyperglycaemia was reconstituted following drug intervention, resulting in a recovery of vascular activity albeit without affecting the sustained hyperglycaemia in STZ-induced diabetic rats.

Discussion

Abnormal vascular activity in STZ-injected rats is associated with activated NADPH oxidase in the vasculature[6] and drug interventions suppress NADPH oxidase, leading to

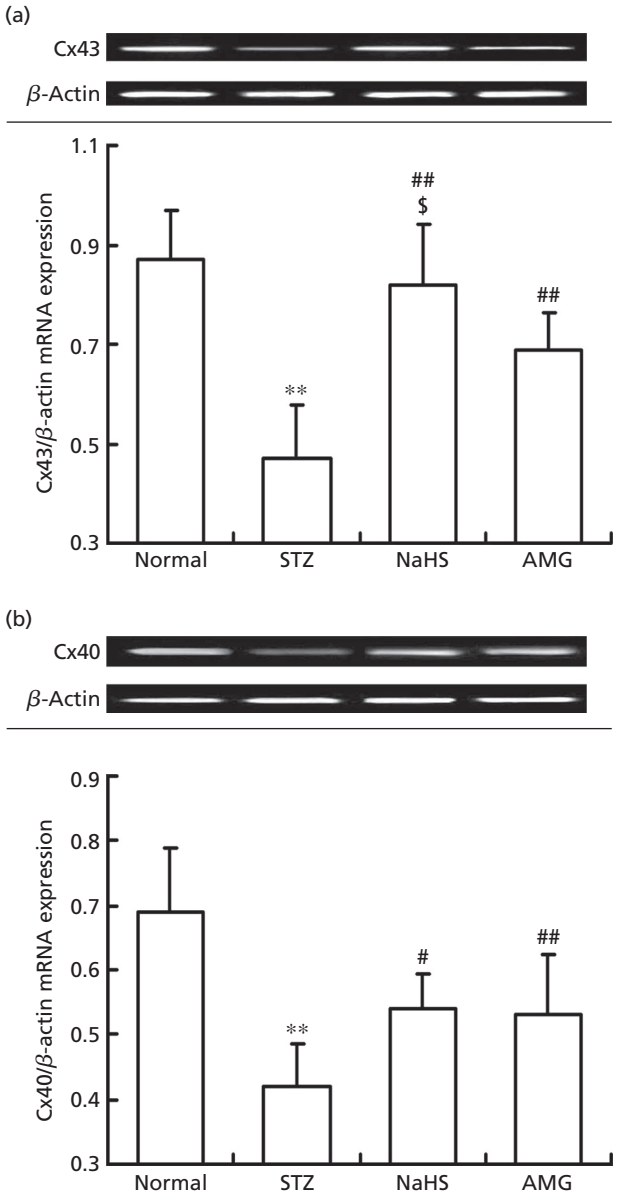

Figure 3 Downregulated expression of Cx43 and Cx40 was found in the vasculature of the thoracic aorta from diabetic rats and was relieved by NaHS and aminoguanidine. Expression of (a) Cx43 and (b) Cx40 is shown in thoracic aorta from normal, diabetic (strptozotocin; STZ), diabetic treated with NaHS and diabetic treated with aminoguanidine (AMG) rats. Data are the means ± SD, $n = 6$. $^{**}P < 0.01$ vs normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs STZ group; $^{\$}P < 0.05$ vs AMG group

amelioration of the vascular abnormality without changing hyperglycaemia significantly. In this study, we demonstrated that a deficiency of intracellular gap junction proteins Cx40 and Cx43 was crucial in mediating diabetic vascular abnormality, which manifested as a mild spasm due to a reduction in NO bioavailability and less vascular relaxation to acetylcholine. Regarding the related pathway, an activated iNOS and PKCε phosphorylation are involved in the vasculature.

Hydrogen sulfide (H_2S) is increasingly recognized as an important gaseous molecule mediating physiological and pathological signals in the cardiovascular and nervous systems.[28] As a strong reductive compound, H_2S reacts effectively with superoxide anion, hydrogen peroxide, peroxynitrite and hypochlorite, and it has been interestingly found that an excess of H_2S in diabetes evidently plays a part in self-protecting mechanisms.[18,29] The small gaseous molecular entity brings about protection of the vasculature from ROS-mediated damage.[30]

A high glucose level exacerbates oxidative stress by producing ROS and AGEs, which are important molecules underlying mechanisms in the pathology of micro- and macrovascular disease.[14] In this study, three subunits of NADPH oxidase were dramatically upregulated in mRNA expression, in keeping with previous findings.[6,31] Abnormal vascular activity directly stems from an activation of NADPH oxidase and an increase of ROS,[32] thus ROS are central to the signal transduction cascade contributing to activated PKC,[33] accounting for mechanisms underlying hyperglycaemia-mediated cellular dysfunction. Given emerging data, an increase in vascular superoxide production is associated with endothelial dysfunction in human vascular disease and has been taken as a key target for therapeutic interventions.[11,12]

NO release in the resting state can be assessed by measuring the bioavailable NO and the maximum NO releasing ability of the endothelium that can be evaluated by using an agonist, such as acetylcholine, is also indirectly quantified by vascular dilatation. The changes caused by hyperglycaemia were reversed by NaHS and aminoguanidine, despite the hyperglycaemia remaining changed. In the pathology of diabetes an induction of iNOS was evident to reinforce an increment in oxidants, which causes insults to the myocardium and vascular endothelium by forming more toxic $ONOO^{\bullet -}$. The findings in this study confirm the antioxidant activity arising from genesis of H_2S, which reacts profoundly with ROS or nitrogen species, limiting their toxic effects on vascular tone.[29,34] Thus, the vascular endothelium can be protected by NaHS as evaluated by NO bioavailability and an improved vasodilative response to acetylcholine, and the beneficial effect by aminoguanidine was also noted in line with findings in the previous report.[10]

GJIC actively participates in cell proliferation, cell differentiation and apoptosis, a process known to occur in cultured endothelial cells. The endothelial integrity is maintained by adhesive interactions at cell–cell and cell–matrix contacts via junction proteins and focal adhesion complexes.[18] Inhibition of GJIC activity induced by high glucose concentrations impairs diffusible transport of small molecules, such as calcium ions, necessary for cell proliferation and maintenance of cellular homoeostasis.[35,36] Downregulated Cx43 and Cx40 appears to be the consequence of upregulated PKC in aortic endothelial cells of diabetic rats, leading to a reduction in cell–cell communication via gap junctions.[37] Upregulated Cx43 and Cx40 in mRNA expression were initiated by NaHS treatment, which demonstrates that the antioxidative effects of H_2S affecting the molecular signalling pathway are critical.[21,38] In addition, H_2S acts as a vasodilator, as demonstrated by its

K_{ATP} channel-opening activity in vasorelaxation following intravenous bolus injections of NaHS.[39]

The effectiveness of aminoguanidine in relieving vascular abnormalities is confirmed again in this study, in line with our previous reports[6,10] and in primate diabetes.[40] NaHS seems to be as effective as aminoguanidine in relieving the changes in diabetes. It is an interesting finding that reversal of the downregulated Cx43 and Cx40 in diabetic vasculature is primary in the pharmacological profile of NaHS and aminoguanidine in ameliorating diabetic vascular disorders.

Conclusions

In this study, diabetic vascular abnormality is relevant to downregulation of Cx43 and Cx40 in the vasculature, contributing to endothelial dysfunction in STZ-injected rats. Downregulated connexin expression occurs in association with upregulation of NADPH oxidases and PKCε. NaHS, a donor of H_2S, effectively relieves diabetic vascular abnormality by normalizing Cx40, Cx43, NADPH oxidase and PKCε, and the efficacy of NaHS appears to be the same as that of aminoguanidine.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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