

Activation of the AMP-Activated Kinase by Antidiabetes Drug Metformin Stimulates Nitric Oxide Synthesis In Vivo by Promoting the Association of Heat Shock Protein 90 and Endothelial Nitric Oxide Synthase

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Metformin, one of most commonly used drugs for the treatment of type 2 diabetes, improves vascular endothelial functions and reduces cardiovascular events in patients with type 2 diabetes, although its mechanisms remain unknown. The current study aimed to elucidate how metformin improves endothelial functions. Exposure of cultured bovine aortic endothelial cells (BAECs) to clinically relevant concentrations of metformin (50–500 $\mu\text{mol/l}$) dose-dependently increased serine-1179 (Ser1179) phosphorylation (equal to human Ser1179) of endothelial nitric oxide (NO) synthase (eNOS) as well as its association with heat shock protein (hsp)-90, resulting in increased activation of eNOS and NO bioactivity (cyclic GMP). These effects of metformin were mimicked or completely abrogated by adenoviral overexpression of a constitutively active 5'-AMP-activated kinase (AMPK) mutant or a kinase-inactive AMPK- α , respectively. Furthermore, administration of metformin as well as 5-aminoimidazole-4-carboxamide ribonucleoside, an AMPK agonist, significantly increased eNOS Ser1179 phosphorylation, NO bioactivity, and coimmunoprecipitation of eNOS with hsp90 in wild-type C57BL6 mice but not in AMPK- α 1 knockout mice, suggesting that AMPK is required for metformin-enhanced eNOS activation in vivo. Finally, incubation of BAECs with clinically relevant concentrations of metformin dramatically attenuated high-glucose (30 mmol/l)-induced reduction in the association of hsp90 with eNOS, which resulted in increased NO bioactivity with a reduction in overexpression of adhesion molecules and endothelial apoptosis caused by

high-glucose exposure. Taken together, our results indicate that metformin might improve vascular endothelial functions in diabetes by increasing AMPK-dependent, hsp90-mediated eNOS activation. *Diabetes* 55:496–505, 2006

AMP-activated protein kinase (AMPK) is a phylogenetically conserved heterotrimer protein consisting of three subunits, α , β , and γ , each of which has at least two isoforms (1–3). AMPK is activated by physiological stimuli such as exercise and by hormones, including adiponectin and leptin, as well as by pathological stresses such as glucose deprivation, hypoxia, oxidant stress, and osmotic shock (1–3). Increases in the ratio of AMP to ATP activate AMPK by a number of mechanisms, including direct allosteric activation and covalent modification due to activation by an AMPK kinase (1–3), which phosphorylates the α -subunit on threonine-172 (Thr172) (4,5). Once activated, AMPK phosphorylates multiple targets both in vivo and in vitro (1–3), including several biosynthetic enzymes such as acetyl-CoA carboxylase, hydroxymethylglutaryl-CoA reductase, glycogen synthase, and both neuronal and endothelial nitric oxide (NO) synthase (eNOS) (2,6).

NO, a free radical gaseous molecule synthesized by the action of the enzyme eNOS, is the most important factor in maintaining vascular homeostasis (7). Endothelium-derived NO promotes vasodilation and inhibits platelet aggregation, leukocyte adherence, and vascular smooth muscle proliferation (7). It has been reported that regulation of eNOS activity is regulated by reversible phosphorylation (8,9) and its interaction with other proteins, such as heat shock protein (hsp)-90 (10,11). For example, AMPK has been demonstrated to phosphorylate eNOS at serine-1179 (Ser1179; equal to human Ser1179) with concomitant activation of eNOS in ischemic heart (7) and in intact human aortic endothelial cells activated with oxidants (peroxynitrite [ONOO⁻]) (12), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (13), and adiponectin (14,15). However, whether AMPK regulates eNOS in vivo remains to be determined.

Metformin, a bioguanide derivative (dimethylbiguanide), is one of the most commonly used drugs for the treatment of type 2 diabetes (16,17). Metformin improves insulin sensitivity, decreases insulin levels, and reduces elevated plasma glucose (16,17). In addition to its insulin-sensitizing effects, metformin has also been shown to have direct vascular effects (18–22). Most importantly, met-

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AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, 5'-AMP-activated kinase; AMPK-CA, constitutively active AMPK kinase; BAEC, bovine aortic endothelial cell; cGMP, cyclic GMP; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFP, green fluorescence protein; hsp, heat shock protein; ICAM, intracellular adhesion molecule; L-NAME, L-nitro-arginine methyl ester; 3-NT, 3-nitrotyrosine; Ser1179, serine-1179; SOD, superoxide dismutase; Thr172, threonine-172; PDK, phosphoinositide-dependent kinase; VCAM, vascular cell adhesion molecule.

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formin has been shown to improve vascular functions and to dramatically reduce cardiovascular end points and mortality for type 2 diabetic patients in large-scale clinical trials (16,17). Despite the long history and success of metformin as a treatment for type 2 diabetes, the mechanism of how metformin improves cardiovascular functions in type 2 diabetes remains to be elucidated.

Recent studies suggest that therapeutic effects of metformin might be mediated by its activation on AMPK (23–25). In addition, we demonstrate that metformin activated AMPK via a c-Src-mediated, phosphoinositide-3 kinase-dependent pathway (12,26). Whether AMPK activation by metformin leads to eNOS activation remains unknown. In the current study, we show that clinically related concentrations of metformin increased eNOS-Ser1179 phosphorylation in an AMPK-dependent manner, which, in turn, increased eNOS activity and NO bioactivity. Furthermore, the ability of metformin to increase NO bioactivity was mimicked by adenoviral overexpression of a constitutively active AMPK and blocked by a kinase-inactive AMPK, suggesting that AMPK is required for the improved endothelial function. Importantly, administration of metformin *in vivo* activated both AMPK and eNOS in the aortas from C57BL/6 wild-type mice but not in those from AMPK- α 1 knockout mice, suggesting that AMPK is required for cardiovascular beneficial effects of metformin. Finally, incubation of bovine aortic endothelial cells (BAECs) with clinically relevant concentrations of metformin dramatically attenuated high-glucose (30 mmol/l)-induced reduction in the association of hsp90 with eNOS, which resulted in increased NO bioactivity with a reduction in overexpression of adhesion molecules and endothelial apoptosis caused by high-glucose exposure. These results strongly suggest that metformin improves endothelial function in diabetes by AMPK-dependent, hsp90-mediated eNOS activation.

RESEARCH DESIGN AND METHODS

Male AMPK-knockout (AMPK^{-/-}) mice (27), which had been cross-bred with C57BL6 mice, were bred from the animal house of the University of Tennessee Medical Center. AMPK-knockout mice were used because previous studies (12,26,28) had demonstrated that the predominant form of AMPK in vascular endothelial cells is AMPK- α 1. Their littermates, C57BL6 mice, were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in temperature-controlled cages with a 12-h light/dark cycle and given free access to water and normal chow. Mice aged 10 weeks were randomly divided into control and treated groups. The mice were abdominally injected with metformin (250 mg/kg) or AICAR (500 mg/kg) for 3 days, and the control mice received 0.9% physiological saline injection, as described previously (26). The animal protocol was reviewed and approved by the institutional animal care and use committee.

BAECs and cell culture media were purchased from Clonetics (Walkersville, MD). Both [³H]L-arginine and [³²P]ATP were obtained from NEN (Boston, MA). Metformin (1,1-dimethyl-biguanide), L-nitro-arginine methyl ester (L-NAME), L-arginine, and calcium ionophore A23187 were obtained from Sigma (St. Louis, MO). Protein A-G agarose, anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase, and antibodies against eNOS were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against AMPK, phospho-eNOS (Ser1179), and phospho-AMPK (Thr172) were obtained from Cell Signaling (Beverly, MA). Fluorescein isothiocyanate (FITC)-labeled anti-vascular cell adhesion molecule (VCAM)-1, phycoerythrin-labeled anti-intracellular adhesion molecule (ICAM)-1, mouse IgG1 control-FITC, and mouse IgG1 control-phycoerythrin were obtained from Antigenix America (Huntington Station, NY). Annexin V-FITC and propidium iodide were from Abcam (Cambridge, MA). Other chemicals and organic solvents of highest grade, if not indicated, were obtained from Fisher Scientific (Morris Plains, NJ).

Cell culture. BAECs and endothelium growth medium were obtained from BioWhittaker (Walkersville, MD). BAECs were grown in endothelium growth medium supplemented with 2% fetal bovine serum (FBS), 100 units/ml

penicillin, 100 μ g/ml streptomycin, and 5.5 mmol/l D-glucose. Cells were incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C and passaged every 3 days by trypsinization. For experiments, BAECs were incubated in complete medium with 2% FBS in 6-well plates, grown to 100% confluence, and maintained in serum-free Dulbecco's modified Eagle's medium overnight, as described elsewhere (12). Cells were treated with metformin for 1 h at concentrations indicated in the figure legends.

To assay the effects of hyperglycemia on the interactions of hsp90 with eNOS, confluent BAECs were exposed to normal medium (5 mmol/l D-glucose), high glucose (30 mmol/l D-glucose), or mannitol (5 mmol/l D-glucose plus 25 mmol/l mannitol) for 48 h with or without metformin (0.5 mmol/l). Metformin was added 30 min before high-glucose exposure.

Adenoviral infection. Ad-GFP, a replication-defective adenoviral vector expressing green fluorescence protein (GFP), was used as control. An adenoviral vector expressing a dominant-negative mutant of AMPK- α 2 (AMPK-DN) was constructed from AMPK- α 2 bearing a mutation altering lysine45 to arginine (K45R), as described previously (12,26,28). To generate an adenoviral vector expressing a constitutively active mutant of AMPK- α 1 (AMPK-CA), a rat cDNA encoding residues 1–312 of AMPK- α 1 and bearing a mutation of Thr172 into aspartic acid (T172D) was subcloned into a shuttle vector (pShuttle CMV [cytomegalovirus]). The *c-myc* epitope tag was fused in frame to the 5'-terminus of the coding sequence. The resulting plasmid was linearized by digesting with *Pme*I and cotransfected into *Escherichia coli* BJ5183 with the adenoviral backbone plasmid pAdEasy-1. Homogenous recombinants were selected with kanamycin. The linearized recombinant plasmid was transfected into transformed human embryonic kidney 293 cells. Recombinant adenoviruses were amplified on 293 cells and purified by two ultracentrifugation steps on cesium chloride gradients. The number of viral particles was assessed by measurement of the optical density at 260 nm.

BAECs were infected with a kinase-dead mutant PDK1 (phosphoinositide-dependent kinase-1; mutation of lysine114 to glycine) (29), Akt dominant-negative adenovirus (30), and Akt constitutively active adenovirus (30). BAECs were infected in medium with 2% FCS overnight. The cells were then washed and incubated in fresh endothelium growth medium without FCS for an additional 12 h before experimentation. Using these conditions, infection efficiency was typically >80%, as determined by GFP expression.

Assays of eNOS activity and NO bioactivity. The eNOS activity in tissue homogenates and intact cells were assayed as described previously (12). Data are reported as the extent of the conversion of [³H]L-arginine to [³H]citrulline that is sensitive to L-NAME and expressed as percent inhibition. NO bioactivity was monitored by assay of cyclic GMP (cGMP) content after being stimulated, using a cGMP kit (Cayman Chemicals, Ann Arbor, MI), as described previously. The results are expressed as picomoles of cGMP per milligram of tricarboxylic acid-precipitable protein solubilized with 1 mol/l sodium hydroxide.

Assay of AMPK activity. AMPK activity was assayed using the SAMS peptides as previously described (26,28). The difference between the presence and absence of AMP is calculated as the AMPK activity.

Immunoprecipitation and Western blotting. Immunoprecipitation and Western blot of AMPK and eNOS were performed as described previously (12,26,28).

Flow cytometric analysis of adhesion molecule expression. Confluent human BAECs cultured in 6-well plates were exposed to normal glucose (5 mmol/l) or high glucose (30 mmol/l) with or without metformin (0.5 mmol/l) for 48 h. After treatment, the cells were subjected to immunofluorescent assessment of VCAM-1 and ICAM-1. Nonspecific labeling was determined by incubating monolayers with mouse IgG₁ control-FITC and with mouse IgG₁ control-phycoerythrin for 60 min. Fluorescent-labeled mouse monoclonal antibodies against adhesion molecules in 1:100 dilutions with PBS containing 0.1% BSA (PBA/BSA) were incubated for 60 min at room temperature under low-light conditions. The cells were dually labeled with the antibodies. Cell monolayers were subsequently rinsed with PBS/BSA and fixed with 1% paraformaldehyde in PBS for 10 min and room temperature. The monolayers were rinsed twice with PBS/BSA and scraped with rubber policeman. Cell suspensions were centrifuged at 1,500 rpm for 5 min, the supernatants discarded, and the pellets resuspended in 0.5 ml PBS/BSA. Cytofluorographic analysis was performed using a Becton Dickinson FACScan (San Jose, CA) with an argon ion laser tuned to 488 nm operating at 15 mW output. Acquisition was set at 5,000 gated cells, and mean fluorescent intensities in the FL-1 (FITC) and FL-2 (phycoerythrin) channels and the percentage of cells were measured in all samples using CellQuest software version 1.2 (Becton Dickinson).

Flow cytometric analysis of the nature of cell death. Flow cytometric determination of apoptosis and necrosis was carried out using annexin V-FITC (green) and propidium iodide (red color), and two-color analyses were performed by flow cytometry. This method relies on the binding of annexin V to phosphatidylserine, which is translocated from the inner membrane leaflet

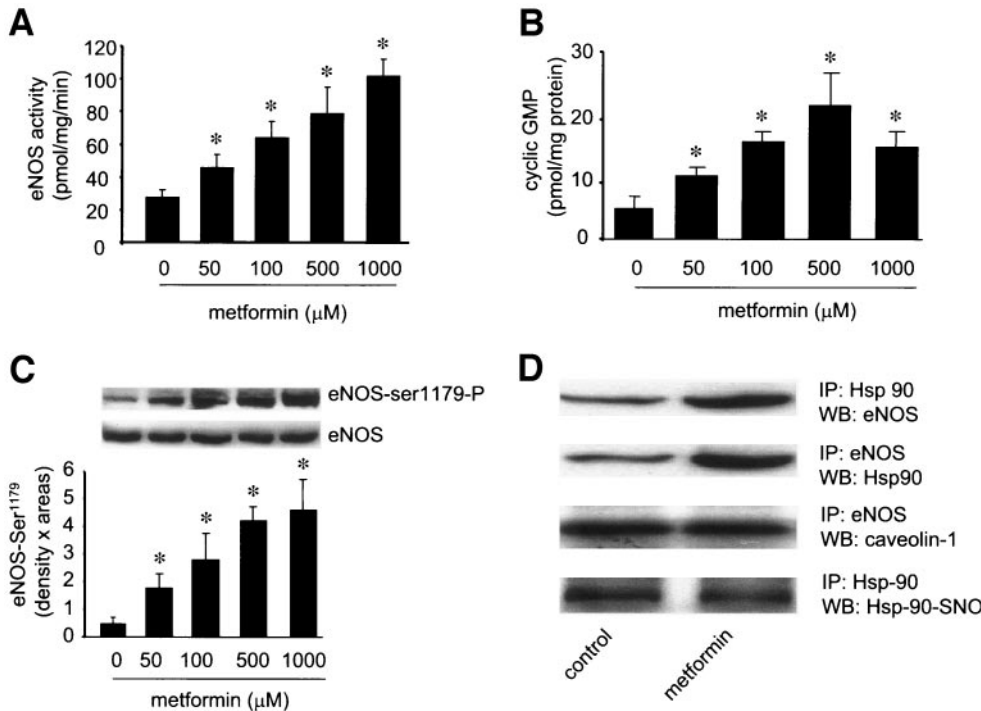


FIG. 1. Metformin increases the eNOS activity, eNOS-Ser1179 phosphorylation, and eNOS-hsp90 association in BAECs. Confluent BAECs were treated with metformin at concentrations indicated for 1 h as described in RESEARCH DESIGN AND METHODS. **A:** Metformin (50 μmol/l to 1 mmol/l) caused a concentration-dependent increase of eNOS activity ($n = 6$). $*P < 0.05$. The eNOS activity was assayed by the conversion of [³H]arginine into [³H]citrulline assay, as described in RESEARCH DESIGN AND METHODS. **B:** Metformin increases the cGMP contents in BAECs. cGMP was assayed as described in RESEARCH DESIGN AND METHODS and expressed as picomoles per milligram protein ($n = 7$). $*P < 0.05$. **C:** Metformin concentration-dependently increased the phosphorylation of eNOS-Ser1179 (eNOS-Ser1179-P) in BAECs. The top panel is a representative blot obtained from five independent experiments. The lower panel represents the summary results of eNOS-Ser1179 phosphorylation ($n = 5$). $*P < 0.05$, control vs. metformin-treated. **D:** Metformin increases the association of eNOS with hsp90. Either hsp90 or eNOS were first immunoprecipitated from the cells and detected in Western blots with the specific antibodies against eNOS, hsp90, caveolin-1, or S-nitrosylated protein (SNO). The blot is a representative of five blots obtained from three to five independent experiments. Asterisks indicate significant difference between the treated groups and controls.

to the outer layer in cells undergoing apoptosis, whereas necrotic cells were stained by both annexin V-FITC and propidium iodide. After treatment with high glucose (30 mmol/l) with or without metformin, cells were harvested, washed once in ice-cold PBS, and resuspended in 1 ml annexin binding buffer (10 mmol/l HEPES, pH 7.4, 140 mmol/l NaCl, and 2.5 mmol/l CaCl₂). Suspensions (100 μl) were transferred into new tubes. Then, 5 μl annexin V-FITC and 10 μl propidium iodide stock solution (50 μg/ml) were added to the cells. After 20 min, 400 μl binding buffer was added to each tube, and samples were analyzed on a FACSCalibur flow cytometer, as described above.

Statistical analysis. Data are the means ± SE. Differences were analyzed using ANOVA followed by appropriate multiple comparison tests. $P < 0.05$ was considered statistically significant.

RESULTS

Activation by metformin of eNOS activity and NO bioactivity in BAECs. NO, generated by eNOS in endothelial cells, is an important factor controlling vascular endothelial function (7). We first determined whether metformin improved endothelial function by increasing eNOS in cultured endothelial cells. Confluent BAECs were treated with metformin (50–500 μmol/l) for 1 h, and eNOS activity was assayed by the conversion of [³H]arginine into citrulline. As shown in Fig. 1A, metformin concentrations dose-dependently increased the conversion of [³H]arginine into citrulline, indicating that metformin increased NO formation from eNOS. Exposure of BAECs to clinically relevant concentrations of metformin (100 μmol/l) for 1 h caused a 40% increase in NO release, as measured by the conversion of [³H]arginine into [³H]citrulline (Fig. 1A).

NO exerts its vasoprotective effects by activating guanylate cyclase to increase cGMP (so-called NO bioactivity). It was interesting to determine whether metformin increased NO bioactivity (cGMP content). After being exposed to metformin, BAECs were stimulated with A23187 (10 μmol/l) for 10 min and were assayed for cGMP. As shown in Fig. 1B, low concentrations of metformin (50–500 μmol/l) dose-dependently increased the cGMP content, indicating that metformin increased NO bioactivity in

vitro. However, despite increasing NO release, high concentrations of metformin (>500 μmol/l) (Fig. 1A) decreased instead of increased the cGMP content, which might have resulted from increased formation of oxidants caused by high concentrations of metformin (26).

Phosphorylation of ser1179 of eNOS is considered one of the important mechanisms leading to eNOS activation (8,9). To determine whether metformin activated eNOS by increasing eNOS ser1179 phosphorylation, we determined eNOS-ser1179 phosphorylation using a specific antibody. As shown in Fig. 1C, metformin dose-dependently increased the detection of eNOS-Ser1179 phosphorylation. In addition, the increased detection of eNOS-Ser1179 was not caused by increased expression of eNOS because metformin up to 1 mmol/l for 48 h did not alter eNOS expression (data not shown).

There is evidence that hsp90 plays an important role in regulating eNOS activity (10,11). We further investigated whether metformin increased eNOS activity by increasing its association with hsp90. The interaction of hsp90 and eNOS was assayed using immunoprecipitation of eNOS or hsp90 with the specific antibodies. Exposure of BAECs to metformin up to 1 mmol/l for 48 h did not alter the expression of hsp90 (data not shown). Compared with control, however, increased amounts of eNOS were detected when hsp90 was immunoprecipitated from metformin-treated BAECs (Fig. 1D). These results were further corroborated by increased detection of hsp90 when eNOS was immunoprecipitated from metformin-treated cells (Fig. 1D). These results suggested that metformin increased the association of hsp90 with eNOS without altering their expression. In contrast, metformin did not alter the association of eNOS with caveolin-1, which binds with eNOS to prevent eNOS activation (Fig. 1D).

There is evidence that S-nitrosylation of hsp90 might

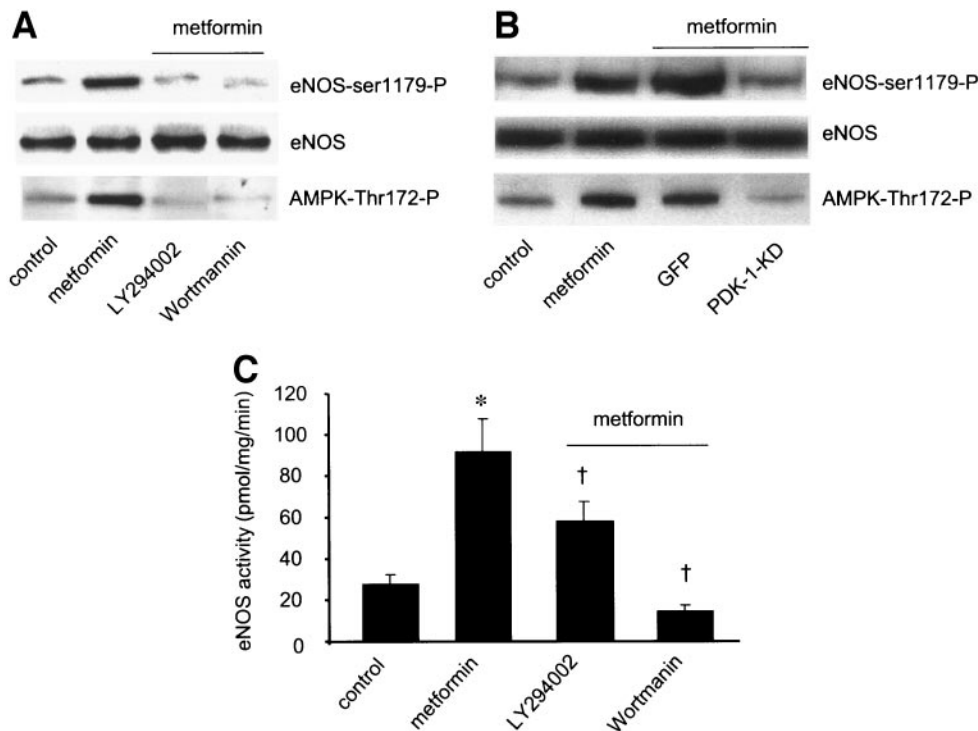


FIG. 2. Activation of eNOS by metformin is phosphoinositide-3 kinase dependent. Cells were incubated with metformin (0.5 mmol/l) for 1 h. After incubation, cellular proteins were extracted immediately and detected in Western blots, as described in RESEARCH DESIGN AND METHODS. **A:** Inhibition of phosphoinositide-3 kinase with wortmannin (100 nmol/l) or LY294002 (50 μ mol/l) attenuates metformin (0.5 mmol/l)-enhanced phosphorylation of AMPK-Thr172 (AMPK-Thr172-P) and phosphorylation of eNOS-Ser1179 (eNOS-Ser1179-P). The blot is a representative of five blots obtained from five independent experiments. **B:** Overexpression of a kinase-dead PDK1 (PDK-1-KD) blocked metformin-up-regulated phosphorylation of eNOS-Ser1179. This blot is a representative of five blots obtained from five independent experiments. **C:** Wortmannin or LY294002 abolished metformin-enhanced [3 H]citrulline formation ($n = 6$). * $P < 0.01$ control vs. metformin ($n = 6$); † $P < 0.01$ metformin plus either wortmannin or LY 294002.

limit its activation on eNOS activity. We then determined whether metformin altered the S-nitrosylation of hsp90. As shown in Fig. 1D, S-nitrosylated hsp90 was weakly detected in control cells. Metformin, however, did not alter the levels of S-nitrosylation of hsp90. Taken together, these data suggest that metformin increased the association of hsp90 with eNOS without affecting the S-nitrosylation of hsp90.

Activation of eNOS by metformin is phosphoinositide-3 kinase-dependent. Several protein kinases, including Akt, AMPK, and protein kinase A, increase eNOS-Ser1179 phosphorylation (12). Phosphoinositide-3 kinase is reported to be the upstream kinase for both Akt and AMPK (8,9,26,28). Thus, it was interesting to investigate whether phosphoinositide-3 kinase pathways contributed to the metformin-induced eNOS activation. As shown in Fig. 2A, inhibition of phosphoinositide-3 kinase, by either wortmannin (100 nmol/l) or LY294002 (50 μ mol/l), prevented metformin-enhanced eNOS Ser1179 phosphorylation. In parallel, either wortmannin or LY294002 also attenuated both eNOS-Ser1179 phosphorylation and NO release (Fig. 2A and C). Overexpression of PDK1 kinase-dead mutant (29), which blocks ONOO⁻-activated phosphoinositide-3 kinase (26,28), also attenuated metformin-enhanced eNOS phosphorylation (Fig. 2D), thus indicating that metformin activates eNOS at least in part via phosphoinositide-3 kinase pathways. Therefore, these data indicate that metformin activates a signaling pathway involving phosphoinositide-3 kinase and PDK1 that leads to eNOS activation.

Activation of eNOS by metformin is mediated by AMPK but independent of Akt. Akt/protein kinase B has been reported to phosphorylate Ser1179 of eNOS in cells stimulated with growth factors (8,9) or hydrogen peroxide (31). In addition, there is evidence that AMPK activation is phosphoinositide-3 dependent (26,28). Because both enzymes are able to phosphorylate eNOS-Ser1179, it was interesting to study the mechanism by

which metformin activated eNOS. Adenoviral overexpression of a dominant-negative mutant of Akt (Akt-DN), which inhibited Akt activity and attenuated vascular endothelial growth factor-enhanced eNOS-Ser1179 phosphorylation (data not shown), did not alter metformin-stimulated phosphorylation of eNOS-Ser1179 (Fig. 3B). In addition, overexpression of a constitutively active Akt mutant (Akt-CA) did not alter metformin-enhanced phosphorylation of eNOS-Ser1179 (data not shown). These results strongly suggested that metformin-increased eNOS-ser1179 phosphorylation might be independent of phosphoinositide-3-Akt pathways.

Activation of AMPK phosphorylates eNOS-ser1179 in hypoxic or ONOO⁻-treated BAECs (28) and in ischemic cardiac myocytes (7) and endothelial cells (32). Studies from others (14) and us (26,28) demonstrated that metformin activated AMPK in a phosphoinositide-3 kinase-dependent fashion. Thus, it was interesting to investigate whether AMPK activation by metformin contributed to eNOS activation. The functional relationship between AMPK activation, eNOS Ser1179 phosphorylation, and NO bioactivity was examined by overexpression of AMPK-DN, a dominant-negative form of AMPK- α 2 that bears a K45R mutation and is a catalytically inactive AMPK- α 2. Overexpression of AMPK-DN, which has a dominant-negative effect on both AMPK- α 1 and - α 2 and blocks both metformin and ONOO⁻-activated AMPK (26,28), significantly attenuated metformin-enhanced phosphorylation of both AMPK-Thr172 and eNOS-Ser1179 (Fig. 3A), indicating that metformin-enhanced eNOS Ser1179 phosphorylation is AMPK dependent. These data were further corroborated by the findings that overexpression of AMPK-DN selectively abolished metformin-enhanced eNOS activity (Fig. 3B). In contrast, overexpression of Akt-CA or Akt-DN did not alter metformin-enhanced eNOS phosphorylation and activity (Fig. 3B), excluding a role of Akt in the activation by metformin. Taken together, these data indicate that

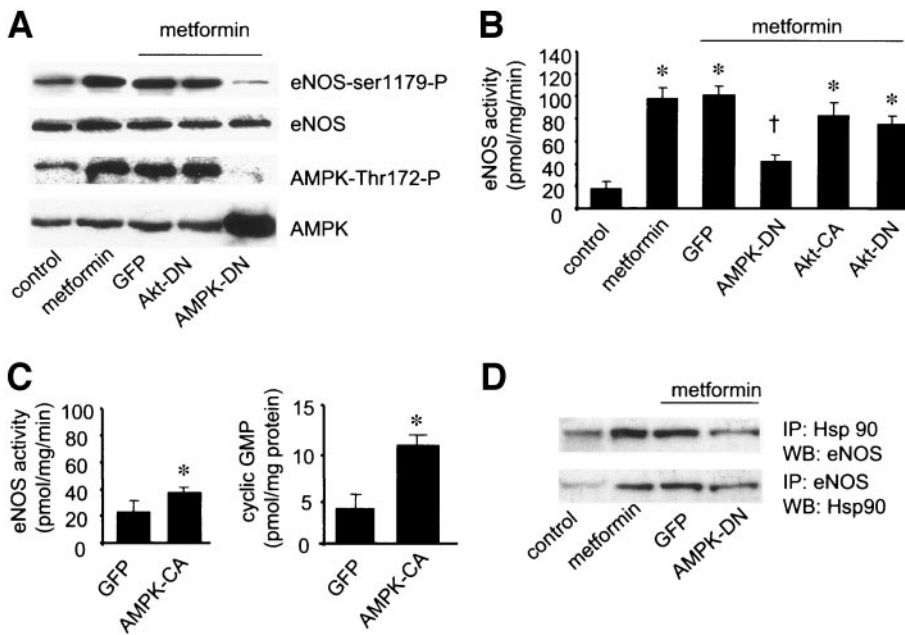


FIG. 3. Activation of eNOS by metformin is AMPK dependent. Confluent BAECs were treated with metformin as described in RESEARCH DESIGN AND METHODS. **A:** Overexpression of AMPK-DN but not Akt-DN inhibits metformin-upregulated phosphorylation of eNOS-ser1179 (eNOS-ser1179-P). Of note is that metformin increased the detection of eNOS-ser1179, which was blocked by overexpressing adenovirus encoding AMPK-DN but not by Akt-DN. The levels of AMPK expression were detected by the antibody against AMPK- α . The blot represents those from four independent experiments. **B:** Metformin-increased NO release is AMPK dependent but not Akt dependent in BAEC. The NO release was assayed by the conversion of [³H]L-arginine to [³H]citrulline as described in RESEARCH DESIGN AND METHODS. Metformin significantly enhances NO release in BAECs ($n = 6$) but is inhibited by AMPK-DN ($n = 6$). * $P < 0.05$, control vs. metformin-treated; † $P < 0.05$. **C:** Overexpression of AMPK-CA enhances metformin-upregulated NO release and cGMP content ($n = 6$). * $P < 0.05$, metformin vs. metformin plus AMPK-CA. **D:** Inhibition of AMPK attenuates the association of eNOS with hsp90 enhanced by metformin. After treatment, hsp90 or eNOS were first immunoprecipitated from the cells and detected in Western blots with the specific antibody. The blot is a representative of five blots obtained from five independent experiments. AMPK-Thr172-P; phosphorylation of AMPK-Thr172.

metformin via AMPK activation but not Akt leads to eNOS activation.

Overexpression of AMPK-CA increases both eNOS phosphorylation and eNOS activity. To establish the role of AMPK in regulating NO bioactivity, we determined the effect of adenovirus-mediated expression of *myc*-tagged AMPK-CA on the changes in eNOS-Ser1179 phosphorylation and NO bioactivity. As expected, after adenoviral infection, the recombinant AMPK- α 1 protein containing a T172D mutation and truncated at residue 312 (~32 kDa) was detected as expected by immunoblotting for the *myc* tag at its NH₂ terminus. In contrast, endogenous AMPK- α 1 and - α 2 protein levels were not obviously affected by the infection. Overexpression of a constitutively active AMPK mutant (AMPK-CA) increased AMPK activity by about twofold. The AMPK activity measured in cells overexpressing AMPK-CA was similar to those stimulated with 500 μ mol/l AICAR (data not shown). As expected, overexpression of AMPK-CA significantly enhanced eNOS activity and cGMP content (Fig. 3C) compared with the cells overexpressing adenovirus encoding GFP. The cells overexpressing AMPK-CA remained sensitive to metformin, as evidenced by the fact that expression of AMPK-CA further enhanced the effect of metformin on eNOS phosphorylation over that observed in GFP-infected cells (data not shown), suggesting that there is a synergistic effect of metformin and AMPK-CA on the phosphorylation of eNOS and NO bioactivity. Thus, activation of AMPK leads to eNOS activation and NO bioactivity

Inhibition of AMPK attenuates metformin-enhanced association of eNOS with hsp90. Association of hsp90 with eNOS is considered to be an important step for eNOS activation (10,11). Thus, we further investigated whether AMPK was involved in metformin-enhanced eNOS association with hsp90. As shown in Fig. 3D, metformin increased the association of eNOS with hsp90. Importantly, inhibition of AMPK with AMPK-DN significantly attenuated the association of hsp90 with eNOS enhanced by metformin, suggesting that AMPK activation leads to the

association of eNOS with hsp90. How AMPK increases the interaction of eNOS with hsp90 remains unknown and is under investigation.

Activation of AMPK and eNOS by metformin in vivo. We further investigated whether metformin increased eNOS activity and NO bioactivity in vivo, and if so, the role of AMPK in the process. To this end, AMPK-knockout and wild-type C57BL/6 mice were administered either metformin or AICAR. After treatment, mouse aortas were immediately isolated and homogenated to assay AMPK activity, eNOS Ser1179 phosphorylation, and eNOS activity. As shown in Fig. 4A and B, administration of metformin, like AICAR, significantly increased the phosphorylation of AMPK-Thr172 and eNOS-Ser1179 in wild-type but not in AMPK-knockout mice (Fig. 4A and B). In parallel, both metformin and AICAR significantly increased eNOS activity (Fig. 4C) and cGMP content (Fig. 4D) in the wild-type but not in AMPK-knockout mice. In addition, both metformin and AICAR increased the association of eNOS with hsp90 in the wild-type but not in AMPK-knockout mice (Fig. 4E). Taken together, these data suggest that AMPK activation is required for metformin-enhanced eNOS activation in vivo.

Metformin attenuates high-glucose-induced inhibition of AMPK with hsp90. Metformin has been shown to improve vascular functions and dramatically reduce cardiovascular end points and mortality for type 2 diabetic patients. We next investigated the effects of diabetes (hyperglycemia) on the interaction of AMPK with hsp90, and, if so, we then determined whether metformin altered the effects of hyperglycemia. Confluent BAECs were exposed to either 5 or 30 mmol/l glucose or 30 mmol/l mannitol for 48 h, and the interactions of hsp90 with AMPK were analyzed by coimmunoprecipitation of hsp90 or eNOS. Exposure of BAECs to 30 mmol/l glucose for 48 h did not alter the expression of either eNOS or hsp90 (data not shown). Compared with cells exposed to 5 mmol/l glucose, however, BAECs exposed to 30 mmol/l glucose but not mannitol for 48 h had a decreased association of

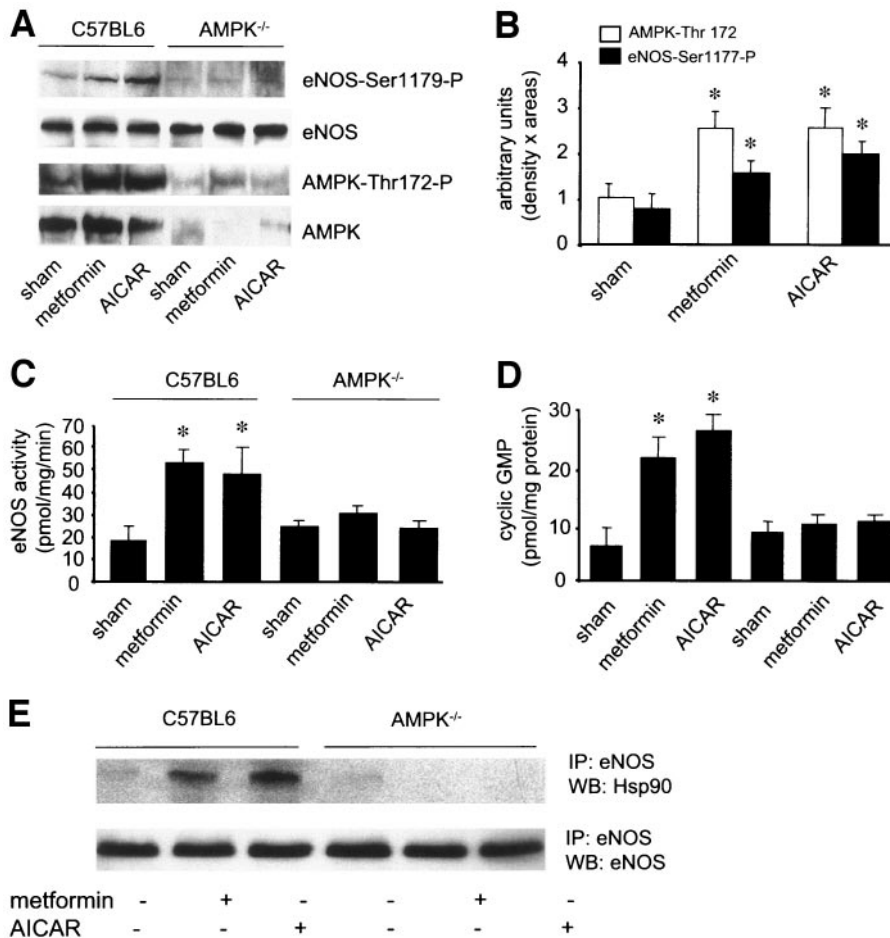


FIG. 4. Metformin activates AMPK and increases NO release in vivo. The mice were abdominally injected with metformin (250 mg/kg) or AICAR (500 mg/kg) for 3 days, and the control mice received 0.9% physiological saline injection, as described previously. Mouse aortas were isolated immediately after the mice were killed. The activities for both AMPK and eNOS were assayed as described in RESEARCH DESIGN AND METHODS (A and B). Administration of metformin and AICAR in mice increases the phosphorylation of both eNOS-ser1179 (eNOS-ser1179-P) and AMPK-Thr172 (AMPK-Thr172-P). Isolated aortas were homogenized, and proteins were separated by SDS-PAGE and Western blotted with the antibodies against AMPK-P and eNOS-P. The blot is a representative of four independent experiments ($n = 4$). * $P < 0.05$, sham vs. metformin-treated. **C:** Increased eNOS activity by metformin and AICAR in vivo. Aortic homogenates (200 μ g) were assayed for the eNOS activity as described in RESEARCH DESIGN AND METHODS. Metformin increases the conversion of [³H]arginine into [³H]citrulline, indicating that metformin increases NO release ($n = 5$). * $P < 0.01$, sham vs. metformin-treated. **D:** Metformin increases cGMP contents in wild-type C57BL6 but not in AMPK-knockout mice in vivo. cGMP contents were assayed as described in RESEARCH DESIGN AND METHODS. Metformin significantly increased AMPK activity ($n = 5$). * $P < 0.01$, sham vs. metformin or AICAR. **E:** Activation of AMPK by metformin or AICAR increases the association of eNOS with hsp90. Isolated mouse aortas were homogenized and eNOS was immunoprecipitated by using a specific antibody against eNOS. Hsp90 was detected in Western blots by using the specific antibody. The blot is a representative of four independent experiments. IP, immunoprecipitation; WB, Western blot.

hsp90 with eNOS (Fig. 5A). Furthermore, metformin (0.5 mmol/l) abolished the effect of high glucose (30 mmol/l) and resulted in increased association of hsp90 with eNOS (Fig. 5A). In parallel, exposure to 30 mmol/l glucose significantly decreased the contents of cGMP compared with 5 mmol/l glucose (Fig. 5B). Interestingly, overexpression of AMPK-DN but not GFP abolished the effect of metformin in cGMP content, suggesting that the effect of metformin might be through AMPK activation (Fig. 5B).

Previous studies (33–35) had demonstrated that exposure of endothelial cells to high glucose resulted in abnormal expression of adhesion molecules such ICAM-1 and VCAM-1. We next investigated whether metformin altered the abnormal expression of both ICAM-1 and VCAM-1 caused by high-glucose exposure. As expected, high glucose (30 mmol/l) significantly increased the protein detection of both ICAM-1 and VCAM-1 (Fig. 5C). Interestingly, metformin (0.5 mmol/l) dramatically decreased the expression of both ICAM-1 and VCAM-1 enhanced by 30 mmol/l glucose (Fig. 5C). Furthermore, overexpression of AMPK-DN but not GFP abolished the effect of metformin in high-glucose (30 mmol/l)-induced expression of both ICAM-1 and VCAM-1 (Fig. 5C), suggesting that metformin might reduce high-glucose (30 mmol/l)-induced expression of adhesion molecules.

Accelerated endothelial apoptosis is one feature of diabetic vascular endothelial dysfunction (33–35). We next investigated whether metformin altered the endothelial apoptosis caused by high glucose. As expected, exposure to 30 mmol/l glucose significantly increased endothelial apoptosis in BAECs. However, metformin (0.5 mmol/l)

significantly inhibited the endothelial apoptosis caused by high-glucose exposure (Fig. 5D). In addition, overexpression of AMPK-DN but not GFP abolished the protective effects of metformin on endothelial apoptosis, suggesting that metformin might inhibit endothelial apoptosis via AMPK activation.

DISCUSSION

The current study demonstrates that metformin, one of most commonly used drugs for treatment of type 2 diabetes, improves vascular endothelial dysfunction by activating AMPK-dependent eNOS activation. Our results demonstrate that not only did metformin increase phosphorylation of AMPK and its downstream target, eNOS Ser1179, but its actions were also mimicked by overexpression of AMPK-CA. In addition, AMPK-DN blocked the increase in eNOS-Ser1179 phosphorylation, eNOS association with hsp90, as well as the increase in eNOS activity and NO bioactivity caused by the antidiabetic drug metformin. Furthermore, administration of metformin increased eNOS ser1179 phosphorylation, eNOS association of hsp90, eNOS activity, and NO bioactivity in the aortas of C57BL/6 mice, whereas these effects were abolished in the mice deficient of AMPK- α 1. These results strongly suggest that the salutary effects of metformin on cardiovascular functions are mediated predominantly by AMPK. Finally, we have demonstrated that metformin abolished the high-glucose-induced decrease in the association of both AMPK and eNOS, which resulted in increased NO bioactivity, decreased expression of adhesion molecule expres-

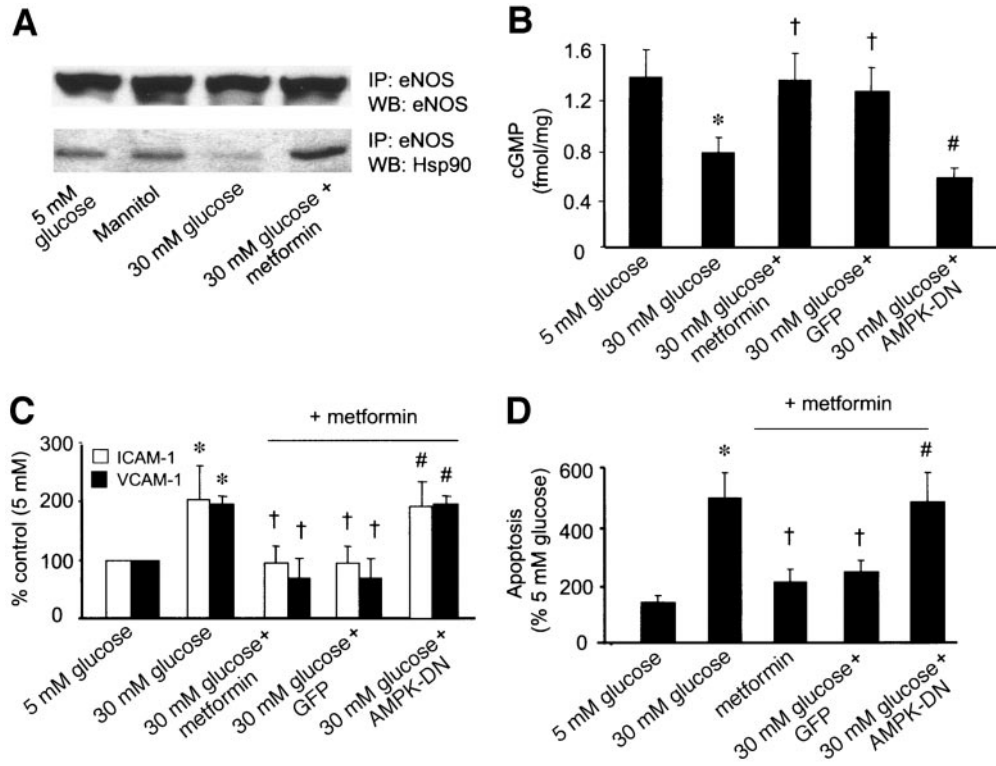


FIG. 5. Metformin increases the association of AMPK-eNOS and attenuates high-glucose-enhanced endothelial apoptosis and overexpression of adhesion molecules. Confluent BAECs, with or without adenoviral infection, were exposed to high glucose, metformin (0.5 mmol/l), or both for 48 h. The coimmunoprecipitation of hsp90 and eNOS and the NO bioactivity (cGMP), endothelial apoptosis, and adhesion molecule expression (ICAM-1 and VCAM-1) were assayed as described in RESEARCH DESIGN AND METHODS. **A:** Metformin (0.5 mmol/l) increases the association of coimmunoprecipitation of hsp90 with eNOS. High-glucose (30 mmol/l) exposure decreased the association of hsp90 with eNOS, whereas metformin reversed the effect of high glucose. At 48 h after incubation, cells were lysed immediately after being washed several times with cold PBS, and proteins were extracted as described in RESEARCH DESIGN AND METHODS. The blot is a representative of three blots from three independent experiments. **B:** Metformin (0.5 mmol/l) increases the NO bioactivity in BAECs exposed to high glucose. Exposure of BAECs to 30 mmol/l glucose decreased NO bioactivity, whereas metformin increased NO bioactivity. Of note is that overexpression of AMPK-DN attenuated the effect of metformin ($n = 5$ or 6). * $P < 0.05$ for 5 mmol/l vs. 30 mmol/l glucose; † $P < 0.05$ for 30 mmol/l glucose vs. 30 mmol/l glucose plus metformin; # $P < 0.05$ for 30 mmol/l glucose plus metformin vs. 30 mmol/l glucose plus metformin but with AMPK-DN. **C:** Metformin (0.5 mmol/l) attenuates high-glucose (30 mmol/l)-induced overexpression of both ICAM-1 and VCAM-1 proteins. Of note is that overexpression of AMPK-DN but not GFP abolished the effects of metformin in high-glucose-induced overexpression of both ICAM-1 and VCAM-1 ($n = 6$ or 7). * $P < 0.05$ for 5 vs. 30 mmol/l glucose; † $P < 0.05$ for 30 mmol/l glucose vs. 30 mmol/l glucose plus metformin; # $P < 0.05$ for 30 mmol/l glucose plus metformin vs. 30 mmol/l glucose plus metformin but with AMPK-DN. **D:** Metformin (0.5 mmol/l) inhibits high-glucose (30 mmol/l)-induced endothelial apoptosis. Of note is that overexpression of AMPK-DN attenuated metformin (0.5 mmol/l)-induced inhibition on endothelial apoptosis in BAECs exposed to 30 mmol/l glucose ($n = 6$ or 7). * $P < 0.05$ for 5 vs. 30 mmol/l glucose; † $P < 0.05$ for 30 mmol/l glucose vs. 30 mmol/l glucose plus metformin; # $P < 0.05$ for 30 mmol/l glucose plus metformin vs. 30 mmol/l glucose plus metformin but with AMPK-DN. IP, immunoprecipitation; WB, Western blot.

sion, and endothelial apoptosis. Because overexpression of AMPK-DN but not GFP abolished the effects of metformin in cells exposed to high glucose, these results strongly suggest that metformin via AMPK improves vascular endothelial function in diabetes, and AMPK might be an important target in treating vascular complications in diabetes.

Unlike traditionally used glucose-lowering reagents, such as sulfonylureas or insulin, metformin improves cardiovascular functions and reduces cardiovascular risks in diabetes (16,17). Metformin has also been shown to improve endothelium-dependent vasorelaxation (18–22), which is largely mediated by NO, a molecule necessary in maintaining vascular homeostasis. The novel finding of the current study is that metformin might improve vascular function through enhanced NO release mediated by AMPK activation of eNOS. Exposure of BAECs to metformin caused a significant increase in the conversion of [³H]arginine into [³H]citrulline, indicating an increase in NO release. Because metformin did not enhance eNOS expression, we further identified that metformin increased NO release by increasing eNOS-Ser1179 phosphorylation and its association with hsp90. BAECs exposed to metformin

and aortic homogenates from metformin-treated mice exhibited increased phosphorylation of AMPK and eNOS. Overexpression of AMPK-DN, but not Akt-DN, blunted eNOS-Ser1179 phosphorylation, hsp90 binding with eNOS, and eNOS activity in BAECs exposed to metformin, whereas overexpression of constitutively active AMPK mutants mimicked the effect of metformin (Fig. 3); these results strongly suggest that metformin activates AMPK, which increases NO release in BAECs exposed to metformin. Because activation of AMPK with AICAR also leads to eNOS-Ser1179 phosphorylation independent of Akt in human aortic endothelial cells (36), these results suggest that activation of AMPK might preferentially phosphorylate eNOS ser1179 in endothelial cells. In addition, overexpression of AMPK-DN blocked metformin-enhanced association of eNOS with hsp90. Association of eNOS with hsp90 is considered an important step in controlling eNOS activity and eNOS uncoupling (8,9). Therefore, increasing the association of eNOS with hsp90 by AMPK might be an important mechanism to increase eNOS activity/decrease eNOS uncoupling. Importantly, metformin, like AICAR, an AMPK agonist, increased the interaction of hsp90 and eNOS, eNOS ser1179 phosphory-

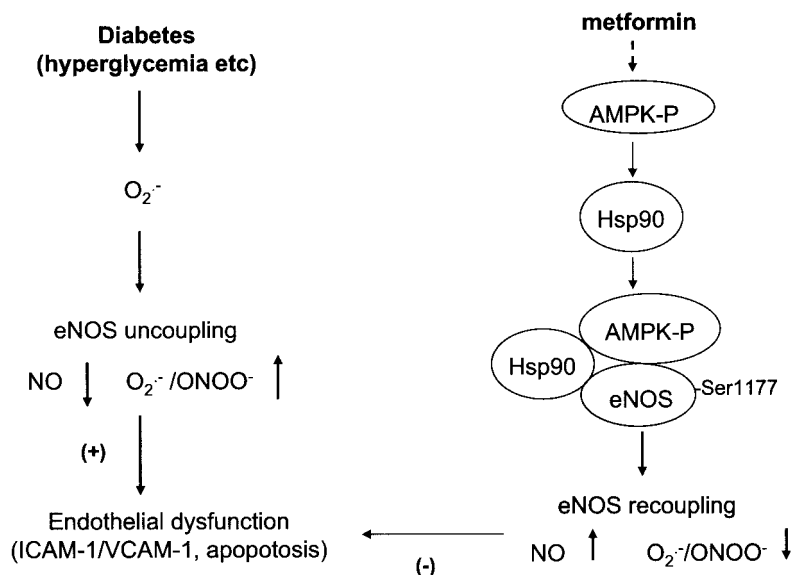


FIG. 6. Proposed scheme of eNOS recoupling by metformin. Metformin recouples eNOS activation by an AMPK-dependent, hsp90-mediated mechanism. Metformin activates AMPK via an Src- and phosphoinositide 3-K-dependent mechanism. Activation of AMPK is associated with phosphorylation of hsp90, which recruits eNOS to form a complex of hsp90-AMPK-eNOS, resulting in an increase in eNOS-Ser1177 phosphorylation. Concurrently, hsp90 reduces eNOS-derived $O_2^-/ONOO^-$ but increases NO (eNOS recoupling). Metformin improves vascular endothelial dysfunction in diabetes through AMPK-dependent, hsp90-mediated pathways. AMPK-P, phosphorylation of AMPK.

lation, and eNOS activity in wild-type but not in AMPK-knockout mice in vivo, suggesting that AMPK activation is required for metformin-enhanced eNOS activation in vivo.

Our previous study demonstrated that metformin activates AMPK, likely via mitochondria-derived oxidants such as $ONOO^-$. $ONOO^-$ is a potent oxidant formed by a direct bimolecular reaction of NO with superoxide anion (O_2^-) at near diffusion-limited rates ($6.7 \times 10^9 \cdot \text{mol/l}^{-1} \cdot \text{sec}^{-1}$) (32,37). This rate constant is three times faster than the enzymatic dismutation of O_2^- catalyzed by superoxide dismutase (SOD) at neutral pH ($K_{SOD} = 2 \times 10^9 \cdot \text{mol/l}^{-1} \cdot \text{sec}^{-1}$) (32,37). Because the concentrations of SOD and NO within tissues are estimated to be $\sim 10 \mu\text{mol/l}$ and 1–20 nmol/l, respectively, in physiological conditions, O_2^- will be predominantly removed by SOD and the formation of $ONOO^-$ will be minimal (32). However, NO levels are up to 100–150 nmol/l in stimulated vessels, and NO concentrations of 0.1 $\mu\text{mol/l}$ up to several micromoles per liter may occur in pathological states of ischemia or inflammation (38–40). In particular, elevated levels of NO caused by upregulation of high-output inducible NOS-II have been reported to occur in diabetes (41,42). Therefore, it is very likely that submicromole to micromole per liter levels of NO , O_2^- , and $ONOO^-$ are formed in stimulated or diabetic tissues, which represents a major potential pathway of NO reactivity (43,33). Thus, the reaction of NO with O_2^- , which was initially viewed as a route for NO inactivation, instead yields the potent oxidizing species $ONOO^-$. Indeed, recent clinical data demonstrated significantly more intense 3-nitrotyrosine (3-NT) staining, a marker for $ONOO^-$, in diabetic patients and animals when compared with normal controls (34). The localization of the 3-NT staining indicates that endothelium-derived NO (in the presence of increased oxidant stress) is inactivated because of the cytotoxic oxidant $ONOO^-$. In addition, increased detection of 3-NT has been described in the human plasma when infused with glucose (35,44). Of particular note is the finding of Frustaci et al. (45) that apoptosis is increased 61- and 85-fold, respectively, in endothelial cells and cardiomyocytes in ventricular myocardial biopsies from diabetic humans. Interestingly, they found that apoptosis in both cell types was strongly associated with positive staining for 3-NT, suggesting a correlation with oxidative stress and $ONOO^-$ generation.

Thus, prevention of $ONOO^-$ formation might be an important target in treating diabetic vascular complications.

In the current study, we provide evidence that activation of AMPK by metformin increases NO formation and NO bioactivity in BAECs exposed to high glucose. In addition, we found that metformin reduced high-glucose-induced abnormal protein expression of adhesion molecules (ICAM-1 and VCAM-1) and prevented endothelial apoptosis. Because overexpression of AMPK dramatically reduced the protective effects of metformin, AMPK activation might be an important target for vascular endothelial dysfunction. Increased association of Hsp90 with eNOS might be an important mechanism for endothelial dysfunction. Indeed, the increased association of hsp90 with eNOS has been demonstrated to “recouple” eNOS activity (increase NO but decrease eNOS-derived O_2^-) both in vitro and in animals (46–47). In addition, AMPK activation might reduce oxidant stress by increasing oxidant stress by other sources or by increasing antioxidant potential. Exposure of cultured human aortic endothelial cells to AICAR dramatically reduced the markers of oxidant stress and prostacyclin synthase nitration caused by 30 mmol/l glucose (Z.X., M.-H.Z., unpublished data). Thus, the beneficial effects of AMPK activation are twofold: increased NO and decreased O_2^- release caused by diabetes, which results in decreased formation of toxic oxidants such as $ONOO^-$ and its consequent toxic effects on endothelium (Fig. 6). Thus, the current study provides important information that AMPK activation could protect endothelial cells against the adverse effects of diabetes by setting in motion events that lead to an increase in NO bioactivity and endothelial function.

In summary, we show that clinically related concentrations of metformin activate eNOS and NO bioactivity in an AMPK-dependent manner. Furthermore, the ability of metformin to increase NO bioactivity was mimicked by adenoviral overexpression of a constitutively active AMPK and blocked by a kinase-inactive AMPK, suggesting that AMPK is required for the improved endothelial function. Importantly, administration of metformin in vivo activated both AMPK and eNOS in the aortas from C57BL/6 wild-type mice but not in those obtained from AMPK-knockout, suggesting that AMPK is required for cardiovascular beneficial effects of metformin. We conclude that metformin

activates AMPK to increase NO release, which contributes to the beneficial effects of metformin in treating vascular diseases.

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