

In Vivo Activation of AMP-Activated Protein Kinase Attenuates Diabetes-Enhanced Degradation of GTP Cyclohydrolase I

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OBJECTIVE—The activation of AMP-activated protein kinase (AMPK) has been reported to improve endothelial function. However, the targets of AMPK in endothelial cells remain poorly defined. The aim of this study was to test whether AMPK suppresses the degradation of GTP-cyclohydrolase (GTPCH I), a key event in vascular endothelial dysfunction in diabetes.

RESEARCH DESIGN AND METHODS—Both human umbilical vein endothelial cells and aortas isolated from streptozotocin-injected diabetic mice were assayed for phospho-AMPK (Thr172), GTPCH I, tetrahydrobiopterin (BH4), and endothelial functions.

RESULTS—Oral administration of metformin (300 mg · kg⁻¹ · day⁻¹, 4 weeks) in streptozotocin-injected mice significantly blunted the diabetes-induced reduction of AMPK phosphorylation at Thr172. Metformin treatment also normalized acetylcholine-induced endothelial relaxation and increased the levels of GTPCH I and BH4. The administration of AICAR, an AMPK activator, or adenoviral overexpression of a constitutively active mutant of AMPK abolished the high-glucose-induced (30 mmol/l) reduction of GTPCH I, biopeterins, and BH4 but had no effect on GTPCH I mRNA. Furthermore, AICAR or overexpression of AMPK inhibited the high-glucose-enhanced 26S proteasome activity. Consistently, inhibition of the proteasome by MG132 abolished high-glucose-induced reduction of GTPCH I in human umbilical vein endothelial cells. Further, aortas isolated from AMPK α 2^{-/-} mice, which exhibited elevated 26S proteasome activity, had reduced levels of GTPCH I and BH4. Finally, either administration of MG132 or supplementation of L-sepiapterin normalized the impaired endothelium-dependent relaxation in aortas isolated from AMPK α 2^{-/-} mice.

CONCLUSIONS—We conclude that AMPK activation normalizes vascular endothelial function by suppressing 26S proteasome-mediated GTPCH I degradation in diabetes. *Diabetes* 58:1893–1901, 2009

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The most important factor for the maintenance of vascular homeostasis is nitric oxide (NO), derived from L-arginine in the catalysis of endothelial nitric oxide synthase (eNOS). Many studies have indicated that diabetes alters the metabolism and function of endothelium in ways that could lead to vascular injury (1). In diabetes, the function of eNOS is altered such that the enzyme produces superoxide anion (O₂⁻) rather than NO (2). This phenomenon is referred to as eNOS uncoupling and has been reported to play a causal role in diabetes-enhanced endothelial dysfunction (3,4). Several studies (5) have suggested that deficiency of tetrahydrobiopterin (BH4), an essential cofactor for eNOS, transforms eNOS into an oxidant-producing enzyme, leading to the production of O₂⁻ and/or peroxynitrite (ONOO⁻).

Intracellular BH4 levels are dictated by a balance of de novo synthesis, BH4 oxidation, and recycling of BH2 to BH4 (6). De novo synthesis of BH4 is controlled by GTP cyclohydrolase I (GTPCH I), a homodecameric protein consisting of 25 kDa subunits in mammalian cells (7). As the first enzyme in the biosynthetic pathway of BH4, GTPCH I is constitutively expressed in endothelial cells and critical for the maintenance of BH4 levels and NO synthesis. Indeed, acute inhibition of GTPCH I uncouples eNOS, induces endothelial dysfunction, and elevates blood pressure in vivo (8). Further, our recent study (9) suggests that hyperglycemia uncouples eNOS by reducing the levels of GTPCH I and BH4.

Proteasomes provide a major pathway of intracellular protein degradation in mammalian cells (10–12). Although proteasomes can degrade proteins by ubiquitin-independent processes, they are mostly involved in the ATP- and ubiquitin-dependent pathway of protein degradation (13). The 26S proteasome complex consists of both the 20S catalytic core, where the proteins are degraded, and 19S complex, a regulatory subunit composed of at least 19 different subunits that form a lid- and a base-like structure; the lid provides the binding sites for poly-ubiquitinated substrates and a deubiquitinating activity involved in the recycling of ubiquitin moieties upon substrate degradation; the base includes six ATPases that interact with the 20S proteolytic core. The ATPases have chaperone functions and are required for the unfolding of substrates and their translocation into the 20S proteolytic chamber (14,15). Therefore, intracellular protein degradation by the proteasome is a highly energy-demanding process and, thus, it is expected that under conditions of energy depletion this process should be tightly regulated. The possible role of the ubiquitin proteasome system in

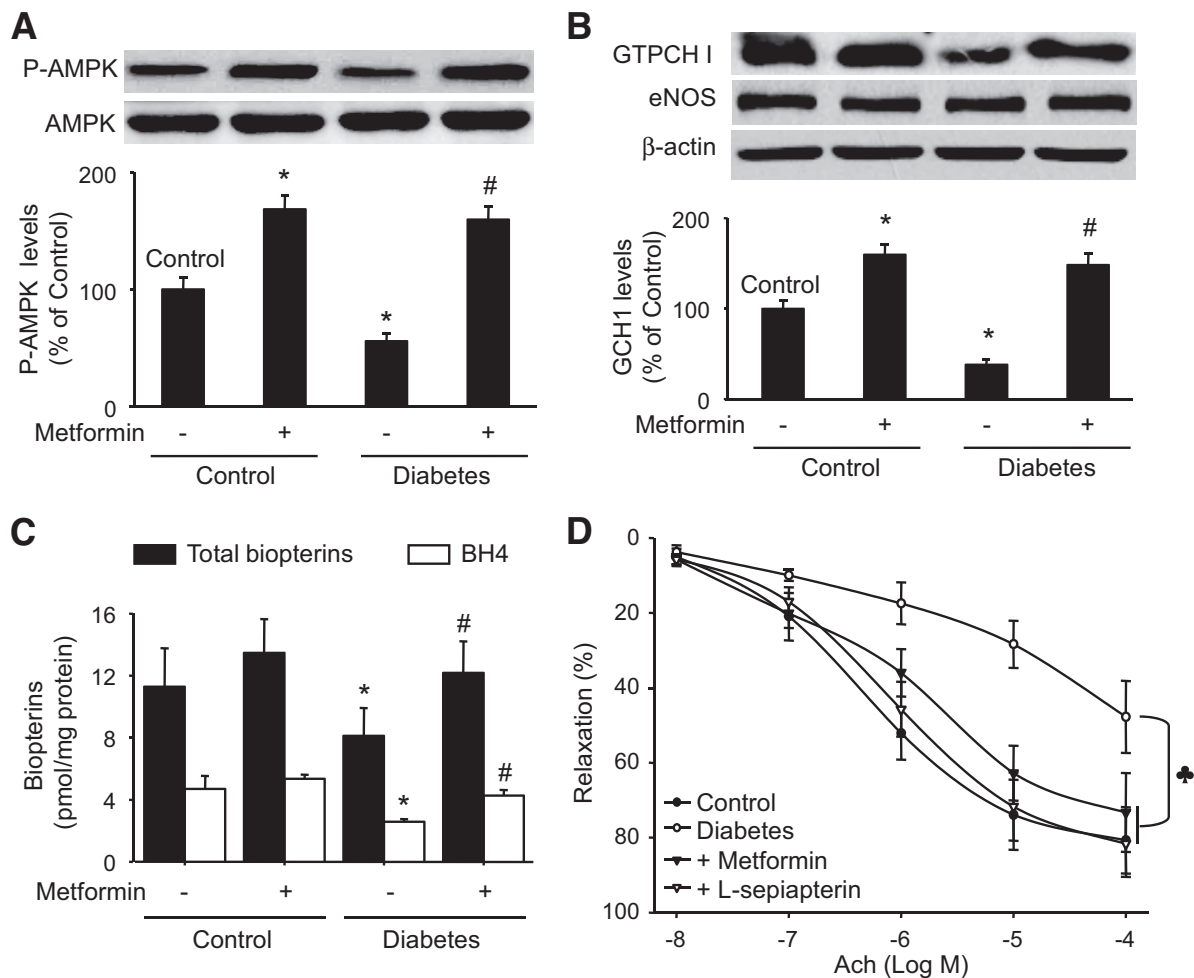


FIG. 1. Activation of AMPK by metformin attenuates diabetes-induced reduction of phospho-AMPK-Thr172 (A), GTPCH I (B), and biopterin (C) levels and improves endothelial function. Control and diabetic mice were fed with metformin ($300 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) for 4 weeks. Mouse aortas were isolated and assayed for phospho-AMPK, GTPCH I, total biopterins, and BH4 levels, as described in RESEARCH DESIGN AND METHODS. The results were obtained from five mice. * $P < 0.05$ versus control, # $P < 0.05$ versus diabetes alone. D: ACh-induced endothelium-dependent relaxation was assayed as described in RESEARCH DESIGN AND METHODS ($n = 5$ per group, ♣ $P < 0.05$). L-sepiapterin ($10 \mu\text{mol/l}$) was added 1 h before the start of the experiments.

the development of atherosclerosis in diabetes has been addressed (16,17).

The AMP-activated protein kinase (AMPK) is a heterotrimeric protein composed of α , β , and γ subunits. The α ($\alpha 1$ and $\alpha 2$) subunit imparts catalytic activity, whereas the other subunits maintain the stability of the heterotrimer complex (18). Activation of AMPK requires the phosphorylation of AMPK at Thr172 in the activative loop of the α subunit (19), and is mediated by at least two kinases, Peutz-Jeghers syndrome kinase LKB1 (20) and Ca^{2+} /calmodulin-dependent protein kinase kinase (21). AMPK is considered an “energy gauge,” which becomes activated when intracellular AMP increases and/or ATP decreases. Recently, Rosa Viana et al. (22) reported that AMPK suppresses proteasome-dependent protein degradation in vitro. As our earlier study (9) demonstrated that proteasome-dependent GTPCH I degradation is key for diabetes-induced endothelial dysfunction, we reasoned that AMPK activation might alleviate diabetic endothelial dysfunction by suppressing proteasome-dependent GTPCH I degradation. Here, we report that pharmacological or genetic activation of AMPK reversed endothelial dysfunction by suppressing GTPCH I degradation.

RESEARCH DESIGN AND METHODS

A full description of materials, animals, and methods used, including cell culture, gene transfection to HUVECs, measurement of biopterins, detections of NO and reactive oxygen species (ROS), streptozotocin (STZ)-induced diabetes in mice, 26S proteasome activity assay, immunoprecipitation and Western blot analysis, reverse-transcription PCR for GTPCH I, and organ chamber, can be found in the online-only data supplement available at <http://diabetes.diabetesjournals.org/cgi/content/full/db09-0267>.

Gene transfection in HUVECs. 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 (AMPK-DN) was constructed from AMPK bearing a mutation altering lysine 45 to arginine (K45R), as described previously (23). To generate an adenoviral vector expressing a constitutively active mutant of AMPK (AMPK-CA), a rat cDNA encoding residues 1–312 of AMPK and bearing a mutation of threonine 172 to aspartic acid (T172D) was subcloned into a shuttle vector (pShuttle CMV [cytomegalovirus]).

STZ-induced diabetes in mice. A low-dose STZ-induction regimen was used to induce pancreatic islet cell destruction and persistent hyperglycemia, as previously described (24) by the Animal Models of Diabetic Complications Consortium (<http://www.amdcc.org>). Hyperglycemia was defined as a random blood glucose level of $>300 \text{ mg/dl}$ for >2 weeks after injection.

Measurement of biopterins. The levels of BH4 and total biopterins were determined by high-performance liquid chromatography, as previously described with some modification (25).

Measurement of superoxide anions and NO. The levels of O_2^- production in cultured cells were detected by using dihydroethidium (DHE) fluorescence

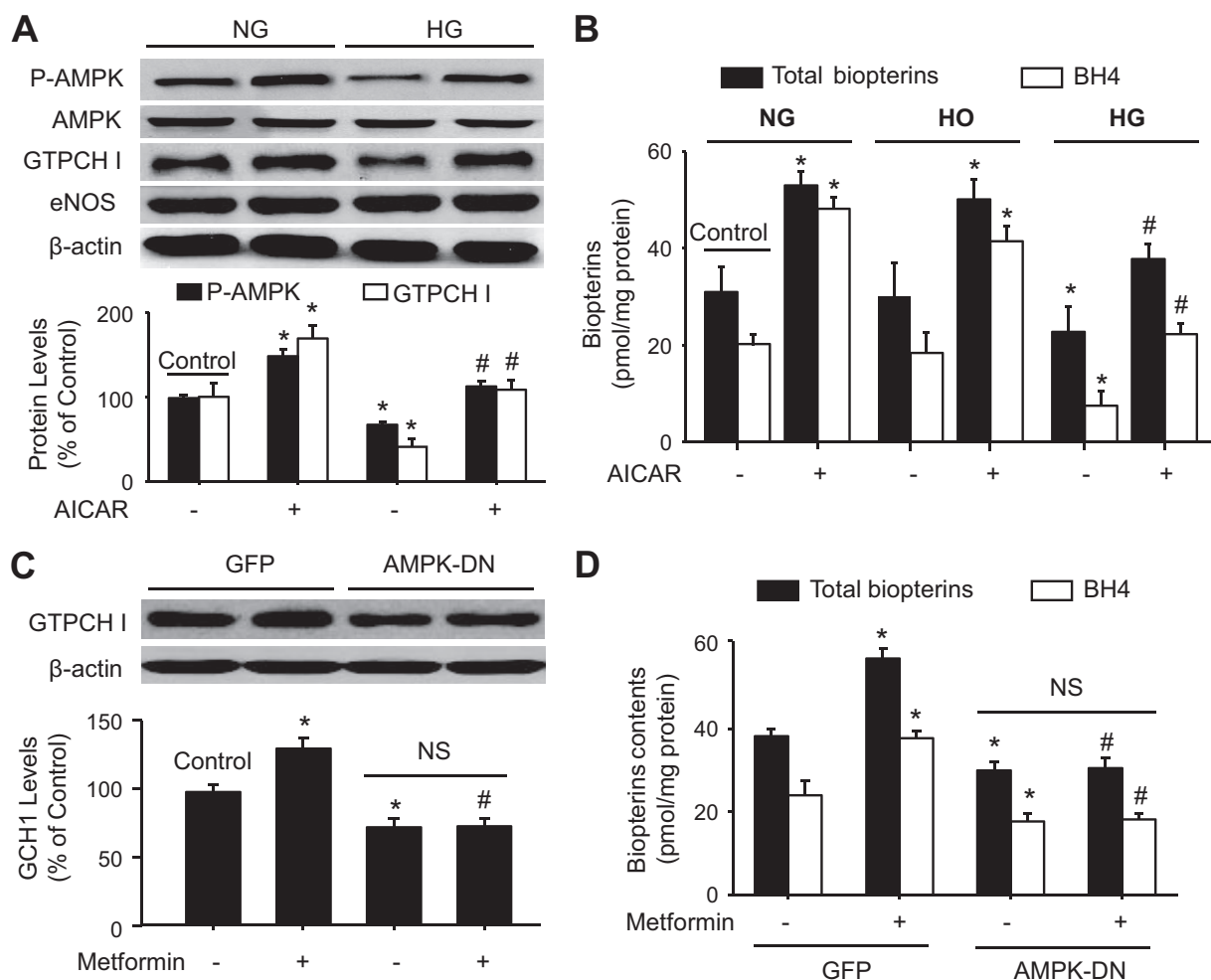


FIG. 2. Activation of AMPK is required for the AICAR/metformin-restored reduction of GTPCH I caused by high-glucose exposure in HUVECs. HUVECs were treated with high glucose (HG) (30 mmol/l D-glucose) or hyperosmotic medium (5 mmol/l glucose plus 25 mmol/l L-glucose) for 24 h in the presence or absence of AICAR (2 mmol/l). HUVECs were collected and assayed for the levels of P-AMPK and GTPCH I protein by Western blot (A) and total bioterins and BH4 (B) ($n = 5$ per group, $*P < 0.05$ vs. control, $\#P < 0.05$ versus high glucose alone). C and D: Overexpression of AMPK-DN abolishes metformin's effects on GTPCH I, bioterins, and BH4. HUVECs were transfected with ad-GFP or ad-AMPK-DN for 24 h and then incubated with or without metformin (2 mmol/l) for 8 h. The results are a summary of three independent experiments. $*P < 0.05$ versus GFP alone, $\#P < 0.05$ versus GFP plus metformin, NS: $P > 0.05$. HO, hyperosmolarity control; NG, normal glucose.

assay. NO release in cultured cells was detected by using the DAF fluorescent probe.

26S proteasome activity assay. The 26S proteasome function was measured, as described previously (26).

Reverse-transcription PCR for GTPCH I. Reverse-transcription PCR was performed according to the manufacturer's protocol (ThermoScript, RT-PCR System, Invitrogen). PCR primer pairs were as described previously (27).

Assays of endothelium-dependent and endothelium-independent vasorelaxation. Organ chamber study was performed, as described previously (28).

Statistical analysis. Data are reported as means \pm SE. All data were analyzed by one- or two-way ANOVA followed by multiple t tests, and $P < 0.05$ was considered statistically significant.

RESULTS

Reduction in the levels of AMPK Thr172 phosphorylation is accompanied by a reduction of GTPCH I in diabetic mice. Data published by our group and others demonstrate that AMPK activation exerts beneficial effects by increasing NO bioactivity via an increase in the phosphorylation of eNOS and/or an increase in the anti-oxidant potential of endothelial cells (29–31). Therefore, it was of interest to investigate the effects of hyperglycemia/diabetes on AMPK activity. As the phosphorylation of Thr172 of AMPK α is required for AMPK activity, and because AMPK-Thr172 is closely related to AMPK activity, we first mea-

sured the levels of phosphorylated AMPK-Thr172 in mouse aortas isolated from mice with STZ-induced diabetes or nondiabetic mice. As depicted in Fig. 1A, the levels of phospho-AMPK (Thr172) were significantly reduced in diabetic mouse aortas relative to nondiabetic mice. In contrast, the level of AMPK α was not different in diabetic and nondiabetic mice (Fig. 1A). Consistent with our earlier report (9), the levels of GTPCH I were significantly reduced in diabetes relative to the control group (Fig. 1B). In contrast, the levels of eNOS were not altered in diabetic mice or metformin-treated mice (Fig. 1B).

Activation of AMPK by metformin attenuates the diabetes-induced reduction of GTPCH I, bioterins, and BH4. Metformin, one of most widely used antidiabetic drugs, markedly reduces cardiovascular incidences and improves endothelial function in patients with diabetes (32). Our earlier studies reported that metformin increases NO bioactivity by activating AMPK (29). We sought to determine whether metformin alters the diabetes-induced reduction of GTPCH I levels. Administration of metformin (300 mg \cdot kg $^{-1}$ \cdot day $^{-1}$) for 4 consecutive weeks in the drinking water, which did not alter the blood glucose levels in control or diabetic mice ($P > 0.05$, data not

shown), significantly increased the levels of both AMPK-Thr172 and GTPCH I in aortas isolated from both diabetic and nondiabetic mice (Fig. 1A and B). Consistently, meformin attenuated the diabetes-induced reduction of both biopterins and BH4 in vivo (Fig. 1C).

Metformin attenuates the diabetes-induced impairment of endothelium-dependent relaxation in vivo.

We next asked whether metformin reverses the diabetes-induced endothelial dysfunction in vivo. Endothelium-dependent relaxation was assayed in aortas isolated from metformin-treated diabetic mice. Compared with nondiabetic mice, the acetylcholine (ACh)-induced endothelium-dependent relaxation in diabetes was markedly reduced (Fig. 1D). Further, metformin alone did not affect the ACh-induced endothelium-dependent relaxation in control mice (data not shown) but abolished the diabetes-induced impairment of endothelium-dependent relaxation (Fig. 1D). In contrast, sodium nitroprusside (SNP)-induced endothelium-independent relaxation was not altered by diabetes or metformin treatment (data not shown).

Supplementation of L-sepiapterin normalizes endothelium-dependent relaxation ex vivo. L-sepiapterin, a precursor of BH4, is converted to BH4 via a salvage pathway (33). We next investigated whether supplementation of L-sepiapterin improved endothelial dysfunction ex vivo. Aorta isolated from diabetic mice were incubated with L-sepiapterin (10 μmol/l) for 1 h in an organ bath. As shown in Fig. 1D, L-sepiapterin, which did not alter the endothelium-dependent relaxation in control mice (data not shown), restored the ACh-induced maximal relaxation (81.6 ± 8.9% vs. 47.8 ± 9.6%, *P* < 0.05) in diabetic mice, implying that diabetes-induced endothelial dysfunction was likely to be because of BH4 deficiency.

AICAR attenuates high-glucose-induced reduction of both GTPCH I and BH4 in HUVECs. As metformin treatment increased the amount of GTPCH I and BH4 in diabetic aortas, we reasoned that metformin improves endothelial function through the inhibition of diabetes-induced GTPCH I degradation. To test this hypothesis, cultured HUVECs were exposed to normal glucose (5 mmol/l), high glucose (30 mmol/l D-glucose), or hyperosmotic control (5 mmol/l D-glucose plus 25 mmol/l L-glucose) in the presence or absence of AICAR (2 mmol/l), an AMPK activator. High glucose did not alter AMPK and eNOS expressions (Fig. 2A). However, consistent with the reduction of both total biopterins and BH4 levels, the levels of AMPK-Thr172 were markedly reduced in high-glucose-exposed HUVECs (Fig. 2A and B). Consistent with our previous report, neither total biopterins nor BH4 levels were altered in the hyperosmotic control group, compared to HUVECs in normal glucose (Fig. 2B). As expected, AICAR treatment increased the level of AMPK phosphorylation at Thr172 in HUVECs exposed to either normal glucose or high glucose without altering AMPK and eNOS expressions (Fig. 2A).

We next determined the effects of high glucose on GTPCH I. As expected, the level of GTPCH I was significantly reduced in high-glucose-exposed cells compared with control cells (Fig. 2A). Consistently, both total biopterins and BH4 levels were lower in high-glucose-treated cells than those in controls. AICAR treatment increased the levels of both total biopterins and BH4 in HUVECs exposed to normal glucose or high glucose (Fig. 2B).

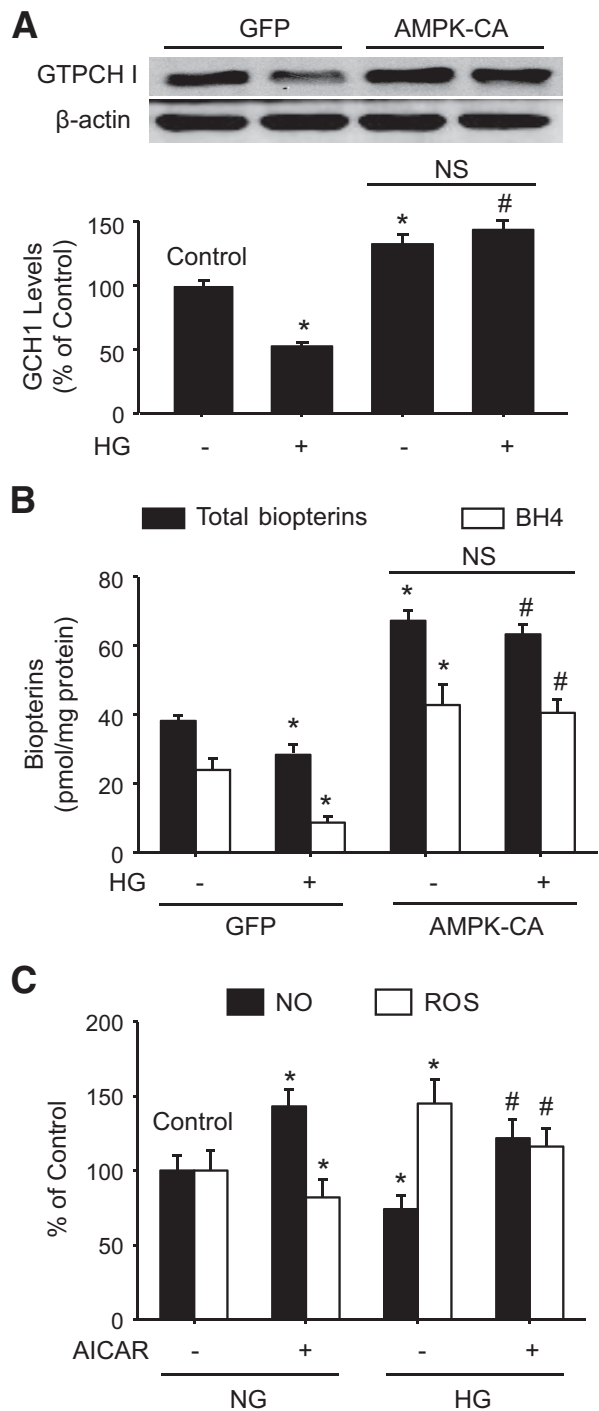


FIG. 3. Overexpression of AMPK-CA abolishes the reduction of GTPCH I levels caused by high glucose (HG) in HUVECs. After 24 h of being infected with ad-GFP or ad-AMPK-CA, HUVECs were incubated with high glucose for an additional 24 h. After the incubation, HUVECs were collected and assayed for GTPCH I protein (A) and total biopterins and BH4 levels (B). **P* < 0.05 versus GFP alone, #*P* < 0.05 versus GFP plus high glucose, NS: *P* > 0.05. C: HUVECs were incubated with high glucose for 24 h with or without AICAR. NO and ROS productions were detected by using the fluorophore 4,5-diamino-fluorescein or dihydroethidine fluorescence, respectively. NG, normal glucose.

Genetic inhibition of AMPK reduces GTPCH I in HUVECs. As high glucose reduced the levels of both AMPK-Thr172 and GTPCH I in HUVECs, we next asked whether genetic inhibition of AMPK would cause a reduction in GTPCH I levels. Toward this end, AMPK was suppressed by adenoviral overexpression of AMPK-DN.

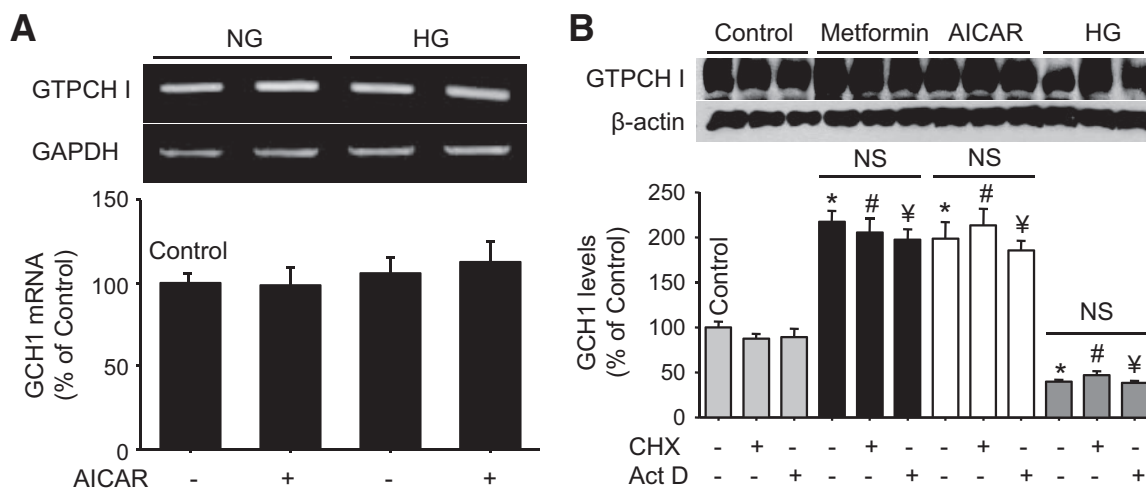


FIG. 4. AMPK activation does not alter GTPCH I expression in HUVECs. **A:** HUVECs were treated with high glucose (HG) for 24 h in the presence or absence of AICAR (2 mmol/l). Cells were subjected to perform RT-PCR analysis for GTPCH I mRNA expression. The picture shown is a representative of three blots of three independent experiments. **B:** HUVECs were treated with metformin, AICAR, or high glucose in the presence or absence of cycloheximide (100 μ g/ml) or actinomycin D (10 μ g/ml) for 24 h, and GTPCH I protein expression was then detected by Western blot. The blot shown is a representative of three independent experiments. * $P < 0.05$ versus control, # $P < 0.05$ versus cycloheximide alone, ¥ $P < 0.05$ versus actinomycin D alone, NS: $P > 0.05$. NG, normal glucose.

Overexpression of AMPK-DN but not GFP significantly reduced the levels of GTPCH I (Fig. 2C), total biopterins (Fig. 2D), and BH4 (Fig. 2D) in HUVECs.

Genetic inhibition of AMPK blocks the effects of metformin on GTPCH I, biopterins, and BH4. To establish the essential role of AMPK in metformin-enhanced GTPCH I protein levels, metformin was administered in HUVECs infected with AMPK-DN or GFP. As shown in Fig. 2C and D, metformin increased GTPCH I, total biopterins, and BH4 levels in GFP-infected HUVECs but not in cells infected with AMPK-DN, implying that AMPK is required for the metformin-induced inhibition of GTPCH I reduction in HUVECs.

Overexpression of AMPK-CA abolishes the reduction of GTPCH I caused by high glucose. We next determined the effects of genetic activation of AMPK on GTPCH I, biopterins, and BH4 by over expressing a constitutively active AMPK (AMPK-CA). As described in Fig. 3A and B, overexpression of AMPK-CA but not GFP abolished the high-glucose-induced reduction of GTPCH I (Fig. 3A), biopterins (Fig. 3B), and BH4 levels (Fig. 3B).

We next determined if AMPK activation altered eNOS-derived NO/ROS production in response to high glucose. As expected, high glucose significantly increased ROS while it suppressed NO production in HUVEC (Fig. 3C). As expected, AICAR significantly increased NO release but decreased ROS, implying that increased GTPCH I and consequent BH4 increase might normalize the function of eNOS in HUVEC.

GTPCH I mRNA is not involved in the AMPK-induced upregulation of GTPCH I protein. Increased levels of GTPCH I could be because of the transcriptional activation of the GTPCH I gene or attributable to the decrease of GTPCH I protein degradation. We first determined whether AMPK activation altered GTPCH I mRNA levels in HUVECs. As shown in Fig. 4A, treatment of HUVECs with AICAR (2 mmol/l) for 24 h did not alter the levels of GTPCH I mRNA in HUVECs exposed to normal glucose or high glucose. In addition, neither cycloheximide nor actinomycin D altered the levels of GTPCH I protein caused by AICAR or metformin (Fig. 4B). Neither cycloheximide nor actinomycin D altered the high-glucose-induced reduction of

GTPCH I levels in HUVECs (Fig. 4B). Taken together, our data suggest that the increase of GTPCH I protein levels caused by AMPK activation is independent of transcriptional activation of the GTPCH I gene.

26S proteasome mediates the reduction of GTPCH I levels in high-glucose-treated HUVECs. The levels of GTPCH I protein are controlled by both GTPCH I synthesis and GTPCH I degradation. Our earlier study demonstrated that high glucose activates the 26S proteasome, resulting in the enhanced degradation of GTPCH I in endothelial cells (9). Co-administration of MG132 (0.5 μ mol/l, 4 h), a potent proteasome inhibitor, abolished the high-glucose-induced reduction of GTPCH I levels (Fig. 5A), confirming that the alteration of GTPCH I levels was because of the activation of the 26S proteasome.

AMPK activation suppresses the increase of 26S proteasome activity caused by high glucose. We next investigated the effects of AMPK activation on 26S proteasome activity in HUVECs. As shown in Fig. 5B, high glucose exposure significantly increased 26S proteasome activity, whereas AICAR treatment significantly reduced 26S proteasome activity in cells exposed to normal or high glucose.

We next determined whether genetic activation of AMPK with AMPK-CA would alter 26S proteasome activity in HUVECs. Infection of AMPK-CA but not GFP reduced 26S proteasome activity in control cells (Fig. 5C). In addition, AMPK-CA but not GFP abolished high-glucose-enhanced 26S proteasome activity in HUVECs. These results suggest that AMPK functions as a physiological suppressor for 26S proteasome-mediated GTPCH I degradation in HUVECs.

Next, we examined whether activation of AMPK affects the ubiquitination of GTPCH I in high-glucose-treated HUVECs. HUVEC were incubated with high glucose for 24 h with or without AICAR. After the incubation, GTPCH I was immunoprecipitated by the specific antibody against GTPCH I. GTPCH I ubiquitination was identified in Western blotting by using the antibody against ubiquitinated protein. As shown in Fig. 5D, AICAR alone significantly increased the level of ubiquitination GTPCH I in HUVEC. Conversely, high glucose significantly decreased the ubiquitination GTPCH I in HUVEC (Fig. 5D). Interestingly, co-administration of high glucose with AICAR abolished

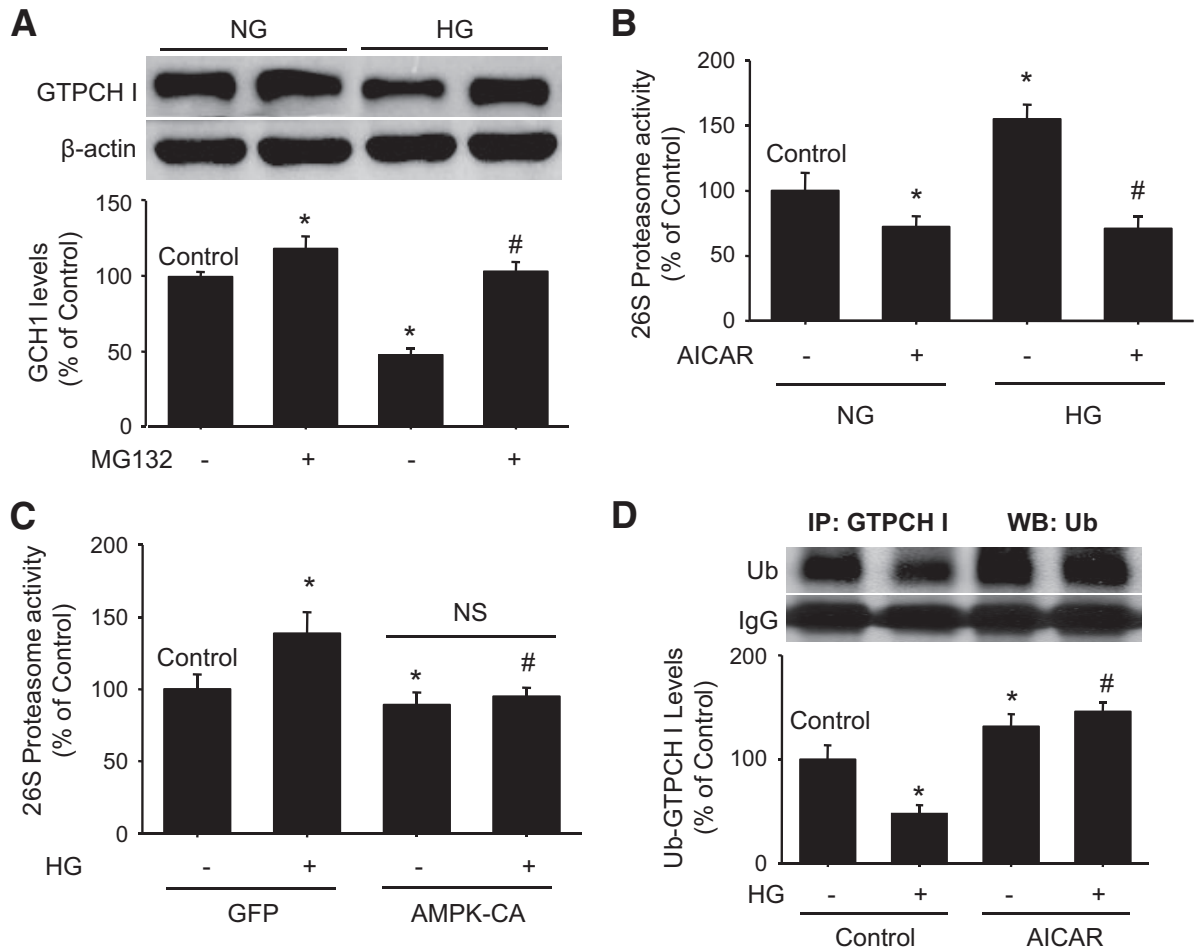


FIG. 5. AMPK activation suppresses 26S proteasome activity in HUVECs. **A:** MG132 blocks the high-glucose (HG)-induced reduction of GTPCH I. HUVECs were treated with high glucose in the presence of MG132 (0.5 $\mu\text{mol/l}$), a proteasome inhibitor. $n = 3$ per group, * $P < 0.05$ versus control, # $P < 0.05$ versus high glucose alone. **B:** Pharmacological activation of AMPK by AICAR suppresses 26S proteasome activity in HUVECs ($n = 3$ per group). 26S proteasome activity was measured using fluorescent proteasome substrates. * $P < 0.05$ versus control, # $P < 0.05$ versus high glucose alone. **C:** Overexpression of AMPK-CA suppresses 26S proteasome activity. After being transfected with GFP or AMPK-CA, HUVECs were incubated with high glucose for 24 h. Cell lysates were assayed for 26S proteasome activity. $n = 3$ per group, * $P < 0.05$ versus GFP alone, # $P < 0.05$ versus high glucose alone, NS: $P > 0.05$. **D:** AICAR attenuates high-glucose-enhanced GTPCH I ubiquitination in HUVECs. HUVECs were incubated with high glucose for 24 h with or without AICAR. After treatments, GTPCH I immunoprecipitated by the specific antibody was Western blotted by the antibody against ubiquitinated protein. The blot is a representative of three blots from three independent experiments. IP, immunoprecipitation; NG, normal glucose; Ub, ubiquitin; WB, Western blot.

the effects of high glucose on the levels of ubiquitinated GTPCH I in HUVEC (Fig. 5D).

AMPK α 2 depletion increases 26S proteasome activity but reduces the levels of GTPCH I, biopterins, and BH4 in isolated aortas. We next determined the effects of AMPK depletion on 26S proteasome activity and the levels of GTPCH I, biopterins, and BH4 in isolated mice aortas. We used AMPK α 1 or - α 2 knockout mice. As shown in Fig. 6A, relative to genetic control mice, C57BL/6, GTPCH I protein levels were markedly lower in AMPK α 2 $^{-/-}$ but not in AMPK α 1 $^{-/-}$ mice aortas, implying that AMPK α 2 subunit plays a key role in the regulation of GTPCH I protein expression. MG132 treatment (0.5 $\mu\text{mol/l}$, 4 h), which only slightly increased GTPCH I levels in control and AMPK α 1 $^{-/-}$ mice, significantly increased GTPCH I levels in the aortas of AMPK α 2 $^{-/-}$ mice.

The deficiency of GTPCH I contributes to the lack of BH4 and eNOS uncoupling in endothelial cells (9,34). We determined the levels of both total biopterins and BH4 in wild-type (WT) and AMPK α 2 $^{-/-}$ mice aortas. As shown in Fig. 6B, both total biopterins and BH4 levels were lower in AMPK α 2 $^{-/-}$ mice aortas than those in WT mice aortas

($P < 0.05$), but injection of AMPK α 2 $^{-/-}$ mice with MG132 (5 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 2 days, intraperitoneally) reversed the effects on both total biopterins and BH4 levels in aortas.

We further detected proteasome activity in AMPK α 2 $^{-/-}$ mice aortas. As shown in Fig. 6C, the 26S proteasome activity was greater in AMPK α 2 $^{-/-}$ mice than that in WT mice. Metformin treatment suppressed 26S proteasome activity in WT mice, but not in AMPK α 2 $^{-/-}$ mice, implying that AMPK α 2 was required for metformin's effects on 26S proteasome in vivo.

Inhibition of the 26S proteasome with MG132 or supplementation of BH4 with L-sepiapterin restores endothelium-dependent relaxation in the aortas of AMPK α 2 $^{-/-}$ mice. To establish a cause-effect relationship between proteasome-dependent GTPCH I degradation and impaired endothelium-dependent relaxation in AMPK α 2 $^{-/-}$ mice, we injected AMPK α 2 $^{-/-}$ mice with MG132 (5 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 2 days, intraperitoneally) or L-sepiapterin (10 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 7 days, intraperitoneally). After the treatments, mouse aortas were isolated and assayed for endothelium-dependent and endothelium-

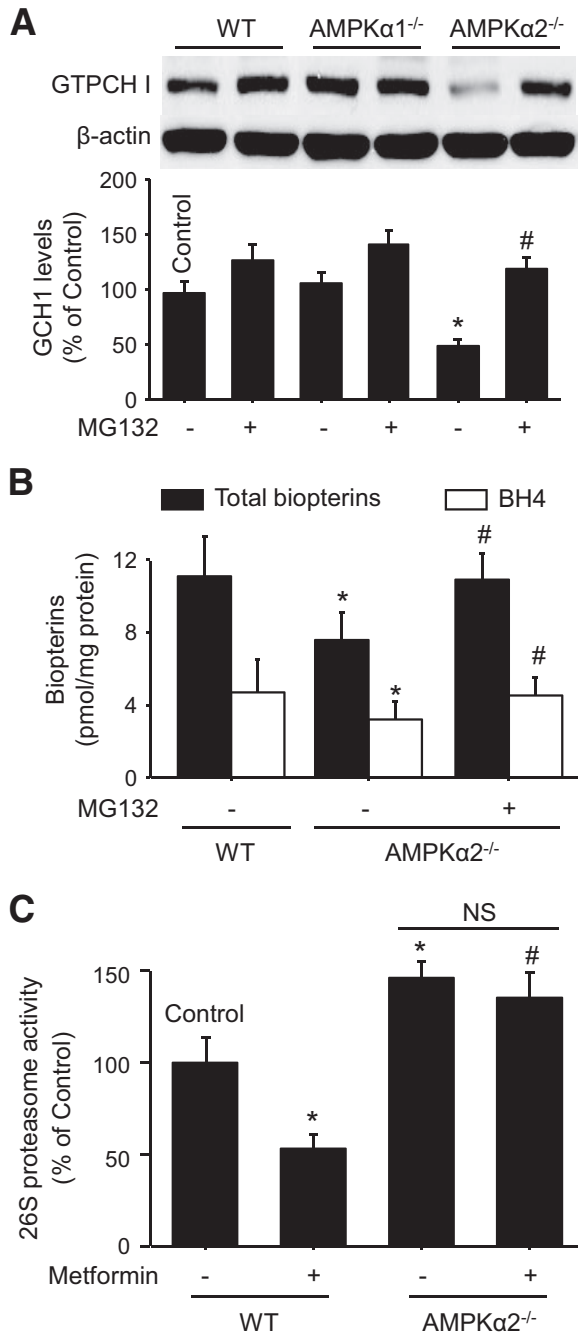


FIG. 6. Decreased levels of GTPCH I, biopeterins, and BH4 are accompanied by increased 26S proteasome activity in isolated aortas from AMPK α 2 knockout mice, in vivo. **A:** After being incubated with or without MG132 (0.5 μ mol/l) for 4 h, aortas isolated from wild-type (WT), AMPK α 1^{-/-}, or AMPK α 2^{-/-} mice were homogenized and assayed for total GTPCH I protein levels by Western blot. The blot shown is a representative of three independent experiments. * P < 0.05 versus control, # P < 0.05 versus AMPK α 2^{-/-} control. **B:** Total biopeterins and BH4 levels in WT and AMPK α 2^{-/-} mice injected with or without MG132 (5 mg \cdot kg⁻¹ \cdot day⁻¹ for 2 days, intraperitoneally). n = 3–5; * P < 0.05 versus WT control, # P < 0.05 versus AMPK α 2^{-/-} control. **C:** 26S proteasome activity. Aortas were treated with metformin (2 mmol/l) for 8 h before the assay. n = 3; * P < 0.05 versus control, # P < 0.05 versus WT plus metformin, NS: P > 0.05.

independent relaxation. As shown in Fig. 7A, the maximal relaxation (Emax) induced by ACh is $83.63 \pm 7.60\%$ in WT aortas. However, Emax of ACh is only $60.9 \pm 5.5\%$ in AMPK α 2^{-/-} aortas (P < 0.05 vs. WT). MG132 did not alter the ACh-induced endothelium-dependent relaxation in WT

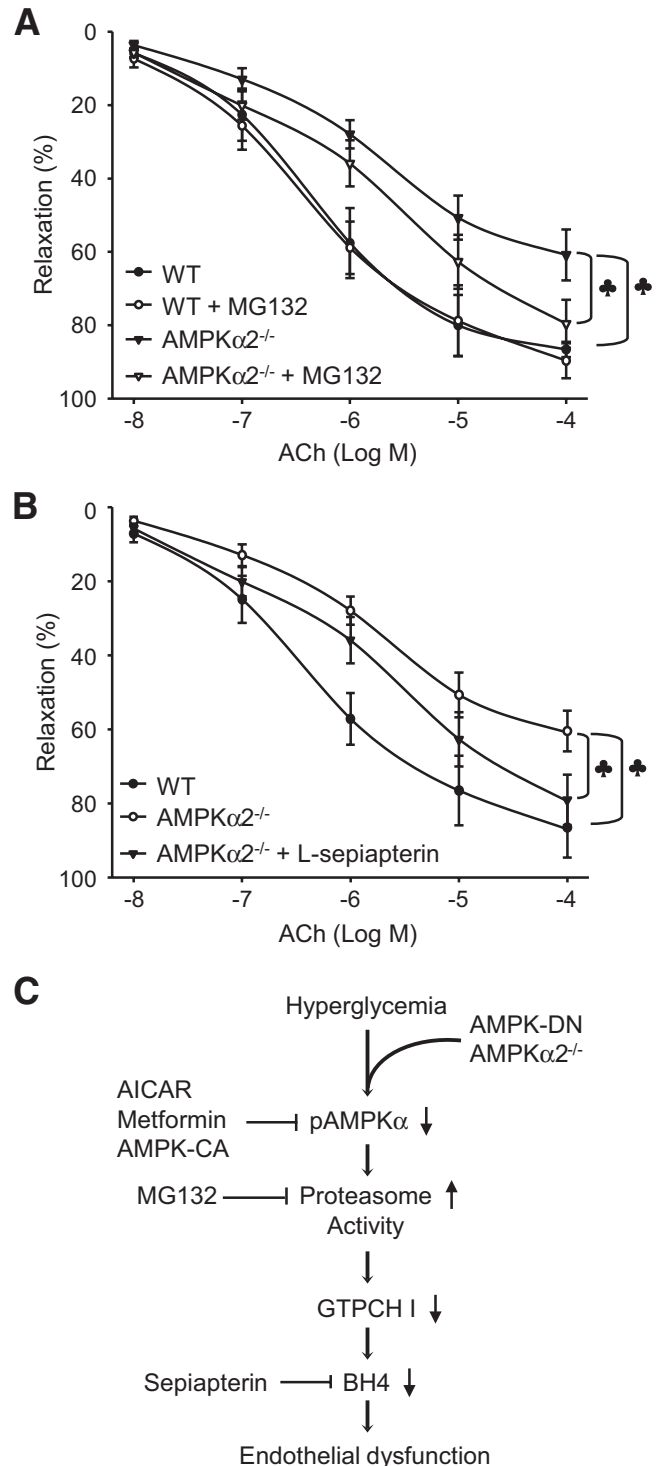


FIG. 7. Inhibition of the proteasome and supplementation with L-sepiapterin improves ACh-induced endothelium-dependent relaxation ex vivo. Male AMPK α 2^{-/-} mice and their genetic controls (C57BL/6 WT mice) were injected with MG132 (5 mg \cdot kg⁻¹ \cdot day⁻¹ for 2 consecutive days) (**A**) or L-sepiapterin (10 mg \cdot kg⁻¹ \cdot day⁻¹ for 7 days) (**B**). After the treatments, isolated mouse aortas assayed for ACh-induced endothelium-dependent relaxation (n = 5 per group, \clubsuit P < 0.05). **C:** Proposed scheme.

mice but improved the relaxation in AMPK α 2^{-/-} mice. As shown in Fig. 7B, L-sepiapterin also mitigated the decrease of ACh-induced Emax in AMPK α 2^{-/-} mice ($73.82 \pm 9.50\%$ vs. AMPK α 2^{-/-}, P < 0.05). SNP-induced, endothelium-independent relaxations in aortas were comparable among

three groups (data not shown). Taken together, these results support the idea that the impaired endothelial function of AMPK α 2^{-/-} mice is because of proteasome-dependent GTPCH I degradation.

DISCUSSION

The major finding of this study is that AMPK inhibition in diabetes or genetic depletion of AMPK leads to abnormal 26S proteasome activity, resulting in the accelerated degradation of GTPCH I, a key enzyme in the de novo synthesis of BH4. The regulation of GTPCH I function can be achieved at both the mRNA and protein levels (stability, activity, and modification) (27). Here, we found that AMPK regulates GTPCH I protein stability in endothelial cells by inhibiting the 26S proteasome. This conclusion is supported by several findings: First, inhibition of the proteasome by MG132 produced suppressive effects similar to AMPK activation on high-glucose-triggered GTPCH I degradation. Second, inhibition of AMPK by high glucose or knockout of AMPK α 2 increased 26S proteasome activity. Third, overexpression of AMPK-CA or AICAR abolished high-glucose-induced upregulation of the 26S proteasome activity. Consistent with our result, Moreno et al. and Viana et al. have reported that two AMPK activators, AICAR and A769662, inhibit 26S proteasome activity in WT mouse embryonic fibroblast cells and human skin fibroblast cells through AMPK-dependent and independent mechanisms (22,35). Fourth, earlier studies by our group and others have demonstrated that GTPCH I protein is ubiquitinated and degraded by the 26S proteasome system under conditions of hyperglycemia or 4-hydroxy-2-nonenal (9,34). Finally, deletion of AMPK α 2 increased the ubiquitination of GTPCH I in the presence of MG132 in HUVECs and mice aortas. However, how AMPK activation suppresses 26S proteasomes remains largely unknown and warrants further investigation.

Metformin, which was shown recently to exert its therapeutic effect in diabetes by activating AMPK (36), is known to improve vascular functions and to dramatically reduce cardiovascular end points and mortality for diabetic patients in large-scale clinical trials (32). Increasing evidence suggests that AMPK activation leads to a series of beneficial metabolic consequences, including augmentation of fatty acid oxidation and glucose uptake in skeletal muscle, as well as inhibition of glucose production, lipogenesis, and cholesterol synthesis in the liver. In addition to producing beneficial metabolic effects, AMPK may regulate endothelial function. There are abundant data to suggest that AMPK is critical for eNOS activation by VEGF, shear stress, estradio, adiponectin, and antidiabetic drugs, including metformin (37). AMPK-dependent eNOS activation is generally ascribed to increased phosphorylation of eNOS at Ser1177 by AMPK, increased association of HSP90 with eNOS, or both. As BH4 is an essential cofactor for the NO synthetic activity of eNOS and GTPCH I deficiency uncouples eNOS (producing superoxide anions instead of NO), AMPK-mediated inhibition of GTPCH I degradation might be essential for maintaining the physiological functions of eNOS. Thus, this study is the first to uncover a novel mechanism (Fig. 7C) by which AMPK activation prevents or ameliorates vascular endothelial function by suppressing proteasome-dependent GTPCH I degradation. Notably, a recent study of obese rats (38) demonstrated that decreased AMPK activity in aortic endothelium is closely associated with

impaired endothelium-dependent relaxation, as well as an increased number of apoptotic endothelial cells, raising the possibility that a decline in AMPK activity contributes to endothelial dysfunction (37). AMPK could be an important target in treating cardiovascular complications in diabetes.

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