Boldine protects endothelial function in hyperglycemia-induced oxidative stress through an antioxidant mechanism

Yeh Siang Lau a, Xiao Yu Tian b, Yu Huang b, Dharmani Murugan a, Francis I. Achike c, Mohd Rais Mustafa a,⁎

⁎ Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia
a Institute of Vascular Medicine, Li Ka Shing Institute of Health Sciences, and School of Biomedical Sciences, Chinese University of Hong Kong, Hong Kong, China
b William Carey University, College of Osteopathic Medicine, 498 Tuscan Avenue, Hattiesburg, MS 39401, USA

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A B S T R A C T

Increased oxidative stress is involved in the pathogenesis and progression of diabetes. Antioxidants are therapeutically beneficial for oxidative stress-associated diseases. Boldine ((S)-2,9-dihydroxy-1,10-dimethoxyaporphine) is a major alkaloid present in the leaves and bark of the boldo tree (Peumus boldus Molina), with known antioxidant activity. This study examined the protective effects of boldine against high glucose-induced oxidative stress in rat aortic endothelial cells (RAEC) and its mechanisms of vasoprotection related to diabetic endothelial dysfunction. In RAEC exposed to high glucose (30 mM) for 48 h, pre-treatment with boldine reduced the elevated ROS and nitrotyrosine formation, and preserved nitric oxide (NO) production. Pre-incubation with β-NADPH reduced the acetylcholine-induced endothelium-dependent relaxation; this attenuation was reversed by boldine. Compared with control, endothelium-dependent relaxation in the aortas of streptozotocin (STZ)-treated diabetic rats was significantly improved by both acute (1 μM, 30 min) and chronic (20 mg/kg daily, i.p., 7 days) treatment with boldine. Intracellular superoxide and peroxynitrite formation measured by DHE fluorescence or chemiluminescence assay were higher in sections of aortic rings from diabetic rats compared with control. Chronic boldine treatment normalized ROS over-production in the diabetic group and this correlated with reduction of NAD(P)H oxidase subunits, NOX2 and p47phox. The present study shows that boldine reversed the increased ROS formation in high glucose-treated endothelial cells and restored endothelial function in STZ-induced diabetes by inhibiting oxidative stress and thus increasing NO bioavailability.

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1. Introduction

Endothelial dysfunction is correlated with hypertension, arteriosclerosis, diabetes and chronic heart failure [1]. It is defined as impairment of endothelium-dependent relaxation and the major factor contributing to this condition is the compromised nitric oxide-cyclic GMP (NO-cGMP) signalling [2]. Excessive production of reactive oxygen species (ROS) interferes with NO signalling and thus plays a pivotal role in the development of vascular complications in diabetes [2,3].

Over the past few decades, hyperglycemia-induced oxidative stress has been increasingly known as a hallmark in diabetic vasculature through several mechanisms such as activation of protein kinase C, polyol pathway and formation of advanced glycation end-product [4]. Hyperglycemia causes the excessive ROS formation, particularly superoxide anion, a radical that result from the reaction of oxygen with a single electron [5,6]. NAD(P)H oxidase, a multi-subunit enzymatic complex, is the major enzymatic sources of superoxide generation in vascular cells [7]. Excess superoxide can react with nitric oxide (NO), forming the toxic peroxynitrite, which in turn uncouples enzymatic nitric oxide synthase (eNOS) by oxidizing the essential NOS redox-sensitive cofactor tetrahydrobipterin and causes eNOS to produce more superoxide anion [1]. This continuous cascade of events reduces the bioavailability of NO and eventually leads to endothelial dysfunction in diabetes [8].

Boldine ((S)-2,9-dihydroxy-1,10-dimethoxy-aporphine) is an aporphine alkaloid found abundantly in the leaves/bark of boldo (Peumus boldus Molina), a widely distributed tree native to Chile. Boldine is also one of the major alkaloids in the bark of a local tree of Phoebe grandis found in the Northern part of Peninsular Malaysia [9,10]. Boldine has been reported to have several pharmacological activities, such as anti-inflammatory, antiplatelet, antithrombotic, antiplatelet, antitumor promoting and cytoprotective effects [11]. The action of boldine has been attributed to its antioxidant activity as it prevents lipid peroxidation in human liver.
microsomes [12], and scavenges hydroxyl radicals [13]. In addition, boldo extracts (Peumus boldus) has been demonstrated in several studies to have anti-inflammatory properties via its ability to interfere with the generation of free radical [14,15]. The methanolic extract of Phoebe grandis of which boldine, is a major compound, was shown to effectively improve the endothelium-dependent relaxations that were diminished by oxidative stress [10]. Although the antioxidant activities of boldine have been extensively studied, it has not been correlated with the hyperglycemia-induced oxidative stress and improving endothelial dysfunction in diabetic animals. Therefore, this study aims to investigate the effect of boldine in abating high glucose-induced ROS formation and improving endothelial dysfunction in streptozotocin-induced diabetic rats.

2. Materials and methods

2.1. Chemicals and materials

Acetylcholine (ACh) chloride, sodium nitroprusside (SNP), serotonin hydrochloride, bis-N-methylacridinium nitrate (lucigenin), diethyliothiocarbamic acid (DETA), diphenylene iodonium (DPI), β-NADPH, 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), boldine, uric acid, β-(+)-glucose, β-mannitol, triton-X, Hepe, phosphate buffer saline (PBS), Tris-base were purchased from Sigma Chemicals Company (St. Louis, MO, USA). Bovine serum albumin (BSA) and Tween-20 were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Kreb’s salts were purchased from BDH Limited and BDH Laboratory Supplies (Poole, UK), respective ly. RPMI 1640 media, foetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (invitrogen, CA, USA). Tempol was purchased from Tocris (Bristol, UK).

2.2. Primary culture of rat aortic endothelial cells

Primary aortic endothelial cells were isolated from adult Sprague Dawley (SD) rats and cultured as previously described [16]. Briefly, the aorta was isolated from the abdominal cavity of the rat after CO2 inhalation. The fat and connective tissues of the rat aorta was cleaned and placed in sterile ice-cold PBS. The aorta was then digested with filtered collagenase type 1A (Sigma, MO, USA) solution at 37 °C for 10 min with gentle shaking. After incubation the cells were centrifuged at 1500 rpm for 10 min and re-suspended in RPMI-1640 containing 10% FBS plus 100 U/ml penicillin and 100 μg/ml streptomycin. After 1 h incubation at 37 °C, the medium was removed and replaced to eliminate the smooth muscle cells. The endothelial cells were incubated in a humidified atmosphere containing 5% CO2 at 37 °C until the cells reached 80% confluence. The identity of RAECs was confirmed by a positive immunofluorescence staining of PECAM-1 (Santa Cruz, CA, USA).

2.3. Measurement of intracellular ROS generation

The amount of intracellular ROS generation was measured with CM-H2DCFDA fluoroscin (Invitrogen, CA, USA) dye that performed under Olympus FV1000 laser scanning confocal system (Olympus America, Inc., Melville, NY, USA). Intracellular ROS was detected once CM-H2DCFDA oxidized to a fluorescent DCF product within the cell. In brief, the isolated primary rat endothelial cells were seeded on circular cover slip and the cells were incubated in normal glucose (NG, 5 mM glucose and 25 mM mannitol as osmotic control of HG) or co-treated with or without boldine (1 μM) in high glucose condition (HG, 30 mM) for 48 h. Tempol (100 μM), a superoxide dismutase (SOD) mimetic compound, was added in the co-cultured as a positive control. At the end of treatment, the cells seeded on the circular cover slips were rinsed twice with normal physiological saline solution (NPSS in mM: NaCl 140, KCl 5, CaCl2 1, MgCl2 1, glucose 10 and HEPES 5) and then incubated at 37 °C for 20 min with CM-H2DCFDA (1 μM). The fluorescence intensity was analyzed using Olympus Fluoview version 1.6 software.

2.4. Detection of nitrotyrosine by immunofluorescence

The confluent cells seeded on coverslip were fixed with 4% formaldehyde for 30 min. The cells were then permeabilized with 0.01% Triton-X 100 and blocked in 5% normal donkey serum (Jackson ImmunoResearch Laboratories, PA, USA) for 1 h. The cells were incubated with mouse anti-nitrotyrosine (1:20; Millipore, MA, USA) overnight at 4 °C. After that, the cells were washed with PBS and incubated with Alexa Fluor 488-conjugated goat-anti-mouse secondary antibody (1:500; Invitrogen, CA, USA) for 2 h. At the end of incubation, the cells were stained with propidium iodide (1:3000; Sigma, MO, USA) to visualize the nucleus. The images were captured under Olympus FV1000 laser scanning confocal system (Olympus America, Inc., Melville, NY, USA) and the immunofluorescence intensity was analyzed using Olympus Fluoview version 1.6 software.

2.5. Detection of NO production in cultured endothelial cells

The experiment was performed as previously described [17]. Basically, the confluent endothelial cells were seeded on the coverslip and followed by high glucose treatment as described in previous section. At the end of treatment, the cells were rinsed with NPSS and incubated with 10 μM DAF-FM diacetate (Invitrogen, CA, USA) for 10 min at 37 °C. NO productions in response to ACh (10 μM) were measured by the level of fluorescence intensity change which were detected under Olympus Fluoview FV1000 laser scanning confocal system (Olympus America, Inc., Melville, NY, USA) mounted on an inverted IX81 Olympus microscope with excitation at 495 nM and emission at 515 nM. The real-time changes in intracellular fluorescence intensity were measured for 15 min and the results were presented as a ratio of fluorescence relative to intensity (F1/F0) before and after addition of ACh.

2.6. Induction of diabetes and chronic treatment

Male Sprague Dawley (SD) (9–10 weeks old) were obtained from the University of Malaya Animal Unit, and housed in a well-ventilated room (temperature: 24+18 °C), and had free access to standard rat chow (Specialty Feeds Pty Ltd., Glen Forrest, Australia) and tap water. All the experimental procedures were approved by the University of Malaya Animal Care and Ethics Committee. Diabetes mellitus was induced in 10–11 weeks SD rats (200–250 g) by injecting a single dose of 60 mg/kg streptozotocin (STZ) freshly dissolved in 0.1 M citrate buffer intraperitoneally. Blood glucose levels were measured using an Accu-check monitor (Roche, Mannheim, Germany) 3 days after diabetes induction. The animals were considered diabetic if the blood glucose level exceeds 17 mmol/L. After 8 week of induction, the rats were injected intraperitoneally with boldine (20 mg/kg/day) or vehicle (20% Tween 20) for 7 days. Body weights and blood glucose levels were recorded.

2.7. Measurement of lipids

Total cholesterol, triglyceride, HDL and non-HDL were determined using the assay kit purchased from BioAssay System, CA, USA (EnzyChrom™ AF Cholesterol Assay Kit; EnzyChrom™ Triglyceride Assay kit; EnzyChrom™ AF HDL and LDL/VLDL Assay Kit, E2HL-100). All assays were according to the manufacture instructions.
2.8. Preparation of aortic rings

At the end of 7 days treatment, the rats were anesthetized with single intraperitoneal dose of pentobarbitone sodium (60 mg/kg body weight). The descending thoracic aorta was isolated and cleaned from surrounding fat and connective tissues. The aorta was cut into rings segments, 3–5 mm long and placed in oxygenated Krebs physiological salt solution (KPSS in mM: NaCl 119, NaHCO3 25, KCl 4.7, KH2PO4 1.2, MgSO4·7H2O 1.2, glucose 11.7, and CaCl2·2H2O 2.5) and some tissues were snap frozen in liquid nitrogen and stored at −80 °C for protein analysis. The fresh aortic rings were maintained at 37 °C and stretched to optimal tension of 9.82 mN in a Multi Wire Myograph System (Danish Myo Technology, Aarhus, Denmark) and continuously oxygenated with 95% O2 and 5% CO2, and the changes of isometric tension in response to different drugs were recorded using the PowerLab LabChart 6.0 recording system (AD Instruments, Australia). The rings were equilibrated for 45 min before being repeatedly stimulated with high KCl solution (high KCl, 80 mM) three times at 4 min intervals to prime the tissues until two consecutive equal contractions were attained. The presence of functional endothelium in pre-contracted aortic rings was confirmed by a relaxant response to acetylcholine (ACH, 10 μM). Thereafter, concentration-relaxation curves of the endothelium-independent relaxant, sodium nitroprusside (SNP, 0.01 mM to 1 μM), and of the endothelium-independent relaxant, sodium nitroprusside (SNP, 0.01 mM to 1 μM) were carried out on 5-HT-precontracted aortic rings.

2.9. In situ detection of vascular superoxide production by laser confocal fluorescence microscopy

The amount of the in situ vascular superoxide formation was determined with using dihydroethidium (DHE, Invitrogen, CA, USA) dye and Olympus FV1000 laser scanning confocal system (Olympus America, Inc., Melville, NY, USA) as described previously [18]. Briefly, aortic rings from the respective groups were frozen in OCT compound (Sakura Finetek, Netherlands) and 10 μm frozen cross sections were obtained. The sections were incubated in dark for 15 min in normal physiological saline solution containing 5 μM DHE fluorescence dye (NPSS: NaCl 140, KCl 5, CaCl2 1, MgCl2 1, glucose 10 and HEPES 5 mM). The fluorescence intensity was measured at excitation/emission of 488/605 nm to visualize the signal. The images were analyzed using the Olympus Fluoview version 2.0 software.

2.10. Vascular superoxide and peroxynitrite production

Lucigenin–enhanced chemiluminescence method was used to estimate the vascular superoxide (O$_2^-$) production as previously described [19,20]. Briefly, aortic rings from each of the vehicle- and boldine-treated ring segments from the 5D and STZ–induced diabetic rats was preincubated for 45 min at 37 °C in 2 ml of Krebs–HEPES buffer (in mM: NaCl 99.0, NaHCO3 25, KCl 4.7, KH2PO4 1.0, MgSO4·7H2O 1.2, glucose 11.0, CaCl2·2H2O 2.5 and Na–HEPES 20.0) in the presence of diethylthiocarbamic acid (DETCA, 1 mM) to inactivate superoxide dismutase and
β-nicotinamide adenine dinucleotide phosphate (NADPH, 0.1 mM) as a substrate for NADPH oxidase. Diphenylene iodonium (DPI; 5 µM) was added for the positive control as an inhibitor of NADPH oxidase. Prior to measurement, a 96-well Optiplate was filled with 300 µl of Krebs–HEPES buffer containing lucigenin (5 µM) and NADPH (0.1 mM) per well and loaded into the Hitex plate CHAMELEON™ V (Finland) in luminescent detection mode to measure the background photo emission over 20 min. At the end of measurement, the rings were dried for 48 h at 65 °C and weighed. The data were expressed as average counts per mg of vessel dry weight.

Levels of peroxynitrite production from aortas of different groups of rats were measured using the luminol-enhanced chemiluminescence method (CHAMELEON™ V, Hitex, Finland) as described [20,21]. This method is similar to that used for superoxide detection with the exceptions that (1) luminol (100 µM) is used instead of lucigenin and (2) aortic rings were incubated in Krebs–HEPES buffer containing 1 mM DETCA and 0.1 mM NADPH in the presence and absence of uric acid (250 mM), a scavenger of peroxynitrite.

2.11. Total nitrates/nitrates measurement

The nitric oxide levels were measured by using Griess reagent kit (Sigma-Aldrich). Total nitrite and nitrate were determined as described by Ansari et al. [31] with slight modification. Briefly, all the nitrates in plasma were converted into nitrates using aspergillus nitrate reductase (20 mU) in the presence of FAD (0.11 mM) and NADPH (100 µM). The incubation was carried out at 37 °C for 90 min in the dark. Equal volume of blood sample and 1× Griess reagent (Sigma, MO, USA) were mixed and the absorbance of the sample is read at 540 nm after 15 min. The reading was compared to the sodium nitrates standard curve.

2.12. Western blot

Aortas and cells were homogenized and lysed in ice-cold RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The lysates were then centrifuged at 20,000 × g for 20 min and supernatants were collected for Western blotting. Protein concentrations of the supernatant were determined by modified Lowry assay (Bio-Rad Laboratories, Hercules, CA, USA). For each sample, 10–30 µg of total tissue protein was separated in 7.5–13% sodium dodecyl sulphate polyacrylamide gel and transferred onto nitrocellulose membranes. The non-specific binding was blocked with 5% non-fat milk or 3% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 (TBs-T) for 1 h at room temperature with gentle shaking. After washing in TBS-T, the blots were incubated with either primary mouse monoclonal antibody of nitrotyrosine (1:500, Millipore), p47phox (1:500, Santa Cruz), NOX-2 (1:1000, Abcam, UK), p67phox (1:500, Santa Cruz) or Rac1 (1:1000, Abcam, UK) overnight at 4 °C. The next day, the membranes were washed three times for 5 min in TBS-T and incubated with respective secondary antibodies conjugated to horseradish peroxidase for 2 h at room temperature. The membranes were developed with Amersham™ ECL plus Western blotting detection system (Amersham, Buckinghamshire, UK). The membrane image was captured under the ChemiDoc-It™ Imaging system (UVP, Cambridge, UK) and

Fig. 2. Boldine reduced high glucose-stimulated nitrotyrosine formation in RAEC. (A) Representative images and summarized results (B) showing the increased nitrotyrosine formation in high glucose-treated RAEC was reversed by the co-treatment with boldine. (C) Western blot showing that the co-treatment with boldine normalized the increased nitrotyrosine formation in high glucose-treated RAEC. Results are means ± SEM of 3 experiments using RAECs from different rats. *P < 0.05, **P < 0.05 compared with HG. *P < 0.05, **P < 0.1 compared with NG.
3.2. Boldine attenuates high glucose-induced increases in nitrotyrosine formation

High glucose stimulation (30 mM, 48 h) elevated formation of nitrotyrosine (an index for increased oxidative stress) in RAEC compared with normal glucose-treated endothelial cells; such increase was inhibited by treatment with boldine or tempol (Fig. 2A and B). Western blotting showed that treatment with boldine and tempol reversed the high glucose-induced up-regulation of nitrotyrosine protein expression in RAEC (Fig. 2C).

3.3. Boldine increases NO bioavailability under high glucose stimulation

The stimulated NO production in RAEC in response to ACh as revealed by the time-dependent increase in the DAF fluorescence intensity was significantly lower after high glucose stimulation compared with normal glucose (Fig. 3A and B). The effect of high glucose was reversed by co-treatment with boldine or tempol in RAEC (Fig. 3).

3.4. Body weight, plasma blood glucose and lipid profile

At the end of treatment, body weight decreased significantly in STZ-induced diabetic rats compared to control rats. The plasma glucose level of diabetic rats was significantly greater than that of control rats. In diabetic rats, there was a slight decreased in blood glucose level after boldine treatment for 7 days (Table 1). STZ-treated rats exhibited a significantly higher plasma triglyceride level compared with control and this level was unaffected after 7-day treatment with boldine, while total plasma cholesterol, HDL and non-HDL levels were similar in all groups of rats (Table 2).

3.5. Boldine reverses β-NADPH-induced impairment of endothelium-dependent relaxations in rat aortas

Pre-incubation of aortic rings with β-NADPH attenuated the acetylcholine-induced endothelium-dependent relaxations. Boldine concentration-dependently (0.01–1 μM) rescued the impaired relaxations (Fig. 4A and B). The vascular oxidative stress induced by β-NADPH was confirmed with the addition of SOD, a superoxide scavenger in functional study. Pre-treatment with SOD prevented the β-NADPH-induced impairment of relaxations (Fig. 4C). By contrast, endothelium-independent relaxations to SNP were comparable in all treatment groups and this suggests the relaxing sensitivity of vascular smooth muscle cells to NO remains unchanged (Fig. 4D).

3.6. Boldine improves endothelium-dependent relaxation in diabetic rat aortas

Endothelium-dependent relaxations to ACh were significantly less in diabetic than in normal rat aortas (Fig. 5A). Acute 30-min treatment with boldine (1 μM) restored the relaxations to the level seen in non-diabetic rat aortas (Fig. 5A). Again neither STZ treatment nor boldine treatment modulated SNP-induced endothelium-independent relaxations (Fig. 5D). The acute beneficial effect of boldine can be confirmed by chronic boldine treatment. STZ-induced diabetic rats were administered with boldine (20 mg/kg, i.p.) for 7 days and aortas were then harvested for functional and biochemical assays. Chronic
treatment with boldine significantly improved ACh-induced relaxations in diabetic rat aortas (Fig. 5C) without affecting endothelium-independent relaxations to SNP (data not shown). The improvement of endothelial function in boldine-treated rats was accompanied by the restoration of the lost plasma nitrates/nitrites level in diabetic rats (Fig. 5D).

3.7. Boldine reduces in situ vascular superoxide formation and inhibits the elevated expression of p47phox in diabetic rat aortas

The intracellular superoxide formation measured by DHE fluorescence was higher in aortic rings from diabetic rats compared with control rats. Seven-day treatment of diabetic rats with boldine reduced the ROS accumulation in the vascular wall of diabetic rat aortas (Fig. 6A and B). Boldine attenuated the increased level of the superoxide-generating p47phox in the diabetic rat aorta (Fig. 6C).

3.8. Boldine reduces vascular superoxide and peroxynitrite in diabetic rat aortas

Production of superoxide and peroxynitrite in the diabetic rat aortas was significantly greater than controls (Fig. 7). This increased superoxide production was abolished by DPI, a NADPH oxidase inhibitor. Uric acid, a direct peroxynitrite scavenger reduced the peroxynitrite level in all groups. Chronic treatment with boldine attenuated the excess aortic generation of superoxide and peroxynitrite in the diabetic rat aortas (Fig. 7).

4. Discussion

The major findings of the present study include that (i) treatment with boldine effectively reduces high glucose-stimulated ROS and nitrotyrosine formation in rat aortic endothelial cells; (ii) acute treatment with boldine reverses the impaired endothelium-dependent aortic relaxations induced by β-NADPH and in STZ-induced diabetic rats; and (iii) the in vitro effect of boldine is confirmed by in vivo treatment as chronic boldine administration improves endothelium-dependent relaxations in aortas from STZ-treated rats. This endothelial cell protection appears to correlate with the increase of NO bioavailability following boldine treatment. Collectively, the present study shows for the first time that boldine treatment attenuates the diabetes-associated oxidative stress and endothelial dysfunction through restoring the NO bioavailability.

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Body weight (g)</th>
<th>Blood glucose (mmol/l)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>After treatment</td>
</tr>
<tr>
<td>Control</td>
<td>231.1 ± 7.8</td>
<td>372.2 ± 13.9</td>
</tr>
<tr>
<td>Control + boldine</td>
<td>230.0 ± 7.1</td>
<td>376.7 ± 10.0</td>
</tr>
<tr>
<td>Diabetes</td>
<td>228.6 ± 6.9</td>
<td>157.1 ± 7.6</td>
</tr>
<tr>
<td>Diabetes + boldine</td>
<td>238.6 ± 6.9</td>
<td>179.4 ± 12.0</td>
</tr>
</tbody>
</table>

Sprague Dawley rats and STZ-induced diabetic rats were treated with boldine (20 mg/kg/daily, i.p.). Results are means ± SEM of 7–9 animals. *P < 0.001 compared with control. *P < 0.05 compared with diabetes.

Fig. 4. Boldine reversed the β-NADPH-induced endothelial dysfunction in rat aortas. (A) Representative traces showing the β-NADPH-induced impairment of ACh-evoked endothelium-dependent relaxation were improved by acute 30-min treatment with boldine which exhibited a concentration-dependent benefit (B). (C) SOD (50 U/ml) produced the same vascular protection as boldine (1 μM) in improving ACh-induced relaxations. (D) SNP-induced endothelium-independent relaxations were similar in all treatment groups. Results are means ± SEM of experiments from 6 different rats. *P < 0.01, **P < 0.001 compared to β-NADPH; *P < 0.001 compared with control.
However, anions concentration normalized present lial exposure glucose-stimulated hypertensive effective endothelial effect induced glutathione NOX2 Effect rats

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + boldine</th>
<th>Diabetes</th>
<th>Diabetes + boldine</th>
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<tbody>
<tr>
<td>Total cholesterol (mg/ml)</td>
<td>0.60 ± 0.09</td>
<td>0.58 ± 0.03</td>
<td>0.71 ± 0.04</td>
<td>0.72 ± 0.07</td>
</tr>
<tr>
<td>Triglyceride (mg/ml)</td>
<td>0.92 ± 0.02</td>
<td>0.85 ± 0.07</td>
<td>1.94 ± 0.28</td>
<td>1.65 ± 0.32</td>
</tr>
<tr>
<td>HDL (mg/ml)</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>Non-HDL (mg/ml)</td>
<td>0.47 ± 0.05</td>
<td>0.52 ± 0.03</td>
<td>0.61 ± 0.04</td>
<td>0.51 ± 0.07</td>
</tr>
</tbody>
</table>

Effect of chronic boldine treatment (20 mg/kg/daily, i.p.) on levels of total cholesterol, triglyceride, HDL and non-HDL in vehicle- or boldine-treated Sprague Dawley (control) rats and STZ-induced diabetic rats. Results are means ± SEM of 6 rats.

P < 0.05 compared with control.

It has previously been shown in the STZ-induced diabetic rats, 8-week treatment with boldine decreased mitochondrial malondialdehyde and carbonyls in the liver, kidney and pancreas, and normalized the elevated Mn-SOD and GSH-peroxidase activity in mitochondria of the pancreas [13]. We have also showed that chronic treatment with boldine restored endothelial function in hypertensive rats through the inhibition of NAD(P)H oxidase [20]. However, there is little information concerning the vasoprotective effect of boldine in metabolic stress-related cardiovascular diseases, in particular endothelial dysfunction in diabetes. The present study provides novel evidence that boldine treatment is effective in reducing oxidative stress and thus restoring endothelial function in STZ-induced diabetic rats.

We first examined the effect of boldine against high glucose-induced ROS over-production in tissue culture. High glucose exposure markedly increases ROS generation in cultured rat aortic endothelial cells (RAEC) which is reversed by boldine at a low concentration of 1 µM but high glucose did not affect the glutathione antioxidant system in RAEC (data not shown). High glucose-stimulated ROS elevation is causally associated with increased expression of NAPDH oxidase subunits including NOX2 and p47phox, which is also reduced by boldine. Superoxide anions react with NO to form peroxynitrite radicals and the latter further lowers bioavailability of NO [22]. We confirmed the contributory role of peroxynitrite by detecting an elevated level of nitrotyrosine in high glucose-treated RAEC resulting from tyrosine nitration mediated by peroxynitrite radicals. The increased superoxide anion may explain a low NO level in RAEC exposed to high glucose. Co-treatment with boldine or tempol, equivalently reduces the elevated nitrotyrosine formation and increases the NO formation in endothelial cells. It is possible that ROS-inhibiting effect of boldine or tempol is sufficient to restore the diminished NO production in high glucose-treated endothelial cells without affecting the eNOS expression (data not shown), thus improving endothelial function.

ROS derived from NAD(P)H oxidase plays a key role in vascular endothelial dysfunction in diabetes [23]. In this study acute exposure to boldine effectively reversed the β-NAPDH-induced impairment of endothelium-dependent relaxations, thus indicating that the anti-oxidative (reduction of ROS) activity of boldine helps to preserve the bioavailability of NO. This finding is further supported by the improvement of endothelial function observed in isolated diabetic aortas that has been given acute and chronic treatment of boldine. The present study, for the first time, reveals the mechanism of vasoprotection of boldine in diabetic aortas is mediated through reducing ROS and increasing NO bioavailability. Further evidence is provided by the findings that (1) elevated superoxide

Fig. 5. Acute and chronic boldine treatment benefited endothelial function in STZ-treated diabetic rats. Acute 30-min treatment of boldine (1 µM) restored ACh-induced endothelium-dependent relaxations (A) but not SNP-induced endothelium-independent relaxations (B) in diabetic rat aortas. Chronic treatment with boldine improved ACh-induced aortic relaxations (C) and the total plasma nitrates/nitrites (D) in STZ-induced diabetic rats. Results are means ± SEM of experiments from 6 to 7 different rats. *P < 0.05, **P < 0.01, ***P < 0.001 compared with diabetes; †P < 0.01, ‡P < 0.001 compared with control.
anion production in aortas of STZ-induced diabetic rats is reversed by chronic boldine treatment and (2) boldine suppressed the upregulated NOX2 and p47phox in the STZ-treated rat aortas and augmented the plasma levels of NO metabolites. In contrast to the changes in endothelial function, the sensitivity of vascular smooth muscle to NO remained unaltered as SNP-induced endothelium-independent relaxation is comparable in all treatment groups (Figs. 4D and 5B). The present results are in line with earlier reports of overexpression of p47phox in high glucose-stimulated ROS generation in human coronary artery endothelial cells, vascular smooth muscle cells, and in diabetic rat arteries [24–26]. Taken together, the present study demonstrates a vascular protective effect of boldine under hyperglycaemic conditions and the concomitant diabetic vasculopathy.

Boldine has been shown to block α1-adrenoceptors in arteries from rats [27] and guinea pigs [28]. This α1-adrenoceptor blocking activity is unrelated to the endothelial cell protection conferred by boldine based on our observation that boldine at 1 μM used in the present study did not inhibit contraction triggered by phenylephrine, an α1-adrenoceptor agonist (data not shown) and this study used another contractile agent, serotonin instead of phenylephrine. In addition, the vascular benefits of chronic treatment with boldine is unlikely to be associated with favourable modulation of metabolic parameters as boldine only slightly reduces plasma glucose levels (p > 0.05) without affecting lipid profile in diabetic rats.

ROS has been implicated in the pathogenesis of β-cell destruction and liver injury in diabetes [29]. Streptozocin causes DNA fragmentation of pancreatic β-cell by stimulation of ROS generation in vitro and in vivo [30]. Jang et al. reported that the plasma glucose lowering effect of boldine was associated with its cytoprotective action on pancreatic β-cell and the prevention of peroxidation products formation. Boldine also attenuates the STZ-induced MDA formation, carbonyl formation and thio oxidation in the pancreas homogenates [13]. The slight glucose lowering effect by boldine observed in this study may be related to its cytoprotective effect against the oxidative damage in pancreatic β-cell as reported by Jang et al. although other contributing factors cannot be ruled out.

In summary, both in vitro (acute) and in vivo (chronic) treatments with boldine augment endothelial function through restoring the NO bioavailability in diabetic rats, thus indicating that boldine could be a potentially effective herb-derived
ingredient in inhibiting oxidative stress and thus preserving endothelial function in hyperglycaemic or diabetic conditions.

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