

Berberine prevents hyperglycemia-induced endothelial injury and enhances vasodilatation via adenosine monophosphate-activated protein kinase and endothelial nitric oxide synthase

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Aims Endothelial dysfunction is a key event that links obesity, diabetes, hypertension, and cardiovascular diseases. The aim of the present study was to examine the protective effect of the alkaloid drug berberine against hyperglycemia-induced cellular injury and endothelial dysfunction.

Methods and results In both cultured endothelial cells and blood vessels isolated from rat aorta, berberine concentration dependently enhanced phosphorylation of endothelial nitric oxide synthase (eNOS) at Ser¹¹⁷⁷ and promoted the association of eNOS with heat shock protein 90 (HSP90), leading to an increased production of nitric oxide. Furthermore, berberine attenuated high glucose-induced generation of reactive oxygen species, cellular apoptosis, nuclear factor- κ B activation, and expression of adhesion molecules, thus suppressing monocyte attachment to endothelial cells. In mouse aortic rings, berberine elicited endothelium-dependent vasodilatations and alleviated high glucose-mediated endothelial dysfunction. All these beneficial effects of berberine on the endothelium were abolished by either pharmacological inhibition of adenosine monophosphate-activated protein kinase (AMPK) or adenovirus-mediated overexpression of a dominant negative version of AMPK.

Conclusion Berberine protects against endothelial injury and enhances the endothelium-dependent vasodilatation, which is mediated in part through activation of the AMPK signalling cascade. Berberine or its derivatives may be useful for the treatment and/or prevention of endothelial dysfunction associated with diabetes and cardiovascular disease.

1. Introduction

The endothelium is a single layer of cells that covers the inner surface of all blood vessels.^{1,2} In addition to forming a physical barrier to protect the vessel wall, the endothelium secretes a large number of bioactive substances involved in the modulation of vascular tone, coagulation, cell proliferation, and inflammation.¹ Endothelial dysfunction, as characterized by decreased bioavailability of nitric oxide (NO) and impaired endothelium-dependent vasodilatations, is intimately associated with insulin resistance and diabetes.^{1–3} Chronic dysfunction of the endothelium is a well-established antecedent of several cardiovascular disorders, including hypertension, atherosclerosis, diabetic vasculopathy, and

myocardial infarction. In addition, dysfunction of peripheral vascular endothelium at arteriolar and capillary levels contributes to the pathogenesis of insulin resistance and the metabolic syndrome by impeding the capillary recruitment and blood flow in skeletal muscle.⁴ In obese subjects and diabetic patients, hyperglycemia, elevated free fatty acids, and pro-inflammatory factors cause a series of pathophysiological changes in the endothelium, including the intracellular accumulation of reactive oxygen species (ROS), endothelial nitric oxide synthase (eNOS) uncoupling, cell apoptosis, and inflammation, which in turn contribute to accelerated damage and dysfunction of the endothelium.⁵ It is now firmly established that endothelial dysfunction is a key mediator that links obesity, diabetes, and its cardiovascular complications. Therefore, therapeutic interventions aiming at alleviation of endothelial dysfunction should represent one of the most effective strategies for the treatment

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and/or prevention of cardiovascular disease associated with metabolic syndrome.

Berberine ($[C_{20}H_{18}NO_4]^+$), an isoquinoline alkaloid originally isolated from the Chinese herb *Coptis chinensis* (Huanglian), is an anti-microbial drug routinely prescribed for the treatment of diarrhoea in many Asian countries. In addition, a growing body of evidence from both animal and clinical investigations suggests that berberine is beneficial in combating against diabetes and cardiovascular disease. In both humans and rodents with diabetes, berberine decreases hyperglycemia and alleviates insulin resistance possibly by the activation of adenosine monophosphate-activated protein kinase (AMPK).^{6–9} It has been used in the treatment of congestive heart failure in humans.^{10,11} In addition, the anti-hypertensive, anti-atherosclerotic and vasculo-protective properties of berberine have been reported in various animal models.^{12–14} However, the mechanisms underlying its cardiovascular effects remain poorly understood.

The present study evaluated the direct endothelial actions of berberine and elucidated the molecular pathways involved in the process. It shows that berberine potently stimulates eNOS activation and endothelial NO production through a mechanism dependent on AMPK. In addition, the present findings demonstrate that berberine protected hyperglycemia-induced endothelial dysfunction by suppressing intracellular ROS accumulation and apoptosis and by inhibiting NF- κ B activation.

2. Methods

2.1 Materials

Anti-phospho-AMPK α (Thr172) and anti-total AMPK α and anti-heat shock protein 90 (HSP90) antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-phospho-eNOS (Ser¹¹⁷⁷), anti-eNOS antibodies, and endothelial cell growth supplement (ECGS) were obtained from BD Transduction Laboratories (Jose, CA, USA). Berberine, phenylephrine, acetylcholine, N^G-nitro-L-arginine methyl ester (L-NAME), and compound C were purchased from Sigma (St Louis, MO, USA).

2.2 Cell culture and adenovirus infection

Human umbilical vein endothelial cells (HUVECs, obtained from American Type Culture Collection) at passages 4–8 were cultured on gelatin-coated flasks in M199 supplemented with 15% foetal bovine serum (FBS, Invitrogen), 0.1 mg/mL heparin, and 0.03 mg/mL ECGS. Cells were infected with recombinant adenoviruses encoding a dominant negative version of AMPK or luciferase (as control) at 50 plaque forming unit/cell as described.¹⁵

2.3 Co-immunoprecipitation and western blot

HUVECs were dissolved in a lysis buffer and were subjected to immunoprecipitation with anti-eNOS antibody as we described previously.¹⁵ Cell lysates were clarified by centrifugation at 15 300 rpm at 4°C for 15 min and were then pre-incubated with 50 μ L protein A/G beads at 4°C for 1 h to remove non-specific bindings. The remaining supernatant was incubated with anti-eNOS antibody at 4°C overnight, and the immuno-complexes were precipitated by adding 50 μ L of protein A/G beads at 4°C for 2 h. Immuno-precipitated complexes or cell lysates were separated by SDS-PAGE, transferred onto nylon membrane and probed with various primary antibodies as indicated in the figures and legends. The proteins were visualized by the chemiluminescence detection.

To quantify the phosphorylation of AMPK, Akt, and eNOS, the membrane was probed with anti-phospho-antibodies specific to

these three proteins, respectively, and then incubated with a stripping buffer (10% SDS, 0.1 M beta-mercaptoethanol, and 1 M Tris-HCl pH 6.7) for 30 min at 50°C. After washing, the membrane was re-probed with anti-total AMPK, eNOS, or Akt. The relative band densities were quantified using the MultiAnalyst software package (Bio-Rad). The results were expressed as phosphorylated proteins relative to total proteins.

2.4 Measurement of nitric oxide content

HUVEC grown in six-well dishes (~90% confluence) were starved in a serum-free medium for 4 h. The cells were treated with various concentrations of berberine dissolved in DMSO. The same volume of DMSO was included in each control group. NO release was determined by the measurement of nitrite (NO₂⁻) and nitrate (NO₃⁻) levels using a Sievers Nitric oxide analyzer (Boulder, USA) as described.¹⁵

2.5 Monocyte adhesion assay

Human THP1 cells were used for the evaluation of monocyte adhesion to endothelial cells. HUVECs were grown to confluence in 24-well plates, and were incubated with 5 or 30 mM glucose, and treated with berberine or other compounds as specified for 48 h. THP1 cells were labelled with 1.5 μ M calcein-AM (Invitrogen, CA, USA) for 1 h at 37°C in RPMI 1640 containing 1% FBS. Labelled THP1 cells (10⁵/100 μ L) were then added to HUVECs and incubated for 1 h at 37°C. After incubation, the medium containing monocytes was aspirated, and the monolayer was gently washed with PBS three times to remove the unbound monocytes. The adhesions to monocytes were determined using an Olympus IX 81 fluorescent microscope with a 20 \times objective. Five randomly selected fields were captured for each experimental condition. The number of adherent THP1 cells was counted using National Institutes of Health Image software, and the values were expressed as adhered cells per field.

2.6 Analysis of NF- κ B activation

HUVECs were grown to confluence in 100 mm culture dishes and were then incubated for 48 h with 5 or 30 mM glucose in the presence of berberine or various inhibitors as indicated. Nuclear and cytoplasmic extracts were prepared using mammalian nuclear protein extraction reagent by following the manufacturer's instructions (Pierce, Rockford, IL, USA). Nuclear protein (30 μ g) was resolved on a 12% SDS-PAGE, transferred to nylon membrane, and probed with anti-human NF- κ B (p65) monoclonal antibody to evaluate its protein abundance. Activation of the NF- κ B p50 subunit was detected on 3 μ g of nuclear protein extracts using a commercial kit (EZ-Detect Transcription Factor Kit; Pierce).

2.7 Quantification of cell surface vascular cell adhesion molecule-1 and inter-cellular adhesion molecule-1 expression

HUVECs grown in 96-well plates were treated with 3.7% formaldehyde (pH 7.4) containing 0.1 M L-lysine monohydrochloride and 0.01 M sodium *m*-periodate. The expression levels of vascular cell adhesion molecule-1 (VCAM-1) and inter-cellular adhesion molecule-1 (ICAM-1) on the cell surface were quantified using *in situ* ELISA as described.¹⁶

2.8 Aortic ring preparation

All experiments were approved by Institutional Animal Research Ethics Committees. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Male Sprague–Dawley rats (250–300 g) were sacrificed by cervical dislocation and exsanguinated. The thoracic aorta was dissected out, removed of adhering connective tissues, and cut into ring segments. Each ring was suspended between two stainless wire hooks in a 10 mL organ chambers filled with Krebs solution that contained

(mM): 119 NaCl, 4.7 KCl, 25 NaHCO₃, 2.5 CaCl₂, 1 MgCl₂, 1.2 KH₂PO₄, and 11 D-glucose. The upper wire was connected to a force-displacement transducer (Grass Instruments, RI, USA) and the lower one fixed to the bottom of the organ bath. The bathing solution was oxygenated by 95% O₂ and 5% CO₂ and maintained at 37°C (pH 7.4). Rings were placed under an optimal resting tension of 15 mN and allowed to equilibrate for 90 min. In some rings, the endothelial layer was mechanically disrupted by gently rubbing the luminal surface of the artery, and the removal of the endothelium was confirmed by the absence of relaxation in response to 1 μM acetylcholine (data not shown).

2.9 Isometric force measurement

All rings were first contracted by 1 μM phenylephrine (to evaluate the contractility) and then relaxed to 1 μM ACh (to assess the endothelial integrity). A sustained contraction was induced by 1 or 0.3 μM phenylephrine in rings with or without endothelium, and berberine was then added cumulatively to evoke relaxations. In experiments examining the role of endothelium-derived NO and that of AMPK, rings were first exposed for 30 min to 100 μM L-NAME or 5 μM compound C, respectively, before contracting them with phenylephrine. Berberine was then applied cumulatively. The effect of the vehicle was also tested. A detailed method for functional study on mouse aortas can be found in Supplementary material online.

2.10 Determination of cyclic guanosine monophosphate content

Aorta rings were incubated with berberine or/and compound C as described above. At the end of the incubation, they were rapidly frozen in liquid nitrogen and then transferred into 1 mL of 80% ethanol. The samples were homogenized with ethanol and incubated for 30 min at 4°C. The samples containing ethanol was dried under a stream of nitrogen at 60°C, and the dried extract was dissolved in an assay buffer for the quantification of the cyclic guanosine monophosphate (cGMP) content using a commercial kit from Amersham Biosciences (Uppsala, Sweden). The amount of cGMP in each thoracic aorta ring was standardized to fmol cGMP mg⁻¹ protein.

2.11 Data analysis

Data are shown as mean ± SEM and *n* refers to the number of samples studied. Several rings prepared from the same artery were examined in parallel, and two consecutive cumulative concentration-response curves for acetylcholine were established in each ring under different treatments. Curves were analyzed by non-linear curve fitting using Graphpad software (version 3.0) and statistical difference between curves was analyzed by two-way ANOVA followed by Bonferroni post-tests. The negative logarithm of the concentration that produced half (pIC₅₀) of the maximal relaxation was determined. *P*-values < 0.05 were regarded as indicating statistically significant difference.

3. Results

3.1 Berberine induces endothelial nitric oxide synthase activation and nitric oxide production through activation of adenosine monophosphate-activated protein kinase

Berberine increased eNOS phosphorylation at the activation site (Ser¹¹⁷⁷) of this enzyme in a time- and concentration-dependent manner (Figure 1A–D). Sixty minutes after treatment with 25 μM of berberine, eNOS phosphorylation at Ser¹¹⁷⁷ increased 2.8-fold over its basal levels, whereas the expression of total eNOS remained unchanged. Co-immunoprecipitation analysis revealed that treatment of cells with berberine enhanced the interaction between eNOS and HSP90 (Figure 1E), an important step for the maximum activation of eNOS activity.¹⁷ The NO production from HUVECs was also elevated significantly after berberine stimulation (Figure 1F). This stimulatory effect of berberine on NO production was abolished by pre-incubation of HUVECs with the eNOS inhibitor L-NAME, indicating that the elevated NO release by berberine is eNOS-dependent.

Treatment of HUVECs with berberine induced phosphorylation of the α-catalytic subunit of AMPK at its activation site (Thr¹⁷²) in a time- and concentration-dependent manner (Figure 2). In contrast, this compound did not affect

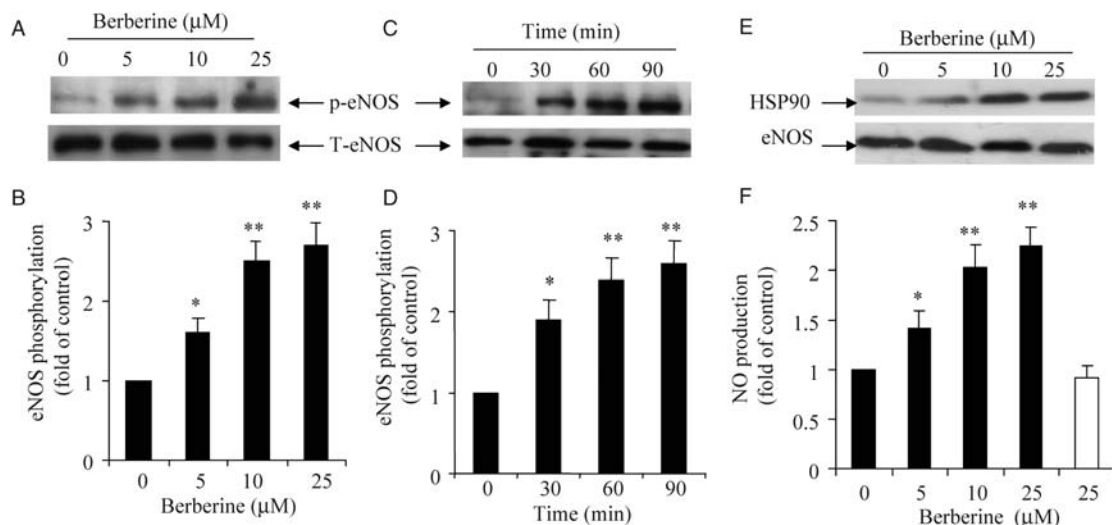


Figure 1 Effects of berberine on the phosphorylation of eNOS, the interaction between HSP90 and eNOS, and on NO production in endothelial cells. HUVECs were stimulated with different concentrations of berberine (A and B) for 60 min or 25 μM berberine for different periods as indicated (C and D). The phosphorylation of eNOS at Ser¹¹⁷⁹ (p-eNOS) and the level of total eNOS protein (T-eNOS) in cell lysates were analyzed by western blotting. (E) Cells were treated without or with different concentrations of berberine for 60 min. Cell lysates were subjected to immunoprecipitation using anti-eNOS antibody. The immunoprecipitated complexes were separated by SDS-PAGE and probed with the anti-HSP90 or eNOS antibody as indicated. (F) NO production in the absence (black bar) or presence (blank bar) of L-NAME was measured and expressed as fold over the control cells treated without berberine. Black bar: **P* < 0.05; ***P* < 0.01 vs. control (*n* = 4–6).

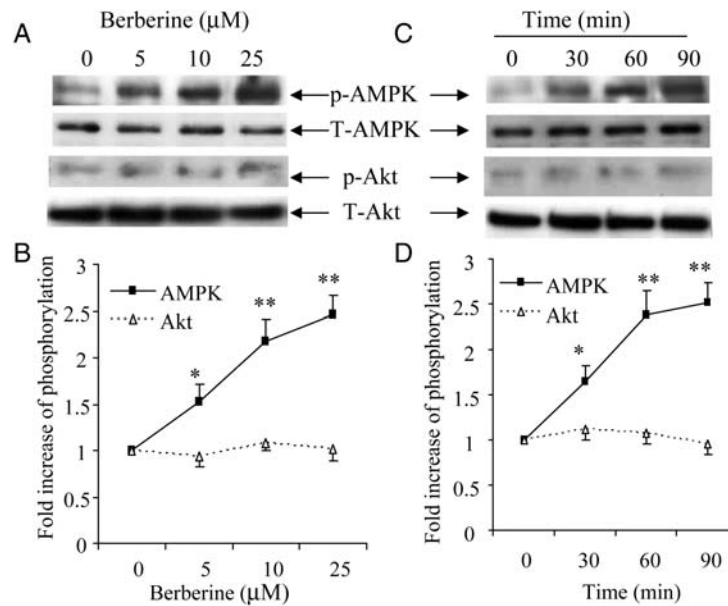


Figure 2 Effects of berberine on phosphorylation of AMPK and Akt at the sites of their activation in HUVECs. Cells were incubated with different concentrations of berberine for 60 min (A and B) or 25 μ M berberine for various periods as specified (C and D). Cell lysates were separated by SDS-PAGE and probed with antibodies against phosphorylated or anti-total AMPK or Akt as indicated. * $P < 0.05$; ** $P < 0.01$ vs. control ($n = 3-5$).

phosphorylation of Akt at either of its two activation sites (Ser⁴⁷³ or Thr³⁰⁸). In addition, berberine did not alter intracellular cAMP levels in HUVECs (data not shown).

Pretreatment of HUVECs with compound C (a selective inhibitor of AMPK) blocked berberine-induced phosphorylation of eNOS at Ser¹¹⁷⁷ and its interaction with HSP90, consequently leading to a markedly reduced production of NO (Figure 3). In cells infected with recombinant adenovirus encoding luciferase (as a control), berberine still induced phosphorylation of eNOS at Ser¹¹⁷⁷, enhanced eNOS-HSP90 interaction, and increased endothelial NO production. In contrast, these effects of berberine were abolished following adenovirus-mediated expression of a dominant negative version of Myc-tagged AMPK (DN AMPK)¹⁵ (Figure 3). The Akt inhibitor SH-5 and the protein kinase A (PKA) inhibitor H89 did not affect berberine-induced phosphorylation of eNOS, eNOS-HSP90 interaction, and NO production.

3.2 Berberine decreases hyperglycemia-induced intracellular reactive oxygen species accumulation and cellular apoptosis through the adenosine monophosphate-activated protein kinase/endothelial nitric oxide synthase signalling pathway

The intracellular concentration of DCF-sensitive ROS in HUVECs was increased ~ 2.5 -fold following incubation with high glucose (30 mM) compared with 5 mM glucose (Figure 4A). Treatment of cells with berberine resulted in a marked reduction of high glucose-induced intracellular ROS generation. The inhibitory effect of berberine on ROS generation was attenuated in cells pretreated with the AMPK inhibitor compound C or by adenovirus-mediated expression of DN AMPK, but not by the Akt inhibitor SH-5 and or the PKA inhibitor H89. Real-time PCR analysis and enzyme activity assays showed that berberine did not affect either the expression or the activity of several key enzymes involved in the anti-oxidant defence response, including glutathione peroxidase, catalase, and superoxide

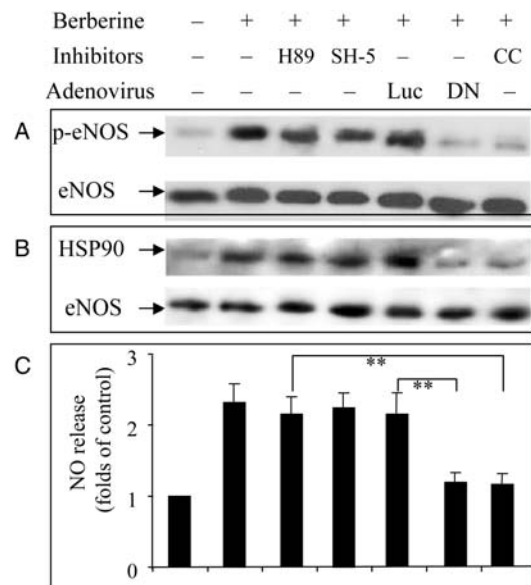


Figure 3 Berberine-induced activation of eNOS and NO release were attenuated by the inhibition of AMPK, but not Akt and PKA in HUVECs. Cells were pretreated with the AMPK inhibitor compound C (CC, 1 μ M), the Akt inhibitor SH-5 (5 μ M), the PKA inhibitor H89 (1 μ M) for 30 min, or infected with recombinant adenovirus expressing luciferase (Luc) or a dominant negative version (DN) of AMPK for 24 h, and then treated with 25 μ M berberine for another hour. The phosphorylation of eNOS at Ser¹¹⁷⁷ (A) and the interaction between eNOS and HSP90 (B), and NO production (C) were determined as in Figure 1. ** $P < 0.01$ ($n = 4-5$).

dismutase (data not shown). In addition, berberine had no obvious effect on the expression of the two membrane subunits (p22^{phox} and gp91^{phox}) or the three cytosolic subunits (p47^{phox}, p67^{phox}, and Rac-1) of the NAD(P)H oxidase, and did not alter the intracellular localization of this enzyme (data not shown).

Following the exposure of HUVECs to high glucose (30 mM) for 72 h, the percentage of apoptosis as assessed by DNA

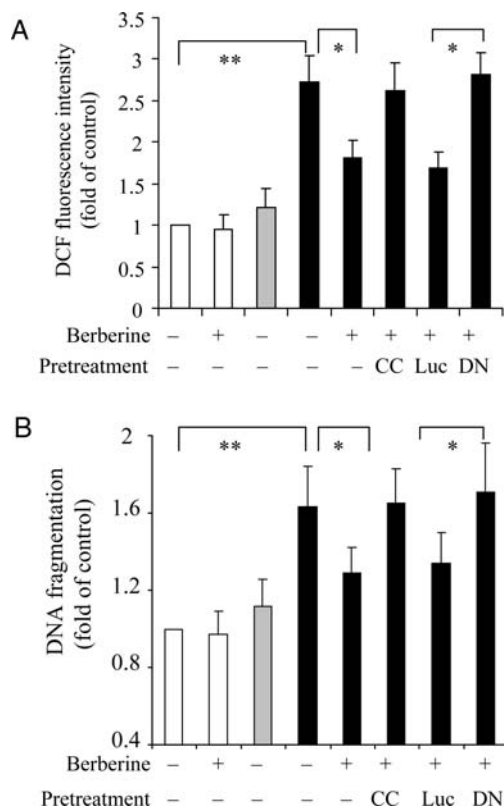


Figure 4 The inhibitory effects of berberine on high glucose-induced ROS accumulation and apoptosis are dependent on AMPK. HUVECs grown in low glucose (5 mM, blank bar), low glucose + 25 mM mannitol (grey bar), high glucose (30 mM, black bar) were pretreated with 1 μ M compound C (CC) for 30 min, or infected with recombinant adenovirus expressing luciferase (Luc) or dominant negative (DN) AMPK for 24 h, and then treated with berberine for another 36 h. (A) Intracellular DCF-sensitive ROS generation in HUVECs; (B) levels of apoptosis determined by DNA fragmentation. * $P < 0.05$; ** $P < 0.01$ ($n = 5-6$).

fragmentation was increased by 62% compared with cells treated with 5 mM glucose and 25 mM mannitol (as an osmotic control) (Figure 4B). The magnitude of increase in high glucose-induced elevation of apoptosis was attenuated significantly following treatment with berberine. This protective effect of berberine on cellular apoptosis was diminished by compound C, the dominant negative version of AMPK, and was partially inhibited by L-NAME.

3.3 Berberine-induced activation of adenosine monophosphate-activated protein kinase and nitric oxide production are involved in the inhibition of monocyte adhesion to endothelial cells

High glucose caused an ~4.5-fold increase in the number of THP1 monocyte cells binding to HUVECs (Figure 5A and B), which was associated with a significant elevation in NF- κ B activation (Figure 5C and D) and the expression of adhesion molecules, including VCAM-1 and ICAM-1 (Figure 5E and F). Treatment with berberine reduced the high glucose-induced NF- κ B activation and the expression of the two adhesion molecules, leading to a significant decrease in the number of adherent THP1 monocytes on HUVECs. The inhibitory effects of berberine on high glucose-induced NF- κ B activation, expression of adhesion molecules, and monocyte

adhesion to endothelial cells were abolished by compound C, but were only partially reversed by L-NAME.

Real-time PCR analysis showed that berberine also suppressed high glucose-induced elevation of several pro-inflammatory cytokines and chemokines, including TNF α , IL1 β , IL8, and MCP1, all of which are the well-established targets of NF- κ B (Supplementary material online, Figure S1).

3.4 Berberine stimulates vasodilatation and prevents high glucose-induced vascular injury in rat aorta

To demonstrate the physiological relevance of the above findings, the effects of berberine on AMPK/eNOS activation and NO-dependent vasodilatation in isolated rat aortic rings were investigated. Consistent with the findings in HUVECs, berberine induced the phosphorylation of AMPK at Thr¹⁷² and eNOS at Ser¹¹⁷⁷ and increased the cGMP levels in the isolated rat aorta (Figure 6A and B). These effects were diminished by pre-incubation with compound C.

In phenylephrine-contracted rings with the endothelium, berberine caused concentration-dependent relaxation with a pIC₅₀ of 5.66 ± 0.04 . Consistent with our previous study,¹⁸ the removal of the endothelium resulted in a partial reduction in berberine-evoked vasorelaxation (Figure 6C and D, see Supplementary material online, Table S1), suggesting that both endothelium-dependent and independent mechanisms are attributable to the vasodilating effect of this compound. Treatment with 100 μ M L-NAME or 3 μ M ¹H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, guanylate cyclase inhibitor) attenuated berberine-induced relaxation to a comparable extent as did by the removal of the endothelium. Compound C at 5 μ M produced the similar inhibitory effects on berberine-induced relaxation as L-NAME or ODQ.

Like in rat aortas, berberine-induced relaxations in mouse aortas were significantly inhibited by 100 μ M L-NAME or by endothelial denudation (Figure 7A) and the relaxation was inhibited by 5 μ M compound C (Figure 7B, see Supplementary material online, Table S2). Exposure (36 h) of mouse aortas to high glucose but not mannitol impaired acetylcholine-induced endothelium-dependent relaxations, which could be largely inhibited by 0.5 μ M berberine (Figure 7C and D, see Supplementary material online, Table S3). Compound C at 5 μ M prevented the effect of berberine on alleviation of high glucose-induced impairment in endothelium-dependent vasodilatation (Figure 7D, see Supplementary material online, Table S3). Incubation with 0.5 μ M berberine did not affect phenylephrine-induced contraction (see Supplementary material online, Figure S2).

4. Discussion

Although both clinical and animal studies demonstrate the therapeutic potential of berberine for the treatment of hypertension, atherosclerosis, and heart disease,^{10-12,14} the underlying mechanism remains elusive. The present study provides both *in vitro* and *ex vivo* evidence showing that the cardiovascular protection by berberine is mediated at least in part by its activation of AMPK. Berberine counteracts several adverse effects of hyperglycemia on the endothelium, including the inhibition of high glucose-induced ROS accumulation and cellular apoptosis, a hallmark of

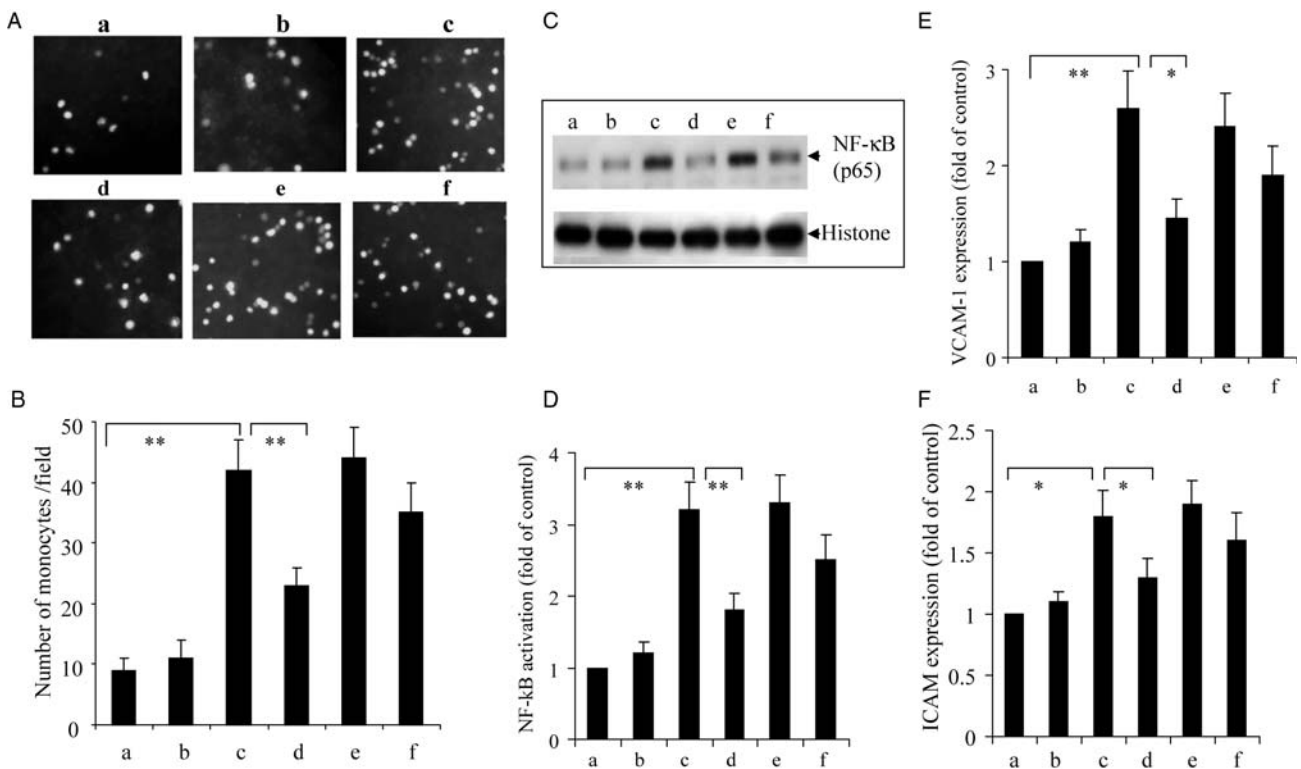


Figure 5 Effects of berberine on high glucose-induced monocyte adhesion, NF- κ B activation and expression of adhesion molecules in HUVECs. Cells were incubated with 5 mM glucose alone (a), 5 mM glucose + 25 mM mannitol (b), 30 mM glucose (c), 30 mM glucose + 25 μ M berberine (d), 30 mM glucose + 25 μ M berberine and 1 μ M compound C (e), 30 mM glucose + 25 μ M berberine and 100 μ M L-NAME (f) for 48 h. (A) Representative micrographs of monocytes adhered to endothelial cells. The adhesion assay was conducted as described in Methods section. (B) Quantification data for monocyte adhered to HUVECs. (C) Representative blot for NF- κ B (p65) expression in nuclear extracts. (D) NF- κ B p50 binding activity as measured by ELISA. (E and F) Cell surface expression of VCAM-1 and ICAM-1. * P < 0.05; ** P < 0.01 (n = 4–6).

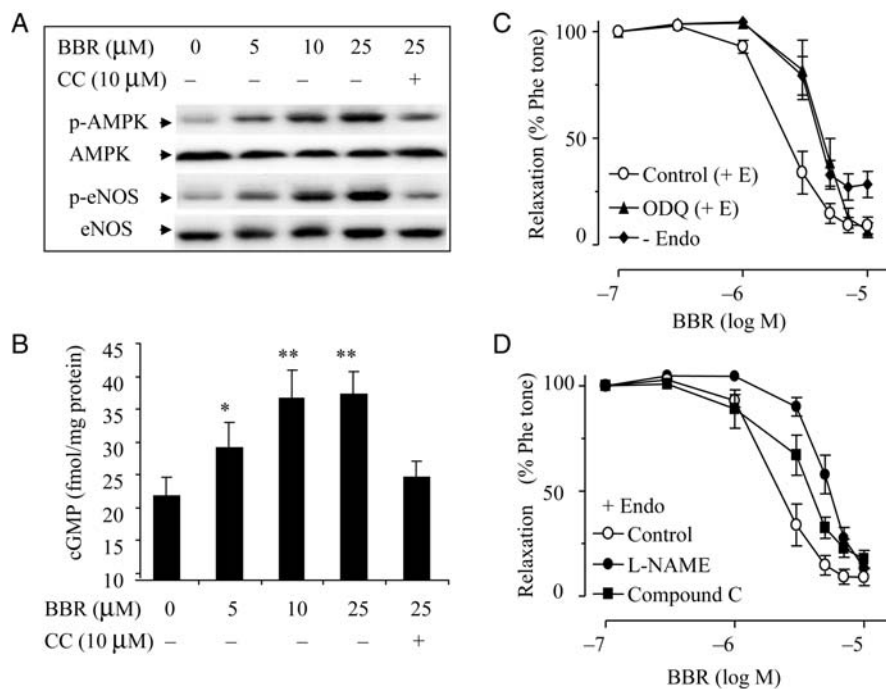


Figure 6 Effects of berberine on phosphorylation of AMPK and eNOS, cGMP levels and vasorelaxations in the isolated rat aorta. Rat aortic rings were incubated with berberine (BBR) at the concentration range of 0.1–10 μ M in the presence or absence of compound C (CC) for 1 h. Tissue extracts were subjected to western blot analysis for phospho and total AMPK or eNOS as indicated (A). The cGMP concentrations in the tissue extracts were determined by immunoassay (B). * P < 0.05; ** P < 0.01 vs. control or berberine+compound C-treated group (n = 5–6). (C and D) The relaxing effect of berberine was attenuated by treatment with L-NAME, ODQ, or compound C in aortas with the endothelium or by removal of the functional endothelium. Data are mean \pm SEM of seven separate experiments in each group.

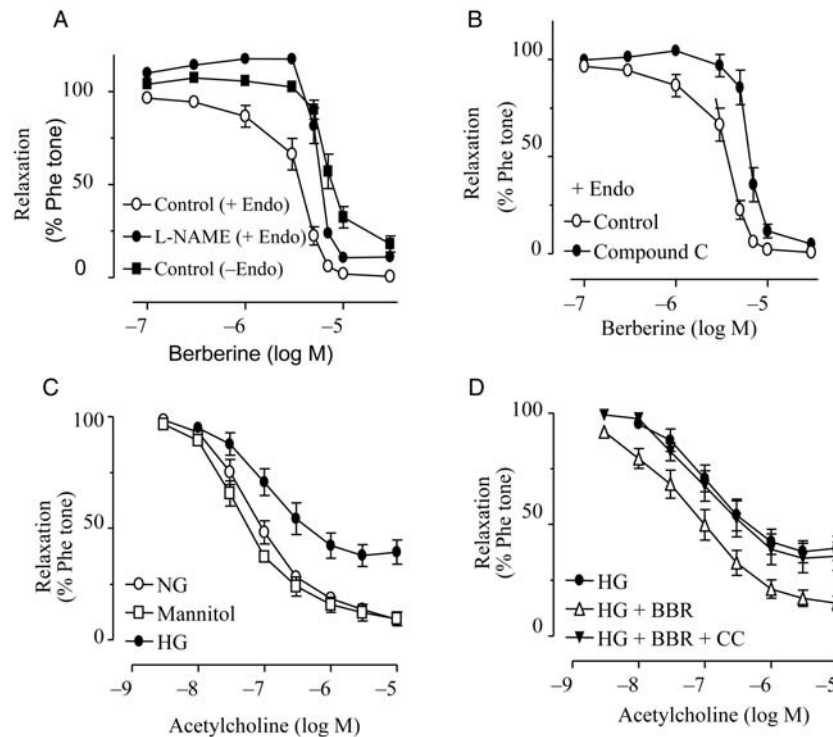


Figure 7 Effect of berberine on vasorelaxation of mouse aorta rings under normal and high glucose conditions. (A and B) Relaxing effects of berberine in mouse aortas were attenuated by treatment with 100 μM L-NAME or by removal of the endothelium or by 5 μM compound C, respectively. (C) Acetylcholine-induced relaxations in mouse aortas were impaired by chronic exposure (36 h) to high glucose (HG) but not mannitol. (D) Acetylcholine-induced relaxation under high glucose condition was improved by 0.5 μM berberine (BBR), whereas this effect of berberine was abolished by 5 μM compound C (CC). NG, normal glucose. Data are mean \pm SEM of 6–10 experiments.

vascular injury. Furthermore, this alkaloid compound suppresses high glucose-mediated NF- κ B activation, the expression of adhesion molecules and monocyte adhesion to endothelial cells, a key event in the early stages of atherosclerosis.⁵ These *in vitro* findings are further supported by our *ex vivo* observation that berberine protects high glucose-induced impairment in endothelium-dependent vasodilatation in mouse aorta. These findings suggest that berberine might be useful for the prevention of vascular dysfunctions associated with obesity and diabetes.

Several protein kinases, including AMPK, Akt, and PKA, have been identified as the upstream kinases of eNOS by phosphorylating eNOS at its activation site (Ser¹¹⁷⁷).¹⁷ The present study demonstrates that berberine-mediated phosphorylation of eNOS, the production of NO and cGMP, and endothelium-dependent vasodilatation are abolished by pharmacological or genetic inhibition of AMPK. In contrast, the pharmacological inhibitors of either Akt or PKA have little effect on the endothelial actions of this alkaloid compound. These findings highlight a central role of AMPK in mediating the endothelial NO production and vasodilatation induced by berberine.

AMPK is a cellular energy sensor that plays a central role in regulating glucose homeostasis and insulin sensitivity. Several animal studies demonstrate that the anti-diabetic effects of metformin^{19,20} are mediated, at least in part, by AMPK activation. In addition to its beneficial metabolic effects, a growing body of evidence suggests that AMPK is an important regulator of vascular homeostasis.²¹ A number of agents and factors with cardiovascular benefits, including statins,²² resveratrol,²³ metformin,²⁴ the PPAR γ agonists thiazolidinediones,²⁵ the PPAR α agonist

fenofibrate,²⁶ estradiol,²⁷ α -lipoic acid,²⁸ high-density lipoprotein,²⁹ adiponectin,^{15,30,31} and VEGF,^{32,33} have been shown to induce AMPK activation in endothelial cells. Besides its role as a direct upstream kinase mediating eNOS phosphorylation at its activation site, AMPK also enhance eNOS activity by promoting the complex formation between eNOS and HSP90, an essential step required for the maximal activation of eNOS.^{15,27}

In addition to increasing the eNOS activity and NO production, AMPK activation has several other additional beneficial effects on the endothelium, such as suppression of hyperglycemia-induced ROS production by promoting mitochondrial biogenesis³⁴ and inhibiting NAD(P)H oxidase activity,³⁵ alleviation of free fatty acids-induced lipotoxicity,³⁶ inhibition of TNF α -induced NF- κ B activation,³⁷ and protection of endothelial cells from apoptosis.^{31,38} Consistent with these observations, the present study shows that the suppressive effects of berberine on hyperglycemia-induced ROS accumulation, apoptosis, and endothelial inflammation are also dependent on AMPK activation. Taken in conjunction, these results suggest an obligatory role of AMPK in conferring the endothelial actions of berberine, and further support the notion that the activation of AMPK by pharmacological interventions may represent a new therapeutic strategy for the treatment of endothelial dysfunction. Notably, L-NAME does not affect the ability of berberine to decrease hyperglycemia-induced ROS accumulation and only causes a partial reduction in berberine-mediated suppression of hyperglycemia-induced NF- κ B and monocyte adhesion, suggesting that AMPK activation by berberine inhibits hyperglycemia-induced ROS production and inflammation in the endothelium through a mechanism

independent of its effects on NO production. Indeed, our gene profiling analysis shows that berberine increases the expression of mitochondrial uncoupling protein 2 (UCP2) (W.Y. and X.A., unpublished results), a well-established anti-oxidant via inhibition of mitochondria ROS production.³⁹ Notably, a recent report shows that both the anti-diabetic drug metformin and AICAR attenuates hyperglycemia-induced oxidative stress in the endothelium through AMPK-dependent upregulation of UCP2 *in vitro* and *in vivo*.⁴⁰ Therefore, it is possible that the suppressive effect of berberine on hyperglycemia-induced ROS accumulation is mediated at least in part by inducing the expression of this anti-oxidant protein.

Two upstream kinases, including LKB1 and calcium/calmodulin-dependent kinase kinase β , has been implicated in the phosphorylation of AMPK α subunit at Thr¹⁷².²¹ However, a recent study shows that activation of AMPK by berberine is not dependent on either of these two upstream kinases.⁷ Notably, berberine has been shown to inhibit mitochondria respiration by inhibiting the respiratory complex I activity in L6 myotube, which in turn activate AMPK by increasing the AMP/ATP ratio.^{7,8} Inhibition of mitochondria respiration by berberine is reminiscent of that observed with metformin⁴¹ and rosiglitazone.⁴² However, unlike metformin,⁴² the pharmacological inhibitors of PI-3-kinase has no effect on activation of AMPK by berberine (W.Y. and X.A., unpublished results), suggesting that these two drugs activate AMPK through distinct mechanisms. The precise mechanisms whereby berberine activates AMPK remain to be elucidated.

In addition to its endothelium-dependent effect, berberine also enhances the endothelium-independent vascular smooth muscle dilatation, and inhibits the endothelium-independent contraction induced by phenylephrine.¹⁸ Our previous study showed that the endothelium-independent vasodilatation by berberine might be attributed to its actions on the inhibition of calcium release and the activation of the potassium channels.¹⁸ Treatment with the putative potassium channel blockers such as tetrapentylammonium ions and 4-aminopyridine results in a significant attenuation of berberine-induced vasodilatation in the endothelium-denuded arteries. Interestingly, a recent study shows that AMPK activates KATP channels,⁴³ which is also an important modulator of vasodilatation. Further study is needed to investigate whether AMPK-dependent activation of the potassium channels plays a part in berberine-elicited vasodilatation.

Besides its beneficial effects on the endothelium, several other pharmacological properties of berberine may contribute to the cardiovascular protection conferred by this compound. In both rodents and patients with type II diabetes, berberine potently decreases hyperglycemia and hyperlipidemia, and alleviates insulin resistance.^{6–8} In hypercholesterolemic patients, berberine lowers serum cholesterol levels by increasing the expression of the hepatic low-density lipoprotein receptor,⁴⁴ and therefore reduces a traditional metabolic risk for cardiovascular disease. Furthermore, berberine attenuates neointima formation by inhibiting proliferation of vascular smooth muscle.¹⁴ These findings, together with the observations of the present study, raise the possibility that berberine or its more biologically available derivatives⁷ might be useful for the treatment and prevention of endothelial dysfunction associated with obesity and diabetes.

Supplementary material

Supplementary Material is available at *Cardiovascular Research* online.

Conflict of interest: None declared.

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