Boldine Ameliorates Vascular Oxidative Stress and Endothelial Dysfunction: Therapeutic Implication for Hypertension and Diabetes

Yeh Siiang Lau, PhD, Wei Chih Ling, BSc, Dharmani Murugan, PhD, and Mohd Rais Mustafa, PhD

(See editorial: Kuala Lumpur Emerging in Vascular Biology by Paul M. Vanhoutte. Journal of Cardiovascular Pharmacology, 2015 65:4;297–298)

Abstract: Epidemiological and clinical studies have demonstrated that a growing list of natural products, as components of the daily diet or phytomedical preparations, are a rich source of antioxidants. Boldine [(*S*)-2,9-dihydroxy-1,10-dimethoxy-aporphine], an aporphine alkaloid, is a potent antioxidant found in the leaves and bark of the Chilean boldo tree. Boldine has been extensively reported as a potent "natural" antioxidant and possesses several health-promoting properties like anti-inflammatory, antitumor promoting, antidiabetic, and cytoprotective. Boldine exhibited significant endothelial protective effect in animal models of hypertension and diabetes mellitus. In isolated thoracic aorta of spontaneously hypertensive rats, streptozotocin-induced diabetic rats, and *db/db* mice, repeated treatment of boldine significantly improved the attenuated acetylcholine-induced endothelium-dependent relaxations. The endothelial protective role of boldine correlated with increased nitric oxide levels and reduction of vascular reactive oxygen species via inhibition of the nicotinamide adenine dinucleotide phosphate oxidase subunits, $p47^{phox}$ and nicotinamide adenine dinucleotide phosphate oxidase 2, and angiotensin II–induced bone morphogenetic protein-4 oxidative stress cascade with downregulation of angiotensin II type 1 receptor and bone morphogenetic protein-4 expression. Taken together, it seems that boldine may exert protective effects on the endothelium via several mechanisms, including protecting nitric oxide from degradation by reactive oxygen species as in oxidative stress–related diseases. The present review supports a complimentary therapeutic role of the phytochemical, boldine, against endothelial dysfunctions associated with hypertension and diabetes mellitus by interfering with the oxidative stress–mediated signaling pathway.

Key Words: boldine, hypertension, diabetes mellitus, oxidative stress, antioxidant, endothelial function

(*J Cardiovasc Pharmacol*™ 2015;65:522–531)

Received for publication August 22, 2014; accepted October 27, 2014.
From the Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia.
Supported by MOE High Impact Research Grant H-20001-00-E000055.
The authors report no conflicts of interest.
Reprints: Mohd R. Mustafa, PhD, Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia (e-mail: rais@um.edu.my).

INTRODUCTION

Free radical–induced oxidative stress is involved in the pathogenesis of a number of diseases, such as diabetes mellitus, hypertension, and atherosclerosis. Endothelial dysfunction, usually assessed as decreased endothelium-dependent vasodilation to vasoactive factors, such as acetylcholine (ACh), and precedes clinical symptoms and obvious vascular pathologies, is widely recognized as a major predictor of disease progression. Such endothelial dysfunction is observed early in the development of the pathology and is due, at least in part, to an excessive vascular formation of reactive oxygen species (ROS) in particular superoxide anion (O_2^-), which is a known scavenger of nitric oxide (NO).

Boldine [(*S*)-2,9-dihydroxy-1,10-dimethoxy-aporphine] is an aporphine alkaloid found abundantly in the leaves and bark of Chilean boldo tree (*Peumus boldus* molina), a widely distributed tree native to central region of Chile.[1] Over the past decade, boldine was also found as one of the major alkaloids in the bark of a local tree in northern part of Peninsular Malaysia.[2] Boldine-containing herbal teas are gaining popularity in South America, and its usage has been extended to some European countries for further pharmaceutical processing into boldine-containing concentrates. Boldine has been extensively reported earlier as a potent "natural" antioxidant and possesses several health-promoting properties like anti-inflammatory, antitumor promoting, antidiabetic, and cytoprotective. These activities of boldine are attributed to its ability to scavenge reactive free radicals.[1]

Boldine given in drinking water (100 mg/kg) prevented oxidative mitochondrial damage and possesses antidiabetic effect.[3] Boldine [20 mg/kg, 7 days, intraperitoneal (ip)] also exhibited both free radical scavenging and antinociceptive activities in mice.[4] Boldine has been indicated to have low toxicity as relatively high doses are required to cause side effects, toxicity, or lethality in several mammalian species, for example, doses of 500 and 1000 mg/kg were required to cause death of mice and guinea pigs, respectively.[1,5]

Epidemiological studies reported that dietary and supplementary intake of antioxidants may reduce coronary artery diseases or cardiovascular events.[6,7] The present article reviews the therapeutic potential of the "natural" antioxidant boldine, as a potential endothelial protective agent against the damaging effects of free radicals in oxidative stress–related diseases, such as hypertension and diabetes mellitus.

ENDOTHELIUM

The endothelium is a complex tissue system that lines the inner surface of the entire vascular system and acts as an interface between blood and smooth muscle cells of the blood vessel wall.[8,9] The vascular endothelium plays a pivotal role in the modulation of normal vascular tone by releasing short-lived vasodilators and vasoconstrictors known as endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs), respectively.[10,11] Besides controlling vascular tone, the endothelium also regulates hemostasis, thrombosis, and inflammatory responses by secreting a variety of procoagulant, anticoagulant, fibrinolytic, and inflammatory factors.[12] Under normal conditions, endothelial cells favorably release anticoagulant and EDRFs rather than other substances. EDRFs, such as NO, vasodilator prostaglandins, and endothelium-derived hyperpolarizing factors, promote vasodilation via stimulation of intracellular guanosine 3′,5′-cyclic monophosphate (cGMP) on the smooth muscle cells.[13,14] However, in pathophysiological conditions as during oxidative stress, the phenotype of endothelial cells is modified to facilitate vasoconstriction, inflammation, and thrombotic events instead of regulating normal vascular tone.[15]

ENDOTHELIAL DYSFUNCTION AND OXIDATIVE STRESS

Under normal physiological conditions, the healthy endothelial cells maintain the balance between vasodilation and vasoconstriction. NO is the most important vasodilator released by the endothelium.[16,17] Conversely, in diseased conditions, endothelium-dependent vasoconstriction becomes more prominent and endothelial dysfunction is exacerbated in the presence of various vasoconstrictors, including endothelins, angiotensin (Ang) II, cyclooxygenase-derived prostanoids, and ROS, particularly O_2^-.[18] Typically, the hallmark of endothelial dysfunction is impaired endothelium-dependent vasodilation because of attenuated NO release and increases in EDCFs.[18–20]

ROS are highly bioactive molecules or chemical species formed by incomplete reduction of oxygen, for example, O_2^-, OH, peroxyl radical, and alkoxyl radical. These molecules possess varying oxidizing potencies. Other molecules like hydrogen peroxide (H_2O_2), hypochlorous acid, ozone, and singlet oxygen also possess oxidizing capability.[21] In the past few decades, O_2^- has been identified as a "primary" ROS, which can further interact with other molecules to generate "secondary" ROS such as H_2O_2 or react rapidly with NO to form peroxynitrite ($ONOO^-$).[22] Eventually, excessive ROS production stimulates a series of oxidative cascade or oxidative stress and reduces the NO bioavailability in the blood vessel walls, leading to impairment of endothelium-dependent relaxations.[23] Oxidative stress is defined as overproduction of pro-oxidant molecules, such as ROS and nitrogen species, which can cause oxidative damage to biomolecules (lipids, proteins, DNA, RNA) and organs.[24] An increase in oxidative stress is typically attributed to either an excessive overproduction of ROS or decreased endogenous antioxidant activity. Such an imbalance plays a critical contributory role in the pathophysiology of endothelial dysfunction. Additionally, ROS has direct vasoconstrictive effect on vascular smooth muscle cells (VSMCs) or indirectly activates the release of EDCFs to the VSMCs through arachidonic acid metabolism. In addition, EDCFs may activate

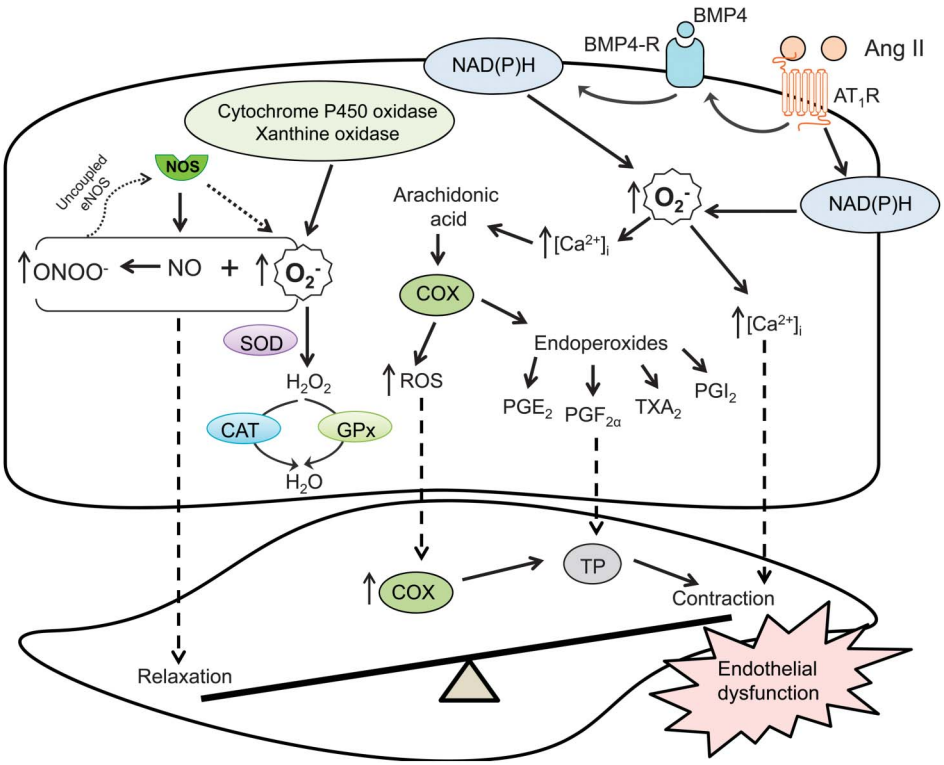

FIGURE 1. Schematic diagram showing that the overproduction of ROS leads to vasoconstriction and consequently to endothelial dysfunction in vascular vessel. The sources of ROS particularly O_2^- include NADPH oxidase, cytochrome P450 oxidase, xanthine oxidase, cyclooxgenase (COX), and uncoupling of eNOS. Various risk factors, such as hypertension and hyperglycemia, also can trigger NADPH-dependent ROS production through synthesis of BMP4 and Ang II peptide. ROS causes an increase in intracellular calcium level and the activation of arachidonic acid metabolism, which eventually result in vascular smooth muscle contraction. The production of ROS also decreases NO bioavailability through the rapid reaction between O_2^- and NO to result in $ONOO^-$ formation and eNOS uncoupling. CAT, catalase; GPx, gluthathione peroxidase; TP, thromboxane A_2 prostanoid.

endoperoxides–thromboxane A_2 prostanoid receptors in VSMCs to evoke vasoconstriction (Fig. 1).[23]

Nicotinamide Adenine Dinucleotide Phosphate Oxidase: Physiological Sources of ROS

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or NOX is a multicomponent enzyme that comprises a backbone, membrane-bound cytochrome b558, which consists of 2 subunits $p22^{phox}$ and $gp91^{phox}$ (NOX2) and other regulatory cytosolic proteins ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac).[25] There are additional 6 NOX isoforms (NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2) that have been identified, and NOX1, 2, 4, and 5 have been found in membranes of vascular cells, including endothelial cells, VSMCs, and fibroblasts.[25–28] NADPH oxidase seems to be the primary source of O_2^- production system in the endothelial cells by transferring one electron from NADPH to molecular oxygen. Furthermore, it can potentially influence the generation of ROS by other oxidant enzymes like xanthine oxidase and mitochondrial enzymes and promotes NO synthase (NOS) uncoupling.[29] An increase of oxidative stress is associated with the upregulation of oxidant enzymes, which later contribute to cardiovascular complication in hypertension and diabetes mellitus.

Endothelial Dysfunction in Hypertension

Hypertension defined as elevated blood pressure can result when total peripheral vascular resistance is increased.[30,31] The risk factors that contribute to the increase of blood pressure include obesity, insulin resistance, high alcohol intake, high salt intake (in salt-sensitive patients), aging, and stress.[31] There are 2 forms of hypertension: primary hypertension and secondary hypertension. Primary or essential hypertension is seen in almost 90% of patients presenting with hypertension with an unknown cause. It is classified as secondary hypertension if the increase in blood pressure is secondary to renal disease, endocrine disorders, or other identifiable causes.[32] Impaired endothelium-dependent relaxation has been well documented in several cardiovascular diseases, such as hypertension and atherosclerosis.[33–35] Similarly, endothelial dysfunction is also a common feature in various animal models of hypertension, including spontaneously hypertensive rats (SHRs), salt-induced hypertension, and renovascular hypertension.[34,36–38]

De Champlain et al[39] demonstrated a greater sensitivity of the vascular tissue of SHRs to oxidative stress is attributed to the excess production of vascular ROS, such as O_2^-. Of note, almost all experimental models of hypertension have been associated with the excessive production of oxidative stress. For example, Ang II– and diet-induced animal models and genetically modified hypertensive rat models have been reported to exhibit increased vascular oxidative stress via the activation of NADPH oxidase.[40,41] Furthermore, an upregulation of NADPH subunit ($p22^{phox}$ or $p47^{phox}$) has been demonstrated in aortas of hypertensive rat models, whereas NADPH oxidase knockout animal attenuated the development of hypertension, suggesting that NADPH oxidase is probably one of the major enzymatic sources of O_2^-.[42–44] Hypertensive patients are also reported to have reduced antioxidant capacity with lower expression of superoxide dismutase (SOD), catalase, and glutathione peroxidase in the whole blood and peripheral mononuclear cells.[45] Elevated blood pressure has also been reported in animal model in which glutathione synthesis was inhibited and extracellular SOD gene deleted.[46]

Excess O_2^- leads to greater vasoconstriction by altering the cellular signal transduction system characterized by an enhanced production of inositol trisphosphate and a reduced cGMP.[39] It is worth noting that apart from reduced endothelium-dependent relaxation, increased endothelium-dependent contraction also significantly contributes to the development of endothelial dysfunctions in hypertension.[11]

Endothelial Dysfunction in Diabetes Mellitus

Diabetes mellitus presents a growing health and socioeconomic problem especially in developing countries like Malaysia, and it is one of the most common cardiovascular disease worldwide, with estimated over 285 million people are affected with diabetes mellitus in 2010, at an annual growth of 2.2%.[47,48] The hyperglycemia in diabetes may result from poor glucose utilization or defective insulin secretion, insulin resistance, or both.[49] There are 2 main types of diabetes mellitus: type 1 and type 2. Type 1 diabetes is caused by pancreatic β-islet cell failure with resulting insulin deficiency, encompasses 5%–10% of diabetes diagnosed, whereas type 2 diabetes, accounting for almost 90% of diabetics, is characterized by insulin resistance.[50] It is noteworthy that an increased risk of developing type 2 diabetes in women has also been reported to be associated with the incidence of gestational diabetes during pregnancy. Gestational diabetes mellitus is defined as glucose intolerance that is first recognized during pregnancy and is at greater risk for the subsequent development of type 2 diabetes mellitus, metabolic syndrome, and cardiovascular diseases.[51,52] ROS generated by hyperglycemia are implicated in the microvascular and macrovascular complications in diabetic patients. These microvascular complications include nephropathy and retinopathy, whereas macrovascular complications include atherosclerotic cardiovascular diseases.[53]

Endothelial dysfunction has been demonstrated in both type 1 and type 2 diabetes, which is associated with decreased level of EDRFs after increased destruction by ROS.[54] Glucose oxidation is believed to be a main source of ROS generation in diabetes followed by glycation and protein kinase C activation.[55,56] Similar to hypertension, excessive oxidative stress has been associated with the increased production of O_2^- via NADPH oxidase and accounts for the endothelial dysfunction in the early stage of the disease.[7] Impaired endothelium-dependent vasodilations had been observed in several animal models of type 1 and type 2 diabetes, including streptozotocin (STZ)-induced diabetes, *db/db* mice, Otsuka Long-Evans Tokishima Fatty rats, and Goto-Kakizaki rats.[57–60]

There are increasing evidences showing that increased production of ROS interferes with NO-cGMP signaling.[54,61] In both diabetic patients and animal models, elevated generation of oxidative stress has been linked to the upregulation or activation of oxidant enzymes, such as NADPH oxidase, and impaired antioxidant enzyme system, such as SOD, catalase, and glutathione peroxidase, leading to excess ROS activity.[28,55,62,63] NADPH-induced O_2^- reacts

rapidly with NO to form the toxic $ONOO^-$ and stimulates uncoupling endothelial NOS (eNOS).[60,64] In consequence, reduced NO bioavailability leads to impaired endothelium-dependent relaxations. It has been recently demonstrated that O_2^- scavengers like tempol and SOD reversed the endothelial dysfunction in STZ-induced diabetic and *db/db* mice.[65,66] In addition, several antioxidants, such as vitamins C and E, have also been reported to prevent the development of endothelial dysfunction in diabetic patients and STZ-induced diabetic animals, suggesting the protective effect of antioxidants in reversing endothelial dysfunction in diabetic patients and animal models.[6,67]

BOLDINE AMELIORATES OXIDATIVE STRESS AND ENDOTHELIAL DYSFUNCTION

Boldine and Oxidative Stress

The degree of oxidative stress is dependent on the fine balances between the generation of ROS and the antioxidant systems. As reducing agents, antioxidants play a crucial role in protecting the cells against the excessive ROS formation by reacting with the ROS and therefore minimizing their action.[68,69] Boldine has been reported to protect the red blood cells against free radical–induced hemolytic damage, even in micromolar concentrations; inhibit spontaneous autoxidation of brain membrane lipids and prevent lipid peroxidation in human liver microsomes; protect lysozyme inactivation against 2,2-azo-bis(2-amidinopropane) dihydrochloride–derived peroxyl radical damage; and scavenge hydroxyl radicals.[3,70–73] Additionally, boldo extracts (*P. boldus*) containing boldine were found to effectively inhibit lipid peroxidation in erythrocytes.[74] Boldine has been shown in several studies to have anti-inflammatory properties via its ability in interfering with the free radical generation.[75,76]

The important enzymatic antioxidants include (1) catalase, responsible to degrade H_2O_2 into H_2O; (2) glutathione peroxidase, protects membrane lipids, proteins, and nucleic acids from oxidation by reducing H_2O_2 into H_2O; and (3) SOD, converts O_2^- into O_2 and H_2O_2. In low-density lipoprotein (LDL) receptor knockout ($LDLR^{-/-}$) mice, treatment with boldine for 12 weeks decreased atherogenic lesion formation and inhibited oxidation of LDL without altering plasma cholesterol, triglycerides, and LDL and high-density lipoprotein levels.[77] In the STZ-induced diabetic models, elevated manganese-SOD and glutathione peroxidase activities in pancreas, liver, or kidney have been demonstrated together with increased production of O_2^-.[78,79] The increased antioxidant status may be because of the activation of the first line of defense system in the host tissues against excessive levels of ROS, suggesting that excessive O_2^- production may trigger the activation of SOD, catalase, and glutathione peroxidase, which convert O_2^- to H_2O_2 and finally to H_2O. Treatment with boldine in drinking water (100 mg/kg, daily) for 8 weeks normalized the elevated manganese-SOD and glutathione peroxidase activity in pancreatic mitochondria, suggesting that the aporphine alkaloid may normalize the enzyme activities through its ROS scavenging actions.[3] In addition, treatment with boldine decreased malondialdehyde level, a reactive lipid peroxidation by-product, and carbonyls in liver, kidney, and pancreatic mitochondria in STZ-induced animals and thus implying that the beneficial effect of boldine is likely to be attributed to its antioxidant property.[3]

Several studies in cell cultures, animal models, and human vessels have shown that oxidative stress is the single most important mechanism implicated in endothelial dysfunction. One of the major ROS strongly implicated in the pathogenesis of endothelial dysfunction is the O_2^-. There are 2 important enzymatic sources of O_2^-: (1) NADPH oxidase enzyme complex, which catalyzes the reduction of molecular oxygen using NADPH substrate as an electron donor, and (2) uncoupling of eNOS, resulting in O_2^- formation instead of NO because of the deficiency of the substrate, L-arginine, and cofactor tetrahydrobiopterin.[80] Increased O_2^- could potentially inactivate NO released by the endothelial cells and in turn form a highly reactive molecule, $ONOO^-$.[50] In our previous study, we have shown that boldine effectively reduced vascular O_2^- and $ONOO^-$ in SHR and diabetic aorta.[81–83] Similarly, increased ROS production by treatment with high glucose in rat/mouse aortic endothelial cells for 48 hours was reduced by cotreatment with tempol.[82,83] Furthermore, upregulated protein expression of nitrotyrosine, a molecular marker of $ONOO^-$, was ameliorated by boldine treatment after high glucose stimulation in rat aortic endothelial cells and in *db/db* diabetic aorta. The dual antioxidative and potent ROS scavenging actions of boldine may explain its therapeutic potential against oxidative stress–associated conditions.

Boldine and Vascular NADPH Oxidase

Similar to SOD, superoxide scavenger, boldine concentration dependently (0.01–1 μM) reversed the β-NADPH–induced impaired relaxation in Sprague-Dawley rat aorta. Treatment of boldine also reduced the expression of $p47^{phox}$ in SHR and type 1 diabetic rat aortas and prevented NOX2 and $p47^{phox}$ expression in rat aortic endothelial cells induced by high glucose for 48 hours. It is worth noting that polyphenols, such as flavonoids, apart from their well-known O_2^- scavenging activity, exert their protective effects by inhibiting NADPH oxidase.[84] The polyphenols have also been demonstrated to improve endothelial function by enhancing NO signaling pathway in vascular endothelial cells in several human and rat arteries.[85,86] Hence, the present finding further demonstrates that boldine not only exhibits potent antioxidant effects but also most likely acts through the inhibition of NADPH-dependent superoxide axis leading to increased bioavailability of NO and improved endothelium-dependent relaxation.

Boldine and Ang II–Induced Bone Morphogenetic Protein-4–dependent Endothelial Dysfunction

Ang II is a bioactive product of renin–Ang system and is a potent vasoconstrictor with pro-inflammatory, mitogenic, and profibrotic actions. There are 2 receptor subtypes for Ang II, denoted as Ang II type 1 receptor (AT_1R) and Ang II type

2 receptor.[87] It is well documented that Ang II acts on the AT_1R, and this receptor subtype is found in both VSMCs and endothelial cells.[88] There are also growing evidences indicating that AT_1R not only activates the classical receptor-coupled calcium signaling pathway but is also a potent stimulator of NADPH oxidase–dependent pathway.[89] Ang II is one of the major regulators of vascular NADPH oxidase to produce O_2^- upon stimulation of AT_1R, triggering the oxidative stress cascade in vascular endothelial cells.[90] Furthermore, we and others have shown that treatment with losartan, an AT_1R blocker, significantly improved ACh-induced vasodilation and reduced the upregulation of NADPH oxidase and oxidative stress in hypertensive and diabetic animal models, supporting that Ang II induced NADPH-dependent ROS generation via AT_1R stimulation.[43,83,91] Chronic treatment with boldine (20 mg/kg, 7 days, oral) reduced AT_1R expression in *db/db* mouse aorta and decreased ROS production, suggesting that reducing Ang II–dependent ROS generation plays a contributive role in the improvement of endothelial function in diabetic *db/db* mice.[83] Pretreatment with boldine reversed the Ang II–induced impairment of endothelium-dependent relaxations by ACh in the aorta in vitro. The endothelial protective actions of boldine most likely involve inhibition of Ang II–dependent ROS production as tempol, a free radical scavenger, restored the ACh-induced relaxation of the aortic rings.[92]

Ang II–induced overproduction of ROS in the blood vessels may be mediated by increased bone morphogenetic protein-4 (BMP4) level. BMP4 is multifunctional growth factor that belongs to the transforming growth factor-β superfamily and is found in calcified atherosclerotic plaques.[93] BMP4 plays an important pathological role in vascular inflammation.[94,95] BMP4 is upregulated after exposure to disturbed flow and oxidative condition in cultured endothelial cells triggering NADPH-dependent ROS generation and inflammatory responses.[96] Bostrom et al[97] have also showed that high glucose promotes BMP4 expression in cultured human aortic endothelial cells that is associated with the vascular calcification. Moreover, BMP4 has been increasingly reported to impair endothelial function in mouse aortas either by increased ROS formation through NADPH oxidase or cyclooxygenase-2, suggesting the possibility that BMP4 may play a pivotal role in vascular diseases such as hypertension, diabetes, and atherosclerosis.[94] However, BMP4 only exerts its pro-oxidant, pro-hypertensive, and pro-inflammatory effects in the systemic arteries, such as aorta, carotid, or coronary arteries, whereas pulmonary arterial endothelial cells are resistant to the adverse effect induced by BMP4.[98] Therefore, BMP4 plays a major contributory role in Ang II–induced oxidative stress and endothelial dysfunction. Recently, we showed that incubation of Ang II for 24 hours in C57/6J mouse aorta caused impairment of the endothelium-dependent relaxations to ACh followed by an upregulation of BMP4 protein expression, and these were reversed by cotreatment with noggin (BMP4 antagonist), losartan (AT_1R inhibitor), or tempol (superoxide scavenger).[83] As expected, treatment with boldine (1 μM) also reversed the endothelial dysfunction and BMP4 upregulation induced by Ang II. Taken together, the results suggest that the endothelial protective effect of boldine may involve the downregulation of the AT_1R-BMP4-ROS generating pathway in the inflamed arteries.

BOLDINE AS A POTENT ANTIOXIDANT: THERAPEUTIC IMPLICATIONS FOR MANAGEMENT OF HYPERTENSION AND DIABETES

Oxidative stress is associated with the imbalance of excessive generation of free radicals, and low levels of antioxidants have received considerable attention in recent years. Increasing evidence indicates that endothelial dysfunctions underlies many of the cardiovascular-related diseases and is closely correlated with oxidative stress. Hence, interventions that can ameliorate oxidative stress and reduce endothelial dysfunction may offer new potential therapeutic opportunities for treating these chronic diseases. The protective role of antioxidants in oxidative stress–related diseases is well documented and forms an important complementary therapy to conventional treatments. Several studies have demonstrated that administration of antioxidants decreased the development of endothelial dysfunction in animal models of hypertension and diabetes mellitus.[67,99] Antioxidants, such as vitamins C and E, effectively improved endothelial function in several animal disease models and clinical studies.[67,100–102]

Impaired endothelium-dependent relaxation and decreased NO bioavailability are commonly observed in animal models of hypertension and diabetes mellitus (both type 1 and type 2). We investigated the endothelial protective effect of boldine treatment in a hypertensive rat model. Endothelial dysfunction in the SHRs is associated with increased systolic blood pressure (SBP) and NADPH oxidase–mediated oxidative stress. Increased NADPH-induced O_2^- production scavenges endothelium-derived NO, leading to reduced NO levels and increased formation of $ONOO^-$ which further diminishes the protective role of NO in the regulation of the vasculature.[81] Repeated treatment of the SHRs with boldine (20 mg/kg, ip) for 7 days attenuated the elevated SBP and improved the endothelium-dependent relaxations to ACh in the isolated aorta from these rats (Table 1). The result provided further support that the endothelial protective effect of boldine in hypertensive rats is at least in part because of the inhibition of NADPH-mediated superoxide production. Protein expression studies denoted that the inhibitory effect of boldine on the NADPH oxidase is associated with downregulation of the membrane-bound regulatory cytosolic protein subunit, $p47^{phox}$. This novel finding of the cellular action of boldine provides further support to the antioxidant actions and therapeutic potential of the alkaloid in the regulation of vascular tone and the maintenance of vascular patency to preserve cardiovascular health.

Similarly, boldine exhibited potent antioxidant effects in both in vitro and animal models of type 1 and type 2 diabetes mellitus.[82,83] As in hypertension, excessive generation of ROS interferes with NO signaling and plays a pathological role in the development of the vascular complications associated with diabetes. In hyperglycemia, glucose autoxidation increases

TABLE 1. Comparison of pEC_{50} and R_{max} Values of ACh-induced Endothelium-dependent and SNP-induced Endothelium-independent Relaxation in Isolated Aorta of Hypertensive and Diabetes Animals Treated With Boldine

	ACh		SNP	
Group	**pEC_{50} (−log M)**	**R_{max} (%)**	**pEC_{50} (−log M)**	**R_{max} (%)**
Hypertensive group[72]				
WKY	6.52 ± 0.48	88.44 ± 1.85	8.01 ± 0.23	100.50 ± 4.90
WKY + boldine	7.68 ± 0.21*	90.38 ± 3.05	8.37 ± 0.07	109.67 ± 2.74
SHR	7.96 ± 0.12*	40.60 ± 5.64*	7.61 ± 0.55	102.00 ± 1.97
SHR + boldine	8.28 ± 0.13†	94.67 ± 5.28†	8.47 ± 0.20	106.80 ± 4.00
Type 1 diabetes mellitus[73]				
SD	6.53 ± 0.48	89.04 ± 1.23	8.38 ± 0.09	106.80 ± 1.55
SD + boldine	6.68 ± 0.08	90.60 ± 2.69	8.05 ± 0.25	106.50 ± 1.85
Diabetes	6.80 ± 0.17	63.75 ± 3.05‡	8.58 ± 0.16	103.30 ± 2.29
Diabetes + boldine	6.70 ± 0.15	88.71 ± 2.08§	8.43 ± 0.21	102.00 ± 3.22
Type 2 diabetes mellitus[74]				
db/m$^+$	6.87 ± 0.07	82.42 ± 2.02	7.34 ± 0.11	95.54 ± 1.92
db/db	ND	26.33 ± 6.97#	7.73 ± 0.08	96.40 ± 0.97
db/db + boldine	6.83 ± 0.11	69.33 ± 4.12**	7.54 ± 0.09	104.30 ± 4.12

Data are the mean ± SEM of 6 separate experiments.
*$P < 0.05$ compared with WKY.
†$P < 0.05$ compared with SHR.
‡$P < 0.05$ compared with SD.
§$P < 0.05$ compared with diabetes.
#$P < 0.05$ compared with *db/m*$^+$.
**$P < 0.05$ compared with *db/db*.
ND, could not be derived.

production of free radicals leading to overproduction of ROS and subsequent inactivation of NO. In both STZ-induced diabetic rat (type 1 diabetes) and *db/db* mice (type 2 diabetes), endothelial dysfunctions were markedly observed by the impaired endothelium-dependent relaxations to ACh in isolated rat or mouse aorta. Acute (30 minutes pretreatment) and repeated treatment (7 days) of boldine significantly reversed the impaired endothelial function in aorta of both diabetic animals (Table 1). As in hypertensive vessels, the endothelial protective actions of boldine are strongly attributed to decreasing ROS formation and enhancing NO bioavailability via increased activity of eNOS in the endothelial cells lining the aortic wall of the diabetic animals. Incubation of rat and mouse aortic endothelial cells grown in high-glucose medium with boldine decreased the generation of O_2^- in these cells, thus protecting NO against degradation. In type 1 diabetic rats, treatment with boldine normalized the overproduction of ROS and this correlated with the downregulation of the NADPH oxidase subunits NOX2 and $p47^{phox}$. Repeated treatment with boldine in the *db/db* mouse decreased protein expression of AT_1R and BMP4 in the vascular wall. This finding suggests that in the type 2 diabetic mice, boldine may additionally have reduced the generation of O_2^- by inhibiting the AT_1R-BMP4-ROS axis and increasing eNOS phosphorylation.

On the other hand, treatment with boldine (20 mg/kg, ip) slightly decreased blood glucose levels in the type 1 diabetic rat but did not have a significant effect in the type 2 diabetic mice. Administration of STZ to rats damages pancreatic beta cells and resulted in diabetes, a mechanism involving increased formation of hydroxyl radicals and other ROS before generation and/or subsequent decay of highly reactive STZ carbonium radicals.[103] Jang et al[3] demonstrated that boldine treatment (100 $mg \cdot kg^{-1} \cdot d^{-1}$) in their drinking water for 8 weeks attenuated the development of hyperglycemia and weight loss induced by STZ. Another study also similarly showed that boldine treatment (50 $mg \cdot kg^{-1} \cdot d^{-1}$) for 10 weeks reduced the hyperglycemia of the type 1 diabetic animals.[104] The glucose-lowering effect by boldine may be strongly correlated with its cytoprotective action against oxidative damage in pancreatic β cells induced by cytotoxic effects of STZ.

Figure 2 and Table 2 summarize the vasculoprotective effect of boldine in animal models of hypertension and diabetes and the protective effects of boldine in animal models in vivo, respectively. Taken together, it seems that boldine may exert positive effects on the endothelium via several mechanisms and mainly by protecting NO from degradation via inhibiting the excessive production and scavenging of O_2^- and additionally by increasing NO bioavailability by promoting eNOS phosphorylation. In addition, boldine attenuates development of STZ-induced diabetes in rats by inhibition of O_2^- production and attenuation of peroxidation-induced product formation. The present results further support the potential use of boldine as a naturally effective antioxidant with protective actions against endothelial dysfunctions associated with oxidative stress as in chronic diseases, such as hypertension and diabetes mellitus.

PHARMACOKINETIC AND TOXICOLOGICAL STUDIES OF BOLDINE

The first reported studies on the biological disposition of boldine were performed by Jimenez and Speisky[105] in vitro using isolated rat hepatocytes and in vivo using Wistar rats,

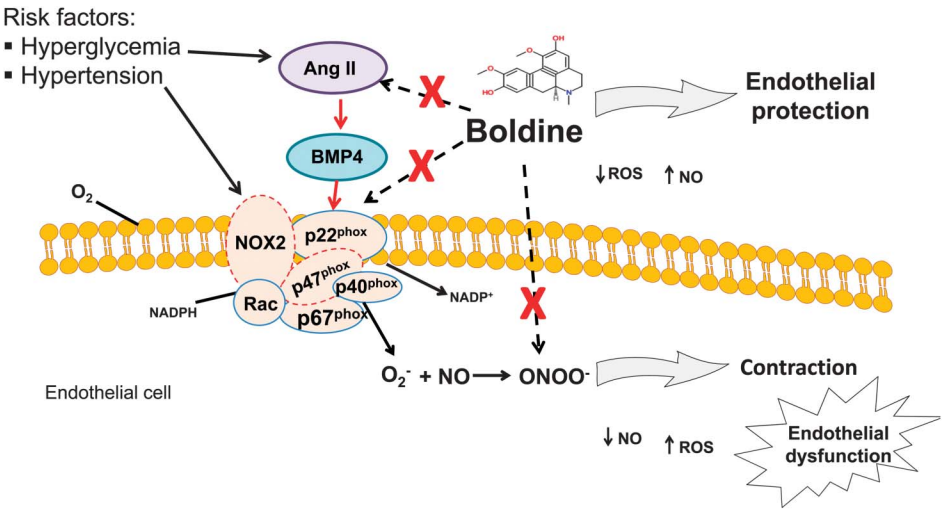

FIGURE 2. Hyperglycemia and hypertension are common risk factors involved in pathophysiology of endothelial dysfunction that is due, at least in part, to an increased NADPH-dependent ROS production. Schematic diagram showing boldine exerts its vascular protection through inhibition of NADPH-dependent ROS production, Ang II–mediated BMP4-induced ROS, and decreased $ONOO^-$ level.

respectively. The results showed that the extracellular and intracellular concentrations of boldine declined time dependently to respective concentrations of 20 and 344 μM at 60 minutes in isolated rat hepatocyte suspension from an initial concentration of 200 μM boldine. This declining concentration of boldine was attributed to the biotransformation process and a lowering of the net flow of boldine molecules into the cells. However, it is worth noting that the intracellular concentration of boldine after 60 minutes was still sufficient to warrant its antioxidant and hepatoprotective activities as it compares with the effective boldine concentration (10–30 μM) needed to protect rat liver microsomal membranes from lipid peroxidation.[71,106] Such antioxidative and cytoprotective effects of boldine were predicted to be sustainable after 150 minutes and with the expected intracellular concentration of boldine of approximately 50 μM.[105] In the in vivo studies, boldine concentration declined rapidly in plasma after a single oral (50 or 75 mg/kg) or an intravenous

TABLE 2. Summary of Protective Effects, Therapeutic Doses, and Route of Administration of Boldine in Animal Models In Vivo

Animal Model	Dose and Duration	Route of Administration	Tissues	Effect	Reference
STZ-induced diabetic rats	100 mg/kg daily for 8 wk	Drinking water	Liver, pancreas, kidney	Attenuation of hyperglycemia and weight loss Decreased malondialdehyde and carbonyl level Normalized manganese-SOD and glutathione peroxidase enzyme activities	Jang et al[3]
STZ-induced diabetic rats	50 mg/kg daily for 10 wk	Oral administration	Kidney	Attenuation of hyperglycemia and high blood pressure Prevention of kidney damage and protection of renal parenchyma	Hernandez-Salinas et al[104]
SHR	20 mg/kg daily for 1 wk	ip Injection	Thoracic aorta	Improved endothelial function Reduced oxidative stress Downregulation of $P47^{phox}$ subunit expression	Lau et al[81]
STZ-induced diabetic rats	20 mg/kg daily for 1 wk	ip Injection	Thoracic aorta	Improved endothelial function Reduced oxidative stress	Lau et al[82]
Diabetic *db/db* mice	20 mg/kg daily for 1 wk	Oral administration	Thoracic aorta	Improved endothelial function Reduced oxidative stress Downregulation of BMP4, nitrotyrosine, and AT_1R expression	Lau et al[83]
LDL receptor knockout mice	1or 5 mg per mouse per day, 5 times a week for 12 wk	Oral administration	Aortic trunk	Decreased in atherosclerotic lesion formation in mouse aorta Inhibited oxidation of LDL	Santanam et al[77]

(10 or 20 mg/kg) administration in rats.[105] After its oral administration, absorption of boldine into the plasma was rapid with a first-order type of kinetics and the maximal plasma concentration was detected between first 15 and 30 minutes. Once absorbed, boldine concentration in the liver increases 3- to 4-fold higher than those detected in brain and almost 10-fold higher than those found in heart. Again, Jimenez and Speisky[105] have also noted that the estimated maximal concentration of boldine that had reached the liver was 72–88 μM, which was 4- to 5-fold greater than the concentrations needed to exert its hepatoprotective activity in vitro.[71,106] Likewise, the boldine concentration found in the heart was approximately 7.2–8.8 μM, which was 7- to 8-fold higher than those used to protect endothelial function in vitro.[82,83] The aorta is the most important blood vessel that carries the oxygen-rich blood from the heart to the entire body. These pharmacokinetic studies demonstrated that repeated oral or ip administration (20 mg/kg) of boldine for 7 days was sufficient to protect against endothelial dysfunction in SHR and diabetic aortas as concur with the effective endothelial protective concentrations (ie, 1 μM) in the isolated aortas.

Boldine is a relatively safe drug judging from the many animal studies and its widespread use as over-the-counter drug supplement. A relatively high boldine concentration is needed to induce lethality in laboratory animal tests. Kreitmair[5] demonstrated that an oral administration of 500–1000 mg/kg of boldine was required to cause death in mice and guinea pigs, respectively. Another study showed that a lower dose of intravenous boldine administration (250 and 50 mg/kg) was needed to induce death in mice and guinea pigs, respectively.[5] Similarly, the LD_{50} of boldine by ip injection in mice was reported as 250 mg/kg, similar to that lethal dose of boldine administered intravenously.[107]

LIMITATION OF THE STUDY AND FUTURE WORKS

Despite mixed outcomes from clinical trials of vitamins C and E, the good safety profile of boldine and its potent antioxidant action should provide new impetus to conduct preliminary investigations of the effectiveness of the alkaloid either for treatment or as prophylaxis in hypertensive and diabetic patients. Combining boldine with current treatments for hypertension and diabetes mellitus would be another area of study given that drug combinations have been shown to be successful for the treatment of hypertension.[108,109] Nevertheless, randomized trial results from human population studied by Heart Protection Study Collaborative Group showed that the combined antioxidant regimen did not produce any significant effect on 5-year mortality from vascular and nonvascular diseases.[110] Similarly, meta-analysis by several clinical trials demonstrated supplementation of beta-carotene and vitamin E did not demonstrate any benefit.[111] Therefore, longer treatment durations of boldine should be studied to investigate its long-term effect. Finally, other potential studies with boldine should also examine other mechanisms, including potential anti-inflammatory and the central actions of the aporphine alkaloid.

REFERENCES

1. O'Brien P, Carrasco-Pozo C, Speisky H. Boldine and its antioxidant or health-promoting properties. *Chem Biol Interact.* 2006;159:1–17.
2. Mukhtar MR, Martin M-T, Domansky M, et al. Phoebegrandines A and B, proaporphine-tryptamine dimers, from Phoebe grandis. *Phytochemistry*. 1997;45:1543–1546.
3. Jang YY, Song JH, Shin YK, et al. Protective effect of boldine on oxidative mitochondrial damage in streptozotocin-induced diabetic rats. *Pharmacol Res.* 2000;42:361–371.
4. Zhao Q, Zhao Y, Wang K. Antinociceptive and free radical scavenging activities of alkaloids isolated from Lindera angustifolia Chen. *J Ethnopharmacol.* 2006;106:408–413.
5. Kreitmair H. Pharmacological effects of the alkaloid of *Peumus boldus* molina. *Die Pharmazie.* 1952;7:507–511.
6. Heitzer T, Finckh B, Albers S, et al. Beneficial effects of alpha-lipoic acid and ascorbic acid on endothelium-dependent, nitric oxide-mediated vasodilation in diabetic patients: relation to parameters of oxidative stress. *Free Radic Biol Med.* 2001;31:53–61.
7. Heitzer T, Schlinzig T, Krohn K, et al. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation*. 2001;104:2673–2678.
8. Luscher TF, Noll G. The pathogenesis of cardiovascular disease: role of the endothelium as a target and mediator. *Atherosclerosis*. 1995;118 (suppl):S81–S90.
9. Mas M. A close look at the endothelium: Its role in the regulation of Vasomotor tone. *Eur Urol.* 2009;8:48–57.
10. Furchgott RF, Vanhoutte PM. Endothelium-derived relaxing and contracting factors. *FASEB J.* 1989;3:2007–2018.
11. Vanhoutte PM, Tang EH. Endothelium-dependent contractions: when a good guy turns bad! *J Physiology.* 2008;586(pt 22):5295–5304.
12. Disse J, Vitale N, Bader MF, et al. Phospholipase D1 is specifically required for regulated secretion of von Willebrand factor from endothelial cells. *Blood*. 2009;113:973–980.
13. Perrella MA, Hildebrand FL Jr, Margulies KB, et al. Endothelium-derived relaxing factor in regulation of basal cardiopulmonary and renal function. *The Am Journal Physiology.* 1991;261(2 pt 2):R323–R328.
14. Vanhoutte PM, Scott-Burden T. The endothelium in health and disease. Texas heart Institute journal/from the Texas heart Institute of St Luke's Episcopal Hospital. *Tex Children's Hosp.* 1994;21:62–67.
15. Pratico D. Antioxidants and endothelium protection. *Atherosclerosis*. 2005;181:215–224.
16. Fenster BE, Tsao PS, Rockson SG. Endothelial dysfunction: clinical strategies for treating oxidant stress. *Am Heart J.* 2003;146:218–226.
17. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980; 288:373–376.
18. Radenkovic M, Stojanovic M, Potpara T, et al. Therapeutic approach in the improvement of endothelial dysfunction: the current state of the art. *Biomed Res Int.* 2013;2013:252158.
19. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res.* 2000;87:840–844.
20. Lerman A, Burnett JC Jr. Intact and altered endothelium in regulation of vasomotion. *Circulation*. 1992;86(suppl 6l):III12–III19.
21. Leonarduzzi G, Sottero B, Poli G. Targeting tissue oxidative damage by means of cell signaling modulators: the antioxidant concept revisited. *Pharmacol Ther*. 2010;128:336–374.
22. Valko M, Leibfritz D, Moncol J, et al. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol.* 2007;39:44–84.
23. Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension*. 2003;42:1075–1081.
24. Uttara B, Singh AV, Zamboni P, et al. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr Neuropharmacol.* 2009;7:65–74.
25. Jiang F, Zhang Y, Dusting GJ. NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair. *Pharmacol Rev.* 2011;63:218–242.
26. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev.* 2007;87: 245–313.

27. Wingler K, Hermans JJ, Schiffers P, et al. NOX1, 2, 4, 5: counting out oxidative stress. *Br J Pharmacol.* 2011;164:866–883.
28. Guzik TJ, Sadowski J, Kapelak B, et al. Systemic regulation of vascular NAD(P)H oxidase activity and nox isoform expression in human arteries and veins. *Arterioscler Thromb Vasc Biol.* 2004;24:1614–1620.
29. Ray R, Shah AM. NADPH oxidase and endothelial cell function. *Clin Sci (Lond).* 2005;109:217–226.
30. Carretero OA, Oparil S. Essential hypertension. Part I: definition and etiology. *Circulation*. 2000;101:329–335.
31. Virdis A, Duranti E, Taddei S. Oxidative stress, vascular damage in hypertension: role of angiotensin II. *Int J Hypertens.* 2011;2011: 916310.
32. Acelajado MC, Calhoun DA. Resistant hypertension, secondary hypertension, and hypertensive crises: diagnostic evaluation and treatment. *Cardiol Clinics.* 2010;28:639–654.
33. Ajay M, Achike FI, Mustafa AM, et al. Direct effects of quercetin on impaired reactivity of spontaneously hypertensive rat aortae: comparative study with ascorbic acid. *Clin Exp Pharmacol Physiol.* 2006;33: 345–350.
34. Benndorf RA, Appel D, Maas R, et al. Telmisartan improves endothelial function in patients with essential hypertension. *J Cardiovasc Pharmacol.* 2007;50:367–371.
35. Jiang J, Valen G, Tokuno S, et al. Endothelial dysfunction in atherosclerotic mice: improved relaxation by combined supplementation with L-arginine-tetrahydrobiopterin and enhanced vasoconstriction by endothelin. *Br J Pharmacol.* 2000;131:1255–1261.
36. Heitzer T, Wenzel U, Hink U, et al. Increased NAD(P)H oxidase-mediated superoxide production in renovascular hypertension: evidence for an involvement of protein kinase C. *Kidney Int.* 1999;55:252–260.
37. Hermann M, Camici G, Fratton A, et al. Differential effects of selective cyclooxygenase-2 inhibitors on endothelial function in salt-induced hypertension. *Circulation*. 2003;108:2308–2311.
38. Lockette W, Otsuka Y, Carretero O. The loss of endothelium-dependent vascular relaxation in hypertension. *Hypertension*. 1986;8(6 pt 2): II61–II66.
39. de Champlain J, Wu R, Girouard H, et al. A ELM, Laplante MA, Wu L. Oxidative stress in hypertension. *Clin Exp Hypertens.* 2004;26:593–601.
40. Lee MY, Griendling KK. Redox signaling, vascular function, and hypertension. *Antioxid Redox Signal.* 2008;10:1045–1059.
41. Touyz RM, Briones AM. Reactive oxygen species and vascular biology: implications in human hypertension. *Hypertension Res.* 2011;34:5–14.
42. Dikalova A, Clempus R, Lassegue B, et al. Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. *Circulation*. 2005;112:2668–2676.
43. Fukui T, Ishizaka N, Rajagopalan S, et al. p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. *Circ Res.* 1997;80:45–51.
44. Zalba G, Beaumont FJ, San Jose G, et al. Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. *Hypertension*. 2000;35:1055–1061.
45. Redon J, Oliva MR, Tormos C, et al. Antioxidant activities and oxidative stress byproducts in human hypertension. *Hypertension*. 2003;41:1096–1101.
46. Welch WJ, Chabrashvili T, Solis G, et al. Role of extracellular superoxide dismutase in the mouse angiotensin slow pressor response. *Hypertension*. 2006;48:934–941.
47. Letchuman GR, Wan Nazaimoon WM, Wan Mohamad WB, et al. Prevalence of diabetes in the Malaysian National health Morbidity Survey III 2006. *Med J Malaysia.* 2010;65:180–186.
48. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Pract.* 2010;87:4–14.
49. Wei M, Ong L, Smith MT, et al. The streptozotocin-diabetic rat as a model of the chronic complications of human diabetes. *Heart Lung Circ.* 2003;12:44–50.
50. Jay D, Hitomi H, Griendling KK. Oxidative stress and diabetic cardiovascular complications. *Free Radic Biol Med.* 2006;40:183–192.
51. Bellamy L, Casas JP, Hingorani AD, et al. Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. *Lancet*. 2009;373:1773–1779.
52. Gunderson EP, Chiang V, Pletcher MJ, et al. History of gestational diabetes mellitus and future risk of atherosclerosis in mid-life: the coronary artery risk development in young adults study. *J Am Heart Assoc.* 2014;3:e000490.
53. Johansen JS, Harris AK, Rychly DJ, et al. Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. *Cardiovasc Diabetol.* 2005;4:5.
54. De Vriese AS, Verbeuren TJ, Van de Voorde J, et al. Endothelial dysfunction in diabetes. *Br J Pharmacol.* 2000;130:963–974.
55. Maritim AC, Sanders RA, Watkins JB 3rd. Diabetes, oxidative stress, and antioxidants: a review. *J Biochem Mol Toxicol.* 2003;17:24–38.
56. Wiernsperger NF. Oxidative stress as a therapeutic target in diabetes: revisiting the controversy. *Diabetes Metab.* 2003;29:579–585.
57. Gupte S, Labinskyy N, Gupte R, et al. Role of NAD(P)H oxidase in superoxide generation and endothelial dysfunction in Goto-Kakizaki (GK) rats as a model of nonobese NIDDM. *PloS One.* 2010;5:e11800.
58. Kim YK, Lee MS, Son SM, et al. Vascular NADH oxidase is involved in impaired endothelium-dependent vasodilation in OLETF rats, a model of type 2 diabetes. *Diabetes*. 2002;51:522–527.
59. Tian XY, Wong WT, Xu A, et al. Rosuvastatin improves endothelial function in db/db mice: role of angiotensin II type 1 receptors and oxidative stress. *Br J Pharmacol.* 2011;164:598–606.
60. Woodman OL, Malakul W. 3',4'-Dihydroxyflavonol prevents diabetes-induced endothelial dysfunction in rat aorta. *Life Sci.* 2009; 85(1-2):54–59.
61. van den Oever IA, Raterman HG, Nurmohamed MT, et al. Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus. *Mediators Inflamm.* 2010;2010:792393.
62. Guzik TJ, Sadowski J, Guzik B, et al. Coronary artery superoxide production and nox isoform expression in human coronary artery disease. *Arterioscler Thromb Vasc Biol.* 2006;26:333–339.
63. Olukman M, Orhan CE, Celenk FG, et al. Apocynin restores endothelial dysfunction in streptozotocin diabetic rats through regulation of nitric oxide synthase and NADPH oxidase expressions. *J Diabetes Complications.* 2010;24:415–423.
64. Zou MH, Cohen R, Ullrich V. Peroxynitrite and vascular endothelial dysfunction in diabetes mellitus. *Endothelium*. 2004;11:89–97.
65. Moien-Afshari F, Ghosh S, Elmi S, et al. Exercise restores endothelial function independently of weight loss or hyperglycaemic status in db/db mice. *Diabetologia*. 2008;51:1327–1337.
66. Serizawa K, Yogo K, Aizawa K, et al. Nicorandil prevents endothelial dysfunction due to antioxidative effects via normalisation of NADPH oxidase and nitric oxide synthase in streptozotocin diabetic rats. *Cardiovasc Diabetol.* 2011;10:105.
67. Keegan A, Walbank H, Cotter MA, et al. Chronic vitamin E treatment prevents defective endothelium-dependent relaxation in diabetic rat aorta. *Diabetologia*. 1995;38:1475–1478.
68. Gomes EC, Silva AN, de Oliveira MR. Oxidants, antioxidants, and the beneficial roles of exercise-induced production of reactive species. *Oxid Med Cell Longev.* 2012;2012:756132.
69. Nordberg J, Arner ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med.* 2001;31: 1287–1312.
70. Jimenez I, Garrido A, Bannach R, et al. Protective effects of boldine against free radical-induced erythrocyte lysis. *Phytother Res.* 2000;14: 339–343.
71. Kringstein P, Cederbaum AI. Boldine prevents human liver microsomal lipid peroxidation and inactivation of cytochrome P4502E1. *Free Radic Biol Med.* 1995;18:559–563.
72. Cassels BK, Asencio M, Conget P, et al. Structure-antioxidative activity relationships in benzylisoquinoline alkaloids. *Pharmacol Res.* 1995;31: 103–107.
73. Speisky H, Cassels BK, Lissi EA, et al. Antioxidant properties of the alkaloid boldine in systems undergoing lipid peroxidation and enzyme inactivation. *Biochem Pharmacol.* 1991;41:1575–1581.
74. Schmeda-Hirschmann G, Rodriguez JA, Theoduloz C, et al. Free-radical scavengers and antioxidants from *Peumus boldus* Mol. ("Boldo"). *Free Radic Res.* 2003;37:447–452.
75. Backhouse N, Delporte C, Givernau M, et al. Anti-inflammatory and antipyretic effects of boldine. *Agents Actions.* 1994;42:114–117.
76. Milian L, Estelles R, Abarca B, et al. Reactive oxygen species (ROS) generation inhibited by aporphine and phenanthrene alkaloids semi-synthesized from natural boldine. *Chem Pharm Bull (Tokyo).* 2004; 52:696–699.

77. Santanam N, Penumetcha M, Speisky H, et al. A novel alkaloid antioxidant, Boldine and synthetic antioxidant, reduced form of RU486, inhibit the oxidation of LDL in-vitro and atherosclerosis in vivo in LDLR(-/-) mice. *Atherosclerosis*. 2004;173:203–210.
78. Kakkar R, Mantha SV, Radhi J, et al. Increased oxidative stress in rat liver and pancreas during progression of streptozotocin-induced diabetes. *Clin Sci (Lond)*. 1998;94:623–632.
79. Wohaieb SA, Godin DV. Alterations in free radical tissue-defense mechanisms in streptozocin-induced diabetes in rat. Effects of insulin treatment. *Diabetes*. 1987;36:1014–1018.
80. Akinci B, Celtik A, Yener S, et al. Prediction of developing metabolic syndrome after gestational diabetes mellitus. *Fertil Steril.* 2010;93: 1248–1254.
81. Lau YS, Machha A, Achike FI, et al. The aporphine alkaloid boldine improves endothelial function in spontaneously hypertensive rats. *Exp Biol Med (Maywood)*. 2012;237:93–98.
82. Lau YS, Tian XY, Huang Y, et al. Boldine protects endothelial function in hyperglycemia-induced oxidative stress through an antioxidant mechanism. *Biochem Pharmacol*. 2013;85:367–375.
83. Lau YS, Tian XY, Mustafa MR, et al. Boldine improves endothelial function in diabetic db/db mice through inhibition of angiotensin II-mediated BMP4-oxidative stress cascade. *Br J Pharmacol.* 2013; 170:1190–1198.
84. Al-Awwadi NA, Araiz C, Bornet A, et al. Extracts enriched in different polyphenolic families normalize increased cardiac NADPH oxidase expression while having differential effects on insulin resistance, hypertension, and cardiac hypertrophy in high-fructose-fed rats. *J Agric Food Chem.* 2005;53:151–157.
85. Zhang H, Zhang J, Ungvari Z, et al. Resveratrol improves endothelial function: role of TNF {alpha} and vascular oxidative stress. *Arterioscler Thromb Vasc Biol.* 2009;29:1164–1171.
86. Rush JW, Quadrilatero J, Levy AS, et al. Chronic resveratrol enhances endothelium-dependent relaxation but does not alter eNOS levels in aorta of spontaneously hypertensive rats. *Exp Biol Med (Maywood).* 2007;232:814–822.
87. Burnier M, Brunner HR. Angiotensin II receptor antagonists. *Lancet*. 2000;355:637–645.
88. Higuchi S, Ohtsu H, Suzuki H, et al. Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology. *Clin Sci (Lond).* 2007;112:417–428.
89. Nguyen Dinh Cat A, Montezano AC, Burger D, Touyz RM. Angiotensin II, NADPH oxidase, and redox signaling in the vasculature. *Antioxid Redox Signal.* 2013;19:1110–1120.
90. Griendling KK, Minieri CA, Ollerenshaw JD, et al. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res.* 1994;74:1141–1148.
91. Bayorh MA, Ganafa AA, Eatman D, et al. Simvastatin and losartan enhance nitric oxide and reduce oxidative stress in salt-induced hypertension. *Am J Hypertens.* 2005;18:1496–1502.
92. Wong WT, Tian XY, Xu A, et al. Angiotensin II type 1 receptor-dependent oxidative stress mediates endothelial dysfunction in type 2 diabetic mice. *Antioxid Redox Signal.* 2010;13:757–768.
93. Dhore CR, Cleutjens JP, Lutgens E, et al. Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. *Arterioscler Thromb Vasc Biol.* 2001;21:1998–2003.
94. Miriyala S, Gongora Nieto MC, Mingone C, et al. Bone morphogenic protein-4 induces hypertension in mice: role of noggin, vascular NADPH oxidases, and impaired vasorelaxation. *Circulation*. 2006;113:2818–2825.
95. Sorescu GP, Sykes M, Weiss D, et al. Bone morphogenic protein 4 produced in endothelial cells by oscillatory shear stress stimulates an inflammatory response. *J Biol Chem.* 2003;278:31128–31135.
96. Sorescu GP, Song H, Tressel SL, et al. Bone morphogenic protein 4 produced in endothelial cells by oscillatory shear stress induces monocyte adhesion by stimulating reactive oxygen species production from a nox1-based NADPH oxidase. *Circ Res.* 2004;95:773–779.
97. Bostrom KI, Jumabay M, Matveyenko A, et al. Activation of vascular bone morphogenetic protein signaling in diabetes mellitus. *Circ Res.* 2011;108:446–457.
98. Csiszar A, Labinskyy N, Jo H, et al. Differential proinflammatory and prooxidant effects of bone morphogenetic protein-4 in coronary and pulmonary arterial endothelial cells. *Am J Physiol Heart Circ Physiol.* 2008;295:H569–H577.
99. Ting HH, Timimi FK, Boles KS, et al. Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Clin Invest.* 1996;97:22–28.
100. Heitzer T, Yla Herttuala S, Wild E, et al. Effect of vitamin E on endothelial vasodilator function in patients with hypercholesterolemia, chronic smoking or both. *J Am Coll Cardiol.* 1999;33:499–505.
101. Mullan BA, Ennis CN, Fee HJ, et al. Pretreatment with intravenous ascorbic acid preserves endothelial function during acute hyperglycaemia (R1). *Clin Exp Pharmacol Physiol.* 2005;32:340–345.
102. Bohm F, Settergren M, Pernow J. Vitamin C blocks vascular dysfunction and release of interleukin-6 induced by endothelin-1 in humans in vivo. *Atherosclerosis*. 2007;190:408–415.
103. Ohkuwa T, Sato Y, Naoi M. Hydroxyl radical formation in diabetic rats induced by streptozotocin. *Life Sci.* 1995;56:1789–1798.
104. Hernandez-Salinas R, Vielma AZ, Arismendi MN, et al. Boldine prevents renal alterations in diabetic rats. *J Diabetes Res.* 2013;2013:593672.
105. Jimenez I, Speisky H. Biological disposition of boldine: in vitro and in vivo studies. *Phytother Res.* 2000;14(4):254–260.
106. Cederbaum AI, Kukielka E, Speisky H. Inhibition of rat liver microsomal lipid peroxidation by boldine. *Biochem Pharmacology*. 1992;44: 1765–1772.
107. Levy-Appert-Collin MC, Levy J. Galenic preparations from *Peumus boldus* leave (Monimiacea) (author's transl) [in French]. *J Pharm Belg.* 1977;32:13–22.
108. Goyal J, Khan ZY, Upadhyaya P, et al. Comparative study of high dose mono-therapy of amlodipine or telmisartan, and their low dose combination in mild to moderate hypertension. *J Clin Diagn Res.* 2014;8: HC08–HC11.
109. McLachlan J, Beattie E, Murphy MP, et al. Combined therapeutic benefit of mitochondria-targeted antioxidant, MitoQ10, and angiotensin receptor blocker, losartan, on cardiovascular function. *J Hypertens.* 2014;32:555–564.
110. MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet*. 2002;360:23–33.
111. Vivekanthan DP, Penn MS, Sapp SK, et al. Use of antioxidant vitamins for preventation of cardiovascular disease: meta-analysis of randomised trials. *Lancet*. 2003;361:2017–2023.