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RESEARCH PAPER

Boldine improves endothelial function in diabetic *db/db* mice through inhibition of angiotensin II-mediated BMP4-oxidative stress cascade

Yeh Siang Lau[1], Xiao Yu Tian[2], Mohd Rais Mustafa[1], Dharmani Murugan[1], Jian Liu[2], Yang Zhang[2], Chi Wai Lau[2] and Yu Huang[2]

[1]*Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia, and* [2]*Institute of Vascular Medicine and Li Ka Shing Institute of Health Sciences, Chinese University of Hong Kong, Hong Kong, China*

Correspondence
Yu Huang, School of Biomedical Sciences, Chinese University of Hong Kong, Hong Kong SAR, China.
E-mail: yu-huang@cuhk.edu.hk
Mohd Rais Mustafa, Department of Pharmacology, University of Malaya, Kuala Lumpur 50603, Malaysia.
E-mail: rais@um.edu.my

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BACKGROUND AND PURPOSE
Boldine is a potent natural antioxidant present in the leaves and bark of the Chilean boldo tree. Here we assessed the protective effects of boldine on endothelium in a range of models of diabetes, *ex vivo* and *in vitro*.

EXPERIMENTAL APPROACH
Vascular reactivity was studied in mouse aortas from *db/db* diabetic and normal mice. Reactive oxygen species (ROS) production, angiotensin AT_1 receptor localization and protein expression of oxidative stress markers in the vascular wall were evaluated by dihydroethidium fluorescence, lucigenin enhanced-chemiluminescence, immunohistochemistry and Western blot respectively. Primary cultures of mouse aortic endothelial cells, exposed to high concentrations of glucose (30 mmol L^{-1}) were also used.

KEY RESULTS
Oral treatment (20 mg $kg^{-1}day^{-1}$, 7 days) or incubation *in vitro* with boldine (1 μmol L^{-1}, 12 h) enhanced endothelium-dependent aortic relaxations of *db/db* mice. Boldine reversed impaired relaxations induced by high glucose or angiotensin II (Ang II) in non-diabetic mouse aortas while it reduced the overproduction of ROS and increased phosphorylation of eNOS in *db/db* mouse aortas. Elevated expression of oxidative stress markers (bone morphogenic protein 4 (BMP4), nitrotyrosine and AT_1 receptors) were reduced in boldine-treated *db/db* mouse aortas. Ang II-stimulated BMP4 expression was inhibited by boldine, tempol, noggin or losartan. Boldine inhibited high glucose-stimulated ROS production and restored the decreased phosphorylation of eNOS in mouse aortic endothelial cells in culture.

CONCLUSIONS AND IMPLICATIONS
Boldine reduced oxidative stress and improved endothelium-dependent relaxation in aortas of diabetic mice largely through inhibiting ROS overproduction associated with Ang II-mediated BMP4-dependent mechanisms.

Abbreviations
Ang II, angiotensin II; BMP4, bone morphogenic protein 4; MAEC, mouse aortic endothelial cells

Introduction

Type II diabetes, a common metabolic disorder, is characterized by hyperglycaemia and hyperinsulinaemia, which impair the functions of both the macro- and microcirculations, thus increasing the risks of developing hypertension and atherosclerosis (Tranche *et al.*, 2005; Senador *et al.*, 2009). Excessive oxidative stress or increased production of reactive oxygen species (ROS) damage endothelial function as an early pathological event leading to cardiovascular diseases (Heitzer *et al.*, 2001). For example, increased formation of NADPH oxidase-dependent superoxide anion has been observed in diabetic animals including *db/db* mice, diet-induced obese mice, Otsuka Long-Evans Tokishima Fatty (OLETF) rats, and Goto-Kakizaki (GK) rats (Kim *et al.*, 2002; Gupte *et al.*, 2010; Tian *et al.*, 2011). Activation of angiotensin II AT_1 receptors (receptor nomenclature follows Alexander *et al.*, 2011) plays a critical role in mediating endothelial dysfunction through AT_1 receptor-dependent NADPH-derived ROS overproduction in arteries of *db/db* diabetic mice (Wong *et al.*, 2010b; Tian *et al.*, 2011), while Ang II is itself a potent vasoconstrictor with pro-inflammatory, mitogenic and profibrotic properties.

Bone morphogenetic protein (BMP), a member of the TGF-β superfamily, activates Smads as the immediate downstream molecules, following binding to BMP receptors (Chen *et al.*, 2004). The BMP family comprises several forms including BMP2, BMP4 and BMP7, which are up-regulated in diabetes and act as pro-inflammatory regulators in blood vessels (Nett *et al.*, 2006; Bostrom *et al.*, 2011). BMP4 impairs endothelial function in mouse aortas either by increased ROS formation through NADPH oxidase or up-regulation of cyclooxygenase-2 (Miriyala *et al.*, 2006; Wong *et al.*, 2010a). An elevated expression of BMP4 and NAPDH oxidase in *db/db* mice suggests a positive involvement of this redox-sensitive pro-inflammatory mechanism in diabetes (San Martin *et al.*, 2007). Therefore, natural products that improve endothelial function in diabetes by favourable modulation of redox-sensitive mechanisms are potentially useful for treating diabetic vasculopathy.

Boldine ((S)-2,9-dihydroxy-1,10-dimethoxy-aporphine) is an alkaloid of the benzylisoquinoline family and found as the major alkaloid in the leaves and bark of the Chilean boldo tree (*Peumus boldus* Molina; O'Brien *et al.*, 2006). Boldine exhibits potent antioxidative properties (Cassels *et al.*, 1995) and, although the pharmacological effects of boldine were reported a decade ago, whether this antioxidant activity benefits vascular function in mouse models of type II diabetes, remains to be elucidated. Therefore, the present study investigated the hypothesis that *in vivo* and *in vitro* treatment with boldine ameliorates endothelial dysfunction in diabetic *db/db* mice by inhibiting the Ang II-mediated, BMP4-dependent, oxidative stress cascade.

Methods

Animals and experimental protocol

All animal care and experimental procedures were approved by the CUHK Animal Experimentation Ethics Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 52 animals were used in the experiments described here. Male diabetic mice (C57BL/KSJ background) lacking the gene encoding for leptin receptor (*db/db*), heterozygote (*db/m*$^+$) and non-diabetic C57 mice were purchased from the Laboratory Animal Service Center of Chinese University of Hong Kong (CUHK). Mice were maintained in a well-ventilated holding room at constant temperature of 24 ± 1°C and received normal chow and tap water *ad libitum*. The *db/db* mice (16–17 weeks old) were randomly assigned to control (vehicle), boldine or tempol treatment groups, and they were treated daily with vehicle (20% ethanol, 0.8 ml kg^{-1}), or boldine (20 mg kg^{-1} day^{-1}) or tempol (20 mg kg^{-1} day^{-1}) by oral administration for 7 days. At the end of the treatment period, mice were killed by CO_2 inhalation.

Artery preparation

The thoracic aorta was isolated, cleaned of surrounding connective tissues, and cut into several ring segments, each 2 mm in length. Rings were suspended in a Multi Wire Myograph System (Danish Myo Technology, Aarhus, Denmark) and bathed in oxygenated Krebs solution containing (in mmol L^{-1}) NaCl 119, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, glucose 11.7 and $CaCl_2.2H_2O$ 2.5. Some arteries were snap-frozen in liquid nitrogen and stored in-80°C for later processing. All rings were maintained at 37°C, stretched to an optimal baseline tension of 3 mN, and continuously oxygenated by 95% O_2 and 5% CO_2. The changes of isometric tension were recorded by a PowerLab LabChart 6.0 recording system (AD Instruments, Bella Vista, NSW, Australia).

Experimental protocol

After equilibration for 30 min, rings were first contracted by 60 mM KCl and washed in Krebs solution three times before phenylephrine (1 μmol L^{-1}) was added to induce a stable contraction. Concentration-response curves for both endothelium-dependent relaxations in response to ACh (3 nmol L^{-1} to 10 μmol L^{-1}) and endothelium-independent relaxations to sodium nitroprusside (SNP, 1 nmol L^{-1} to 10 μmol L^{-1}) were obtained.

Detection of ROS formation in en face endothelium and cryostat sections of mouse aortas

The level of oxidative stress in *en face* endothelium and cryostat sections of mouse aorta was assessed by confocal microscopy using dihydroethidium (DHE) dye. The aortic segments and cryostat sections (5 μm) of mouse aortas were pre-incubated in DHE (5 μmol L^{-1}, Invitrogen, Carlsbad, CA, USA) for 15 min in normal physiological saline solution (NPSS, composition in mmol L^{-1}: NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, glucose 10 and HEPES 5; Tian *et al.*, 2012b). At the end of incubation, aortic DHE dye was washed away and the fluorescence intensity at one optical section of the rings was visualized using a Olympus FV1000 laser scanning confocal system (Olympus, Tokyo, Japan). The fluorescence intensity was measured with 515 nm excitation and 585 nm emission

and the images were analysed using Olympus Fluoview software (version 2.0, Olympus).

Detection of vascular superoxide formation

The amount of superoxide anion formation was determined using the lucigenin-enhanced chemiluminescence method (Lau *et al.*, 2013). Briefly, isolated mouse aortic rings were pre-incubated for 45 min at 37°C in 2 mL of Krebs–HEPES buffer (in mmol L^{-1}: NaCl 99.0, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.0, $MgSO_4$ 1.2, glucose 11.0, $CaCl_2$ 2.5 and Na-HEPES 20.0) in the presence of diethylthiocarbamic acid (1 mmol L^{-1}) to inactivate superoxide dismutase (SOD) and β-nicotinamide adenine dinucleotide phosphate (0.1 mmol L^{-1}), as substrate for NADPH oxidase. The rings were then transferred into vials containing 300 uL of Krebs-HEPES buffer containing 10 µmol L^{-1} lucigenin. Repeated measurements were made over 10 min in 1 min intervals using a luminometer (GloMax® 20/20 Luminometer, Madison, WI, USA). The data were expressed as average counts per mg of tissue dry weight.

Detection of AT_1 receptors by immunohistochemical staining

The localization of AT_1 receptors in mouse aortas was determined by immunohistochemistry (Wong *et al.*, 2010a). Briefly, aortic rings were fixed in 4% paraformaldehyde at 4°C overnight and then were dehydrated and embedded in paraffin on the following day. The paraffin block was cut into 5 µm thick sections on microtome (Leica Microsystems, Nussloch, Germany), followed by re-hydration. Sections were then treated with 1.4% H_2O_2 in absolute methanol for 30 min at room temperature to block the activity of endogenous peroxidase. To avoid false negative staining, sections were boiled in 0.01 mol L^{-1} sodium citrate buffer (pH 6) for 15 min to unmask antigenic sites in the specimens. After washing in PBS, sections were blocked with 5% donkey serum (Jackson Immunoresearch, West Grove, PA, USA) for 1 h and incubated with primary mouse monoclonal antibody to the AT_1 receptor (1: 50, Abcam, Cambridge, UK) overnight in a humidified chamber at 4°C. At the end of incubation period, sections were incubated with biotin-SP conjugated goat anti-mouse secondary antibodies (1:200, Jackson Immunoresearch) for 1 h at room temperature and then for 30 min with streptavidin-HRP conjugate (1:200, Zymed Laboratory, San Francisco, CA, USA). Sections were washed in PBS three times and colours were developed using 3,3′-diamonobenzidine peroxidase substrate kit (Vector Lab, Burlingame, CA, USA). The nuclei were counterstained with haematoxylin and sections without primary antibody served as negative controls. Images were captured using Leica DMRBE microscope coupled to SPOT-RT cooled CCD colour digital camera and SPOT Advanced software (Version 3.5.5, Diagnostic Instruments, Sterling Heights, MI, USA).

Organ culture of isolated aortas

The isolated aortic rings were cultured in DMEM (Gibco, Gaithersberg, MD, USA) supplemented with 10% FBS (Gibco), 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin. Aortas from *db/db* mice were incubated for 12 h in the presence or absence of boldine (1 µmol L^{-1} in 0.001% DMSO), tempol (SOD mimetic, 100 µmol L^{-1}), noggin (BMP4 antagonist, 100 ng ml^{-1}) or losartan (AT_1 receptor antagonist, 3 µmol L^{-1}). Aortic rings from C57 mice were incubated in normal glucose (NG, 5 mmol L^{-1} glucose and 25 mmol L^{-1} mannitol as osmotic control of high glucose), high glucose (30 mmol L^{-1}) and co-treatment with either boldine (1 µmol L^{-1}) or tempol (100 µmol L^{-1}) for 36 h in an incubator (5% CO_2; 37°C). In another set of experiments, rings were treated with Ang II (0.5 µmol L^{-1}) for 24 h and thereafter transferred to wire myographs for functional examinations.

Primary culture of mouse aortic endothelial cells (MAECs)

Primary MAECs were isolated from two male C57BL/6J mice (5–6 weeks old; Tian *et al.*, 2012a). In brief, the aorta was isolated from anaesthetized mice after a single intraperitoneal injection of pentobarbital sodium (40 mg kg^{-1}) and perfusion of heparin (100 U mL^{-1}) through the circulation from the left ventricle. The aortas were dissected in sterile ice-cold PBS to remove adipose and connective tissues and then incubated for 10 min in collagenase type 1A (Sigma, St. Louis, MO, USA) solution at 37°C with gentle shaking. Detached endothelial cells were centrifuged at 800 *g* for 10 min and the cell pellets were re-suspended and cultured in endothelial cell growth medium (EGM, Gibco, Invitrogen) containing 20% FBS, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin, in addition to endothelial cell growth supplement (50 µg mL^{-1}, BD Transduction Laboratory, San Diego, CA, USA). The cells were kept in a humidified atmosphere containing 5% CO_2 at 37°C for 45 min and culture medium was then replaced and cells allowed to grow into confluence. Cells from passages between 1 and 3 were used for the present study. The endothelial cells were verified by positive staining for PECAM-1 (Santa Cruz, Santa Cruz, CA, USA) and negative staining for the smooth muscle marker, β-actin (DakoCytomation, Carpinteria, CA, USA).

Measurement of intracellular ROS formation in MAECs

Intracellular ROS production in MAECs was measured by a fluorogenic probe, CM-H_2DCFDA and the fluorimetric signal was captured on an Olympus FV1000 laser scanning confocal system (Olympus, America Inc.). Briefly, the confluent MAECs were seeded on circular cover slips and incubated in a low serum medium (EGM with 2% FBS) for 4 h. The cells were then exposed to high glucose (30 mmol L^{-1}) for 36 h with or without co-treatment with boldine (1 µmol L^{-1}) or tempol (100 µmol L^{-1}). At the end of treatment, cells were washed twice in NPSS and preloaded with CM-H_2DCFDA (1 µmol L^{-1}) for 20 min at 37°C before the fluorescence signal was measured at 488 nm excitation and 520 nm emission.

Western blotting

Aortas and MAECs were homogenized in ice-cold 1X RIPA buffer containing leupeptin 1 µg mL^{-1}, aprotonin 5 µg mL^{-1}, PMSF 100 µg mL^{-1}, sodium orthovanadate 1 mmol L^{-1}, EGTA 1 mmol L^{-1}, EDTA 1 mmol L^{-1}, NaF 1 mmol L^{-1}, and β-glycerolphosphate 2 mg mL^{-1}. The lysates were centrifuged at 20 000 g for 20 min and the supernatant was collected for Western blotting. Protein concentrations of the supernatant were determined by a modified Lowry assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples of protein (15µg)

loaded in each lane were separated on 7.5 or 10% SDS-polyacrylamide gel and then transferred to an immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) at 100 V. The blots were blocked for non-specific binding with 2% BSA or 5% non-fat milk in Tris-buffered saline containing 0.1 % Tween 20 (TBS) for 1 h at room temperature with gentle shaking. After rinsing in TBS-T, the blots were incubated with either primary polyclonal antibody to phosphorylated eNOS at Ser^{1177} (p-eNOS-Ser^{1177}; 1:500, Cell Signaling Technology, Inc., Beverly, MA, USA), monoclonal anti-eNOS (1:500, BD Transduction Laboratory), anti-nitrotyrosine (1:1000, Milipore), anti-AT_1 receptor (1:1000, Abcam, Cambridge, UK) or anti-BMP4 (1:500, Sigma). After overnight incubation at 4°C, the membranes were washed three times and incubated with the respective secondary antibodies conjugated to HRP for 2 h at room temperature. The membranes were developed with Amersham™ ECL plus Western blotting detection system (Amersham, Bukinghamshire, UK) and images were captured under ChemiDoc-It® Imaging system (UVP, Cambridge, UK). The densitometric analysis was performed using VisionWorks®LS analysis software and the respective protein expression levels were normalized to the housekeeping proteins β-actin or GAPDH

Data analysis

Results are shown as means ± SEM from *n* mice. Concentration–response curves were fitted to a sigmoidal curve using non-linear regression with the aid of the statistical software GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA, USA). Data were analysed for statistical significance using Student's *t*-test for unpaired observations and, for comparison of more than two groups, one-way ANOVA followed by Bonferroni's multiple comparison test (Prism 5.0, GraphPad Software). A value of $P < 0.05$ was taken statistically significant.

Materials

ACh chloride, SNP, boldine, L-NAME, phenylephrine, angiotensin II (Ang II) were purchased from Sigma. Tempol was purchased from Tocris (Bristol, UK). Noggin and BMP4 were purchased from R&D System, Inc (Minneapolis, MN, USA). Losartan was purchased from Cayman (Ann Arbor, MI, USA). Noggin and BMP4 were dissolved in PBS plus 0.1% BSA and 4 mmol L^{-1} HCl respectively. Losartan was dissolved in DMSO. Boldine was dissolved in DMSO for *in vitro* study or ethanol (20%) for oral feeding. Other drugs were dissolved in double distilled water.

Results

Boldine improves endothelial function in diabetic db/db *mice*

Figure 1 shows that the endothelium-dependent relaxations to ACh (Figure 1A) but not endothelium-independent relaxations to SNP (Figure 1B) in aortic rings from *db/db* mice were impaired, compared with those from *db/m*$^+$ mice. Oral administration of boldine (20 mg kg^{-1} day^{-1}) or tempol (20 mg $kg^{-1}day^{-1}$) to *db/db* mice for one week, reversed this impairment (Figure 1A & Table 1), without affecting SNP-induced

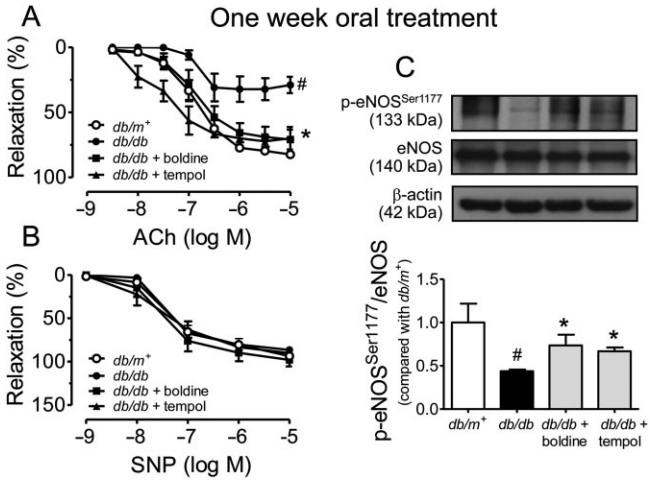

Figure 1

ACh-induced endothelium-dependent (A) and SNP-induced endothelium-independent relaxations (B) in aortic rings from *db/db* mice orally treated for 1 week with vehicle (20% EtOH), boldine (20 mg kg^{-1} kg^{-1} day^{-1}), or tempol (20 mg kg^{-1} kg^{-1} day^{-1}). (C) Chronic boldine treatment increased the level of eNOS phosphorylation at Ser^{1177} in aortas of *db/db* mice. Results are shown as mean ± SEM of six separate experiments. $^{\#}P < 0.05$ compared with *db/m*$^+$ mice; $^{*}P < 0.05$ compared with *db/db* mice.

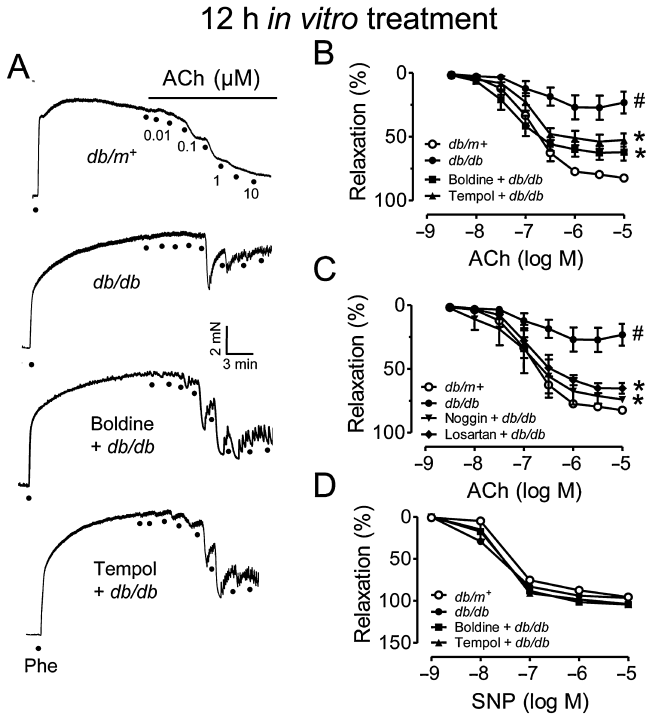

Figure 2

In vitro exposure to boldine (1 μmol L^{-1}) or tempol (100 μmol L^{-1}) for 12 h reversed the impaired ACh-induced relaxations in *db/db* mouse aortas (A & B) without affecting SNP-induced relaxations (D). Treatment (12 h) with noggin (100 ng mL^{-1}) or losartan (3 μmol L^{-1}) also restored the impaired relaxation in these aortas (C). Results are means ± SEM of six separate experiments. $^{\#}P < 0.05$ compared with *db/m*$^+$ mice; $^{*}P < 0.05$ compared with *db/db* mice.

responses in aortic rings (Figure 1B). Such treatment did not affect the plasma lipid profile or glucose levels (data not shown). In addition, treatment with either boldine or tempol restored the decreased phosphorylation of eNOS at Ser^{1177} in *db/db* mouse aortas (Figure 1C). Likewise, incubation *in vitro* with boldine (1 μmol L^{-1}) or tempol (100 μmol L^{-1}) for 12 h also reversed the impaired ACh-induced relaxations in *db/db* mouse aortas (Figure 2A & B), without affecting SNP-induced relaxations (Figure 2C). The impaired relaxations were also reversed by treatment with the AT_1 receptor antagonist, losartan (3 μmol L^{-1}) or the BMP4 antagonist, noggin (100 ng mL^{-1}; Figure 2C).

Table 1

The agonist sensitivity (pEC_{50}) and percentage of maximum response (R_{max}) of ACh-induced endothelium-dependent relaxations in aortic rings isolated from *db/m*$^+$ and *db/db*

	Ach	
Group	**pEC_{50} (log M)**	**R_{max} (%)**
db/m$^+$	6.60 ± 0.06	82 ± 2.0
db/db	4.35 ± 0.47#	29 ± 6.3#
db/db + boldine	6.28 ± 0.12*	71 ± 7.7*
db/db + tempol	6.81 ± 0.15*	70 ± 8.7*

Results are means ± SEM of six separate experiments. #$P < 0.01$ compared with *db/m*$^+$.
*$P < 0.01$ compared with *db/db*.

Boldine reduces vascular oxidative stress in db/db *mice*

Daily treatment with boldine or tempol for one week normalized the elevated ROS accumulation in *en face* endothelium (Figure 3A), across the vascular wall (Figure 3B), and superoxide anion levels (Figure 3C) in aortas from *db/db* mice, as shown by DHE fluorescence, using the lucigenin enhanced-chemiluminescence method. The Western blot results showed that the elevated protein expression of BMP4 (Figure 4A) and nitrotyrosine (another oxidative stress index, Figure 4B) in *db/db* mouse aortas was reversed by this chronic treatment with boldine or tempol.

Boldine reduces the expression of AT_1 receptors in db/db *mouse aortas*

Immunohistochemistry staining showed the presence of AT_1 receptors in both endothelial cells and smooth muscle cells in mouse aortas and chronic treatment with boldine or tempol reduced the expression of these receptors in the aortas from *db/db* mice (Figure 5A). Such changes were confirmed by the Western blot data (Figure 5B).

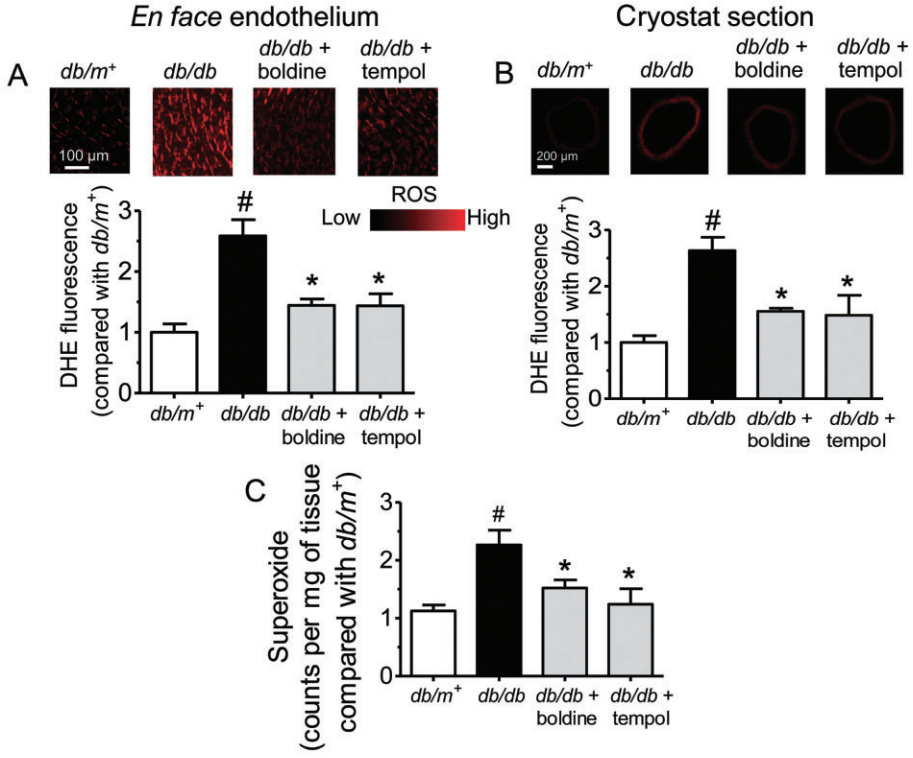

Figure 3

Chronic boldine treatment reversed the elevated ROS in *en face* endothelium (A) and cryostat section of *db/db* mouse aortas (B) as indicated by changes in DHE fluorescence and inhibited the increased generation of superoxide anion in these aortas (C) as detected by the lucigenin-enhanced chemiluminescence method. Results are means ± SEM of four to six separate experiments. #$P < 0.05$ compared with *db/m*$^+$ mice; *$P < 0.05$ compared with *db/db* mice.

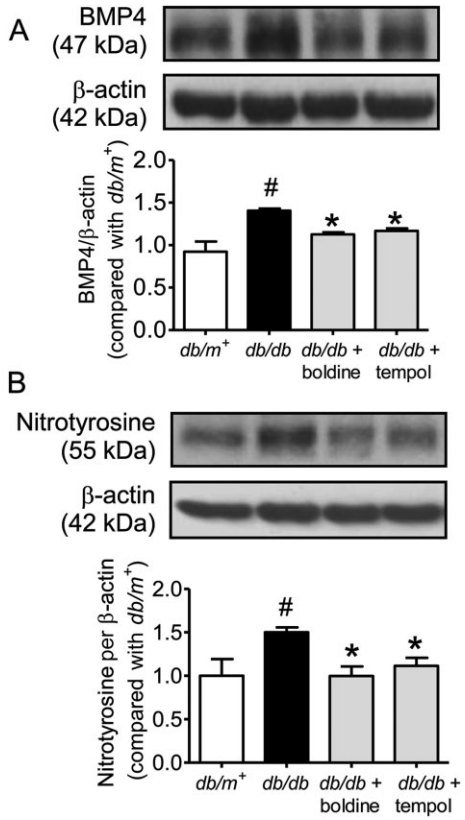

Figure 4

Western blotting showing the up-regulation of expression of BMP4 (A) and nitrotyrosine (B) in aortas from *db/db* mice with and without receiving 1 week treatment with boldine or tempol. Results are means ± SEM of four separate experiments. $^{\#}P < 0.05$ compared with *db/m*$^{+}$ mice; $^{*}P < 0.05$ compared with *db/db* mice.

Boldine protects against Ang II-induced BMP4-dependent endothelial dysfunction

Ang II (500 nmol L^{-1}) attenuated the ACh-induced relaxations in aortas from non-diabetic C57 mice (Figure 6A) and this impairment was reversed by co-treatment with the BMP4 inhibitor noggin or the AT_1 receptor antagonist losartan (Figure 6B). Ang II elevated the expression of BMP4 in cultured mouse aortas and this effect was reversed by noggin or losartan (Figure 6D). Like noggin or losartan, both boldine and tempol also reversed the impairment of ACh-induced relaxations (Figure 6C) and up-regulation of BMP4 expression in Ang II-treated aortas from non-diabetic mice (Figure 6E).

Boldine reverses high glucose-induced endothelial dysfunction in mouse aortas

Exposure to high glucose (36 h) attenuated ACh-induced relaxations and this effect was reversed by co-treatment with boldine (1 μmol L^{-1}) or tempol (100 μmol L^{-1}; Figure 7A). Treatment with high glucose for 36 h raised the ROS production in MAECs as indicated by changes of the DCF-DA fluorescence intensity, while ROS elevation was prevented by pre-treatment (8 h, data not shown) and co-treatment (Figure 7B&C) with boldine or tempol. In addition, high glucose-induced reduction in eNOS phosphorylation in MAECs was reversed by co-treatment with boldine or tempol (Figure 7D).

Discussion

The present study provides experimental evidence that *in vivo* treatment with boldine effectively restored the impaired endothelium-dependent relaxations in aortas of *db/db* mice and reduced the expression of several oxidative stress markers, BMP4, nitrotyrosine and AT_1 receptors in diabetic mouse arteries. The renin-angiotensin system and associated oxidative stress play a crucial role in maintaining endothelial dysfunction in diabetic mice (Wong *et al.*, 2010a) while BMP4 is a novel important mediator of endothelial dysfunction in hypertension (Wong *et al.*, 2010a; 2013; Tian *et al.*, 2012b). To further elucidate the inhibitory effect of boldine on Ang II-mediated vascular dysfunction, Ang II was used to trigger ROS generation and thus reduce the bioavailability of NO in the vascular wall. As expected, treatment with Ang II *in vitro* impaired the endothelium-dependent relaxations accompanied by raised BMP4 expression in aortas from non-diabetic mice. Co-treatment with boldine reversed the harmful effects of Ang II on relaxations and BMP4 up-regulation. It appears that boldine reduced Ang II-induced BMP expression mainly through limiting ROS generation in the inflamed arteries as the known ROS inhibitor tempol produced the same benefits as boldine. In addition, reducing expression of AT_1 receptors and the associated ROS overproduction also played a positive role in the improvement of endothelial function in diabetic *db/db* mice induced by boldine.

Boldine had been shown to ameliorate the development of diabetes in streptozotocin-treated rats by inhibiting oxidative stress-associated tissue damage and restoring antioxidant enzyme activities (Jang *et al.*, 2000). We have recently shown that treatment with boldine reversed endothelial dysfunction in hypertensive rats through suppression of NADPH oxidase-mediated ROS overproduction (Lau *et al.*, 2012) and protected endothelial function in high glucose-induced oxidative stress through inhibiting the expression of NADPH oxidase (Lau *et al.*, 2013). In addition, boldine reduced carrageenan-induced guinea pig paw oedema probably through inhibiting the biosynthesis of pro-inflammatory prostaglandins (Backhouse *et al.*, 1994). However, the effect of boldine on endothelial dysfunction and vascular inflammatory response in type II diabetes remained undefined. The present study provides novel findings on the protective effect of boldine in endothelial cells through the inhibition of Ang II-mediated BMP4 up-regulation and ROS overproduction, under hyperglycaemic conditions.

The renin-angiotensin system and associated NADPH oxidase-derived ROS mediate endothelial dysfunction in the mouse model of type II diabetes through decreasing the bioavailability of NO (Wong *et al.*, 2010b). The present study shows that the impaired endothelium-dependent relaxations in *db/db* mouse aortas or in high glucose-treated mouse aortas were reversed by both *in vivo* and *in vitro* treatment with boldine or tempol. The improved relaxations were accompanied by restored phosphorylation of eNOS. Further support comes from experiments on cultures of mouse endothelial

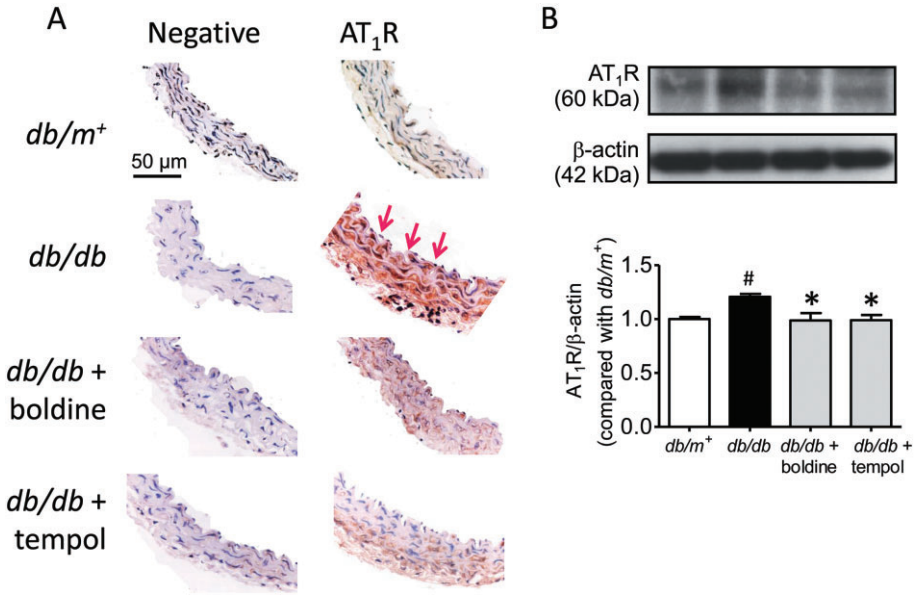

Figure 5

One week treatment with boldine or tempol reduced the expression of AT_1 receptors (AT_1R) in *db/db* mouse aortas as detected by immunohistochemistry (A) and Western blotting (B). Arrows indicate the endothelial layer of the artery. Results are shown as mean ± SEM of three to four separate experiments. $^{\#}P < 0.05$ compared with *db/m*$^{+}$ mice; $^{*}P < 0.05$ compared with *db/db* mice.

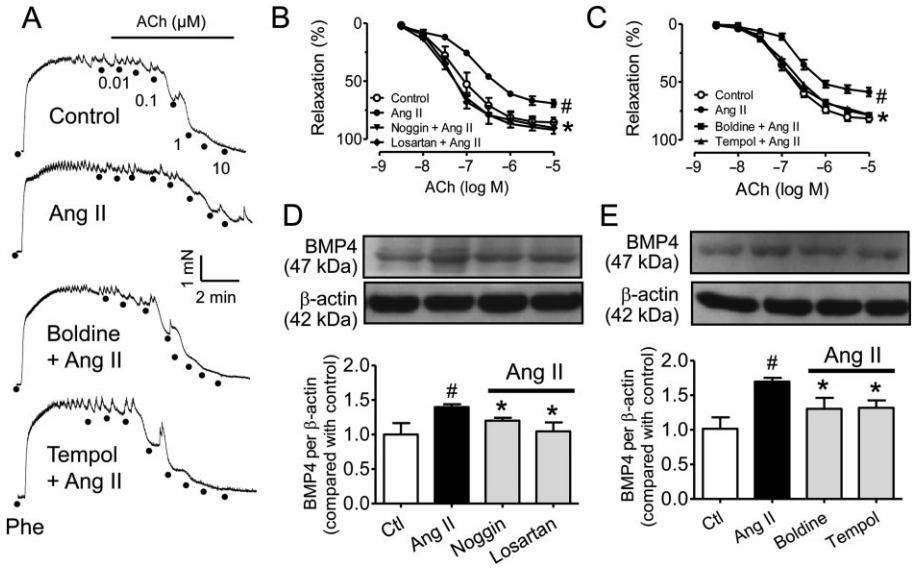

Figure 6

Ang II-induced impairment of ACh-induced relaxations was reversed by *in vitro* treatment with boldine (1 μmol L^{-1}, A & C), tempol (100 μM, A & C), noggin (100 ng ml^{-1}, A&B) and losartan (3 μmol L^{-1}, A & B) in aortas from non-diabetic mice. These four inhibitors normalized Ang II-induced increase in BMP4 expression (D & E). Results are means ± SEM of four to six separate experiments. $^{\#}P < 0.05$ compared with control; $^{*}P < 0.05$ compared with Ang II.

cells in which the high glucose-induced reduction in eNOS phosphorylation was reversed by treatment with boldine or tempol *in vitro*.

BMP4, an important matrix cytokine stimulates the expression of adhesion molecules and induces endothelial dysfunction through NADPH oxidase-dependent mechanisms (San Martin *et al.*, 2007). Up-regulation of BMP4 in *db/db* mouse aortas may involve ROS-dependent vascular inflammation (San Martin *et al.*, 2007). The present results demonstrate that endothelial dysfunction in diabetic mice was accompanied by augmented oxidative/nitrosative stress and BMP4 up-regulation. Treatment with boldine restored

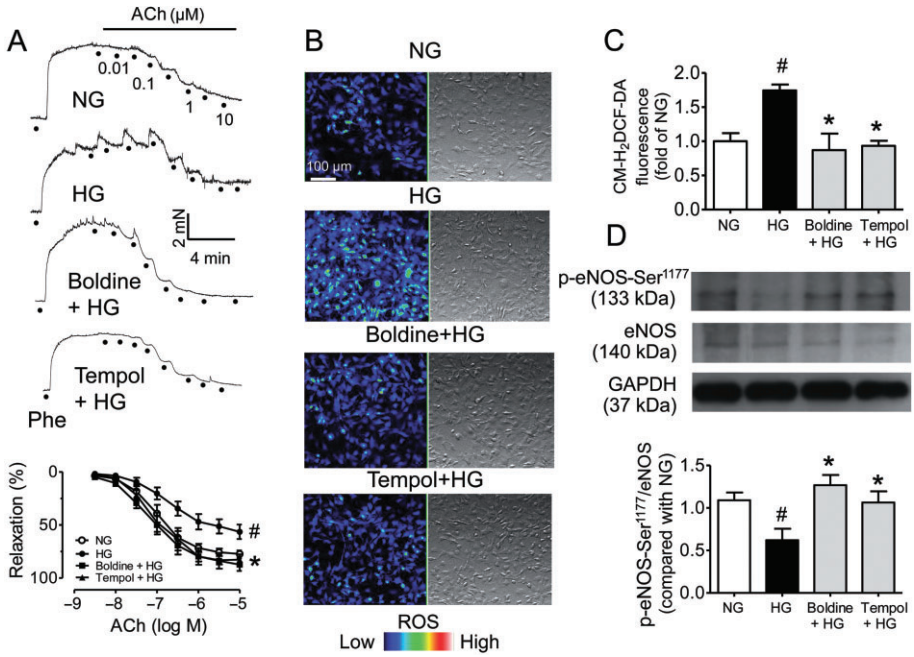

Figure 7

In vitro treatment with boldine or tempol reversed high glucose-induced impairment of ACh-induced relaxations in non-diabetic mouse aortas (A) and normalized the elevated ROS production in high glucose (HG)-treated MAECs (B & C). (D) Boldine and tempol increased the eNOS phosphorylation in HG-treated MAECs. NG: normal glucose (5 mmol L^{-1} glucose and 25 mmol L^{-1} mannitol as osmotic control for HG). Results are means ± SEM of four to six separate experiments. $^{\#}P < 0.05$ compared with NG; $^{*}P < 0.05$ compared with HG.

the impaired endothelial function in *db/db* mice and inhibited ROS overproduction and BMP4 up-regulation. Furthermore, the increased expression and activity of the renin-angiotensin system is likely to be associated with up-regulated expression of BMP4 and the latter causes ROS overgeneration, thus impairing endothelial function in diabetes, while boldine protects endothelial function through inhibiting this AT_1 receptor-BMP4-ROS axis. In the present study, boldine reversed the Ang II-induced impairment of ACh-induced relaxations and BMP4 over-expression *in vitro* and *in vivo* treatment with boldine normalized the increased expression of AT_1 receptors in *db/db* mouse aortas. The harmful effects of Ang II were inhibited by treatment with the AT_1 receptor blocker losartan or the BMP4 antagonist noggin, suggesting that the improved endothelial function in *db/db* mice is probably attributable to boldine-induced inhibition of the Ang II-mediated BMP4 and associated oxidative stress in the vascular wall. A recent study also indicates a pathophysiological role of BMP4 in Ang II-induced cardiomyocyte hypertrophy as Ang II stimulates BMP4 expression in cultured cardiac fibroblasts (Sun *et al.*, 2013). However, the present study cannot discount the possible involvement of other BMP isoforms such as BMP2, BMP6 and BMP7 in oxidative stress and vascular inflammation in diabetic mice, which deserves future investigation.

Taken together, our present results demonstrate that boldine is effective in inhibiting the AT_1 receptor-mediated cellular signalling cascade and in ameliorating endothelial dysfunction in diabetic mice. Our findings further suggest a therapeutic potential of boldine-containing medicinal herbs in alleviating diabetic vasculopathy.

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Conflict of interest

None declared.

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