In Vivo Activation of AMP-activated Protein Kinase Attenuates Diabetes-enhanced Degradation of GTP Cyclohydrolase I

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Running title: AMPK suppresses GTPCH I degradation

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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 (HUVECs) and aortas isolated from streptozotocin (STZ)-induced diabetic mice were assayed for phospho-AMPK (Thr172), GTPCH I, tetrahydrobiopterin (BH4), and endothelial functions.

Results: Oral administration of metformin (300 mg/kg/day, 4 weeks) in STZ-injected mice significantly blunted the diabetes-induced reduction of AMPK phosphorylation at Thr172. Metformin treatment also normalized acetylcholine (ACh)-induced endothelial relaxation and increased the levels of GTPCH I and BH4. The administration of AICAR, an AMPK activator, or adenoviral over expression of a constitutively active mutant of AMPK abolished the high glucose (30 mM)-induced reduction of GTPCH I, biopetinens, and BH4 but had no effect on GTPCH I mRNA. Furthermore, AICAR or over expression of AMPK inhibited the HG-enhanced 26S proteasome activity. Consistently, inhibition of the proteasome by MG132 abolished HG-induced reduction of GTPCH I in HUVECs. Further, aortas isolated from AMPKα2−/− 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 AMPKα2−/− mice.

Conclusions: We conclude that AMPK activation normalizes vascular endothelial function by suppressing 26S proteasome-mediated GTPCH I degradation in diabetes.

Abbreviations: ACh, acetylcholine; ActD, actinomycin D; AICAR, 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; AMPK-CA, adenovirus encoding constitutively active mutant of AMPK; AMPK-DN, adenovirus encoding dominant-negative mutant of AMPK; BH4, tetrahydrobiopterin; CHX, cycloheximide; DM, diabetes mellitus; eNOS, endothelial nitric oxide synthase; DAF : the fluorophore 4,5-diamino- fluorescein (DAF-2); DHE : Dihydroethidine ; GTPCH I, GTP Cyclohydrolase I; GFP, green fluorescence protein; HUVECs, human umbilicus vein endothelial cells; HG, high glucose; MAECs, mouse aortic endothelial cells; MG132, proteasome inhibitor (Z-Leu-Leu-Leu-al); NG, normal glucose; NO, nitric oxide; SNP, sodium nitroprusside; STZ, streptozotocin; U46619, 9,11-dideoxy-11-9-epoxymethano-prostaglandin F2
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 mellitus alters the metabolism and function of endothelium in ways that could lead to vascular injury (1). In diabetes mellitus, the function of eNOS is altered such that the enzyme produces superoxide anion (O$_2^-$·) 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 have suggested that deficiency of tetrahydrobiopterin (BH4), an essential cofactor for eNOS, transforms eNOS into an oxidant-producing enzyme, leading to the production of O$_2^-$· 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 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 (α1 and α2) subunit imparts catalytic activity, while the other subunits maintain the stability of the heterotrimeric 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
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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.

MATERIALS 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 ROS, streptozotocin-induced diabetes mellitus in mice, 26S proteasome activity assay, immunoprecipitation and western blot analysis, reverse-transcription polymerase chain reaction for GTPCH I, and organ chamber, can be found in the online-only Data Supplement available at http://diabetes.diabetesjournals.org.

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]).

Streptozotocin-induced diabetes mellitus in mice: A low-dose streptozotocin (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 mg/dL for >2 weeks after injection.

Measurement of biopterins: The levels of BH4 and total biopterins were determined by HPLC, as previously described with some modification (25).

Measurement of superoxide anions and NO: The levels of O$_2^-$ production in cultured cells was detected by using dihydroethidium (DHE) fluorescence 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 polymerase chain reaction for GTPCH I: Reverse-transcription polymerase chain reaction was performed according to the manufacturer’s protocol (ThermoScript™, RT-PCR System, Invitrogen, USA). Polymerase chain reaction 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 mean ± SEM. All data were analyzed by 1- or 2-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
demonstrates 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 AMPKα is required for AMPK activity, and because AMPK-Thr172 is closely related to AMPK activity, we first measured the levels of phosphorylated AMPK-Thr172 in mouse aortas isolated from STZ-induced diabetes or non-diabetic mice. As depicted in Figure 1A, the levels of phosphor-AMPK (Thr172) were significantly reduced in diabetic mouse aortas, relative to non-diabetic mice. In contrast, the level of AMPKα was not different in diabetic and non-diabetic mice (Figure 1A). Consistent with our earlier report (9), the levels of GTPCH I were significantly reduced in diabetes, relative to the control group (Figure 1B). In contrast, the levels of eNOS were not altered in diabetic mice or metformin-treated mice (Figure 1B).

Metformin attenuates the diabetes-induced impairment of endothelium-dependent relaxation in vivo: We next asked whether metformin reverses the diabetes mellitus–induced endothelial dysfunction in vivo. Endothelium-dependent relaxation was assayed in aortas isolated from metformin-treated diabetic mice. Compared to non-diabetic mice, the ACh-induced endothelium-dependent relaxation in diabetes was markedly reduced (Figure 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 (Figure 1D). In contrast, 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 µM) for 1 hour in an organ bath. As shown in Figure 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 due to BH4 deficiency.

AICAR attenuates HG-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 (NG, 5 mM), high glucose (HG, 30 mM D-glucose), or hyperosmotic control (5 mM D-glucose plus 25 mM L-glucose) in the presence or absence of AICAR (2 mM), an AMPK activator. HG did not alter AMPK and eNOS expressions (Figure 2A). However, consistent with the reduction of both total biopterins and BH4 levels, the levels of AMPK-Thr172 were markedly reduced in HG-exposed HUVECs (Figure 2A and 2B). Consistent with our previous report, both total biopterins and BH4 levels were not altered in the hyperosmotic control group, compared to HUVECs in normal glucose (Figure 2B). As expected, AICAR treatment increased the level of AMPK phosphorylation at Thr172 in HUVECs exposed to either NG or HG, without altering AMPK and eNOS expressions (Figure 2A).

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

**Genetic inhibition of AMPK reduces GTPCH I in HUVECs:** As HG 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 over expression of AMPK-DN. Over expression of AMPK-DN, but not GFP significantly reduced the levels of GTPCH I (Figure 2C), total biopterins (Figure 2D), and BH4 (Figure 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 Figure 2C and 2D, metformin increased GTPCH I, total biopterins, and BH4 levels in GFP-infected HUVECs, but not in cells infected with AMPK-DN, implying AMPK is required for the metformin-induced inhibition of GTPCH I reduction in HUVECs.

**Over expression 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 Figure 3A and 3B, over expression of AMPK-CA, but not GFP abolished the HG-induced reduction of GTPCH I (Figure 3A), biopterins (Figure 3B), and BH4 levels (Figure 3B).

We next determined if AMPK activation altered eNOS-derived NO/ROS production in response to HG. As expected, HG significantly increased ROS while it suppressed NO production in HUVEC (Figure 3C). As expected, AICAR significantly increased NO release but decreased ROS, implying that increased GTPCH 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 due to the transcriptional activation of the GTPCH I gene or to the decrease of GTPCH I protein degradation. We first determined whether AMPK activation altered GTPCH I mRNA levels in HUVECs. As shown in Figure 4A, treatment of HUVECs with AICAR (2 mM) for 24 hours did not alter the levels of GTPCH I mRNA in HUVECs exposed to NG or HG. In addition, neither
cycloheximide nor actinomycin D altered the levels of GTPCH I protein caused by AICAR or metformin (Figure 4B). Neither cycloheximide nor actinomycin D altered the HG-induced reduction of GTPCH I levels in HUVECs (Figure 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.

The 26S proteasome mediates the reduction of GTPCH I levels in HG-treated HUVECs: The levels of GTPCH I protein are controlled by both GTPCH I synthesis and GTPCH I degradation. Our earlier study demonstrated that HG activates the 26S proteasome, resulting in the enhanced degradation of GTPCH I in endothelial cells (9). Co-administration of MG132 (0.5 µM, 4 hours), a potent proteasome inhibitor, abolished the HG-induced reduction of GTPCH I levels (Figure 5A), confirming that the alteration of GTPCH I levels was due to the activation of the 26S proteasome.

AMPK activation suppresses the increase of 26S proteasome activity caused by HG: We next investigated the effects of AMPK activation on 26S proteasome activity in HUVECs. As shown in Figure 5B, HG exposure significantly increased 26S proteasome activity, while AICAR treatment significantly reduced 26S proteasome activity in cells exposed to NG or HG.

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 (Figure 5C). In addition, AMPK-CA, but not GFP abolished HG-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 HG-treated HUVECs. HUVEC were incubated with HG for 24 hours with or without AICAR. After the incubation, GTPCH I were immunoprecipitated by the specific antibody against GTPCH I. GTPCH I ubiquitination was identified in western blotted by using the antibody against ubiquitinated protein. As shown in Figure 5D, AICAR alone significantly increased the level of ub-GTPCH I in HUVEC. Conversely, HG significantly decreased the ub-GTPCH I in HUVEC (Figure 5D). Interestingly, Co-administration of HG with AICAR abolished the effects of HG on the levels of Ub-GTPCH I in HUVEC (Figure 5D).

AMPKα2 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 AMPKα1 or α2 knockout mice. As shown in Figure 6A, relative to genetic control mice, C57BL6, GTPCH I protein levels were markedly lower in AMPKα2−/− but not in AMPKα1−/− mice aortas, implying that AMPKα2 subunit plays a key role in the regulation of GTPCH I protein expression. MG132 treatment (0.5 µM, 4 hours), which only slightly increased GTPCH I levels in control and AMPKα1−/− mice, significantly increased GTPCH I levels in the aortas of AMPKα2−/− 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 WT and AMPKα2−/− mice aortas. As shown in Figure 6B, both total biopterins and BH4 levels were lower in AMPKα2−/− mice aortas than those in WT mice aortas (P<0.05), but injection of AMPKα2−/− mice with MG132 (5 mg/kg/day, 2 days, I.P.) reversed the effects on both total biopterins and BH4 levels in aortas.
We further detected proteasome activity in AMPKα2−/− mice aortas. As shown in Figure 6C, the 26S proteasome activity was greater in AMPKα2−/− mice than that in WT mice. Metformin treatment suppressed 26S proteasome activity in WT mice, but not in AMPKα2−/− mice, implying that AMPKα2 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 AMPKα2−/− mice:** In order to establish a cause-effect relationship between proteasome-dependent GTPCH I degradation and impaired endothelium-dependent relaxation in AMPKα2−/− mice, we injected AMPKα2−/− mice with MG132 (5 mg/kg/day, 2 days, I.P.) or L-sepiapterin (10 mg/kg/day, 7 days, I.P.). After the treatments, mouse aortas were isolated and assayed for endothelium-dependent and endothelium-independent relaxation. As shown in Figure 7A, the maximal relaxation (Emax) induced by ACh is 83.63 ± 7.60% in WT aortas. However, Emax of ACh is only 60.9 ± 5.5 in AMPKα2−/− aortas (P<0.05 vs. WT). MG132 did not alter the ACh-induced endothelium-dependent relaxation in WT mice, but improved the relaxation in AMPKα2−/− mice. In Figure 7B, L-sepiapterin also mitigated the decrease of ACh-induced Emax in AMPKα2−/− mice (73.82 ± 9.50% vs. AMPKα2−/−, 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 AMPKα2−/− mice is due to 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, modification) (27). Here we found that the 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 similar suppressive effects as AMPK activation on HG-triggered GTPCH I degradation. Second, inhibition of AMPK by HG or knockout of AMPKα2 increased 26S proteasome activity. Third, overexpression of AMPK-CA or AICAR abolished HG-induced upregulation of the 26S proteasome activity. Consistent with our result, Daniel Moreno and Rosa Viana PF 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 AMPKα2−/− 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 endpoints 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
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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 either 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 (Figure 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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All authors have full access to the data and take full responsibility for the integrity of the data. We have read and agreed to the manuscript as written.
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REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Activation of AMPK by metformin attenuates diabetes-induced reduction of phosphor-AMPK-Thr172 (A), GTPCH I (B), and bioperins (C) levels and improves endothelial function. Control and diabetic mice were fed with metformin (300 mg/kg/day) for 4 weeks. Mouse aortas were isolated and assayed for phospho-AMPK, GTPCH I, total bioperins, and BH4 levels, as described in Materials and Methods. The results were obtained from 5 mice. *P<0.05 vs. control, #P<0.05 vs. diabetes alone. (D) ACh-induced endothelium-dependent relaxation was assayed as described in Materials and Methods (n=5 per group, ♦ is indicated P<0.05). L-sepiapterin (10 µM) was added 1 hour prior to the start of the experiments.

**Figure 2.** Activation of AMPK is required for the AICAR/metformin-induced reduction of GTPCH I caused by HG exposure in HUVECs. HUVECs were treated with HG (30 mM D-glucose) or hyperosmotic medium (HO, 5 mM glucose plus 25 mM L-glucose) for 24 hours in the presence or absence of AICAR (2 mM). HUVECs were collected and assayed for (A) the levels of P-AMPK and GTPCH I protein by western blot, (B) total bioperins, and BH4. (n=5 per group, *P<0.05 vs control, #P<0.05 vs. HG alone). (C and D) Overexpression of AMPK-DN abolishes metformin’s effects on GTPCH I, bioperins, and BH4. HUVECs were transfected with ad-GFP or ad-AMPK-DN for 24 hours and then incubated with or without metformin (2 mM) for 8 hours. The results are a summary of three independent experiments. *P<0.05 vs. GFP alone, #P<0.05 vs. GFP plus metformin, NS indicated P>0.05.

**Figure 3.** Over expression of AMPK-CA abolishes the reduction of GTPCH I levels caused by HG in HUVECs. After 24 hours of being infected with ad-GFP or ad-AMPK-CA, HUVECs were incubated with HG for an additional 24 hours. After the incubation, HUVECs were collected and assayed for GTPCH I protein (A), and total bioperins and BH4 levels (B). *P<0.05 vs. GFP alone, #P<0.05 vs. GFP plus HG, NS indicated P>0.05. (C) HUVECs were incubated with HG for 24 hours with or without AICAR. NO and ROS productions were detected by using DAF or DHE fluorescence, respectively.

**Figure 4.** AMPK activation does not alter GTPCH I expression in HUVECs. (A) HUVECs were treated with HG for 24 hours in the presence or absence of AICAR (2 mM). Cells were subjected to perform RT-PCR analysis for GTPCH I mRNA expression. The picture shown is a
Figure 5. AMPK activation suppresses 26S proteasome activity in HUVECs. (A) MG132 blocks the HG-induced reduction of GTPCH I. HUVECs were treated with HG in the presence of MG132 (0.5 µM), a proteasome inhibitor. n=3 per group. *P<0.05 vs. control, #P<0.05 vs. MG132 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 vs. control, #P<0.05 vs. HG alone. (C) Over expression of AMPK-CA suppresses 26S proteasome activity. After being transfected with GFP or AMPK-CA, HUVECs were incubated with HG for 24 hours. Cell lysates were assayed for 26S proteasome activity. n=3 per group, *P<0.05 vs. GFP alone, #P<0.05 vs. HG alone, NS indicated P>0.05. (D) AICAR attenuates HG-enhanced GTPCHI ubiquitination in HUVECs. HUVEC were incubated with HG for 24 hours with or without AICAR. After treatments, GTPCHI immunoprecipitated by the specific antibody was western blotted by the antibody against ubiquitinated protein. The blot is a representative of 3 blots from 3 independent experiments.

Figure 6. Decreased levels of GTPCH I, biopeterins, and BH4 is accompanied by increased 26S proteasome activity in isolated aortas from AMPKα2 knockout mice, in vivo. (A) After being incubated with or without MG132 (0.5 µM) for 4 hours, aortas isolated from WT, AMPKα2+/−, or AMPKα2−/− 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 vs. control, #P<0.05 vs. AMPKα2−/− control. (B) Total biopeterins and BH4 levels in WT and AMPKα2−/− mice injected with or without MG132 (5 mg/kg/day, 2 days, I.P). n=3-5; *P<0.05 vs. WT control, #P<0.05 vs. AMPKα2−/− control. (C) 26S proteasome activity. Aortas were treated with metformin (2 mM) for 8 hours before the assay. n=3; *P<0.05 vs. control, #P<0.05 vs. WT plus metformin, NS indicated P>0.05.

Figure 7. Inhibition of the proteasome and supplementation with L-sepipeterin improves ACh-induced endothelium-dependent relaxation, ex vivo. Male AMPKα2−/− mice and their genetic controls (C57BL6 WT mice) were injected with MG132 (5 mg/kg/day for 2 consecutive days) (A) or L-sepiapterin (10 mg/kg/day for 7 days) (B). After the treatments, isolated mouse aortas assayed for ACh-induced endothelium-dependent relaxation (n=5 per group, ♣ is indicated as P<0.05.). (C) Proposed scheme.
Figure 1

A. P-AMPK levels (% of Control)

B. GTPCH I levels (% of Control)

C. Total biopterins and BH4

D. Relaxation (%) vs. Ach (Log M)
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Figure 2

A. P-AMPK, AMPK, GTPCH I, eNOS, β-actin

B. Total biopterins, BH4

C. GFP, AMPK-DN

D. Total biopterins, BH4
Figure 3

AMPK suppresses GTPCH I degradation
AMPK suppresses GTPCH I degradation

Figure 4

A.

GTPCH I

GAPDH

GCH1 mRNA

(% of Control)

0 50 100 150

NG HG

Control - + - +

AICAR

B.

GTPCH I

β-actin

GCH1 levels

(% of Control)

0 50 100 200 250

Control Metformin AICAR HG

NS NS

NS

CHX Act D

- + - + - + - + - + - + - + - + - +
AMPK suppresses GTPCH I degradation

Figure 5

A. GTPCH I and β-actin levels in NG and HG conditions. MG132 treatment effects are also shown.

B. 26S proteasome activity in Control and AICAR-treated conditions, comparing NG and HG.

C. 26S proteasome activity in HG with GFP and AMPK-CA overexpression.

D. Immunoprecipitation (IP) and Western Blot (WB) analysis of Ub-GTPCH I levels under Control and AICAR conditions.
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Figure 6

A. GTPCH I levels (% of Control) with WT, AMPKα1^+ and AMPKα2^-/

B. Total biopterins and BH4 with MG132, WT and AMPKα2^-/

C. 26S proteasome activity (% of Control) with Metformin, WT and AMPKα2^-/
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Figure 7

A. 

B. 

C. 

Hyperglycemia → AMPK-DN AMPKα2<sup>−/−</sup> → pAMPKα → MG132 → Proteasome Activity → GTPCH I → Sepiapterin → BH4 → Endothelial dysfunction