C-peptide activates AMPKα and prevents ROS-mediated mitochondrial fission and endothelial apoptosis in diabetes mellitus

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ABSTRACT

Vasculopathy is a major complication of diabetes mellitus; however, molecular mechanisms mediating the development of vasculopathy and potential strategies for prevention have not been identified. We have previously reported that C-peptide prevents diabetic vasculopathy by inhibiting reactive oxygen species (ROS)-mediated endothelial apoptosis. To gain further insight into ROS-dependent mechanism of diabetic vasculopathy and its prevention, we studied high glucose-induced cytosolic and mitochondrial ROS production and its effect on altered mitochondrial dynamics and apoptosis. For the therapeutic strategy, we investigated the vasoprotective mechanism of C-peptide against hyperglycemia-induced endothelial damage through the AMP-activated protein kinase α (AMPKα) pathway using human umbilical vein endothelial cells and aorta of diabetic mice. High glucose (33 mM) increased intracellular ROS through a mechanism involving inter-regulation between cytosolic and mitochondrial ROS generation. C-peptide (1 nM) activation of AMPKα inhibited high glucose-induced ROS generation, mitochondrial fission, mitochondrial membrane potential collapse, and endothelial cell apoptosis. Additionally, the AMPK activator 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside and the anti-hyperglycemic drug metformin mimicked protective effects of C-peptide. C-peptide replacement therapy normalized hyperglycemia-induced AMPKα dephosphorylation, ROS generation, and mitochondrial disorganization in aorta of diabetic mice. These findings highlight a novel mechanism by which C-peptide activates AMPKα and protects against hyperglycemia-induced vasculopathy.
INTRODUCTION

C-peptide and insulin are co-secreted in equimolar amounts into the circulation from the pancreatic β-cells of Langerhans (1). C-peptide deficiency is a prominent attribute of type 1 diabetes mellitus (DM) (1). Deficiencies of C-peptide and insulin may also occur in the late stages of type 2 DM due to progressive loss of β-cells (2,3,4). Recent evidence demonstrates a beneficial role for C-peptide in diabetic neuropathy (1,5,6), nephropathy (1,6,7), and vascular dysfunction (1,5) and inflammation (1). C-peptide protects against diabetic vascular damage by promoting nitric oxide (NO) release (8) and suppressing nuclear factor-kappa B (9), which suppresses leukocyte-endothelium interactions (8,9). C-peptide may prevent atherosclerosis by inhibiting vascular smooth muscle proliferation and migration (10) and reducing venous neointima formation (11). However, the molecular mechanism by which C-peptide prevents diabetic complications is not understood well enough to permit its clinical implementation.

Generation of reactive oxygen species (ROS) in response to high glucose is the leading cause of endothelial damage and diabetic vasculopathy (12). Protein kinase C (PKC)-dependent nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is considered to be a major cytosolic mediator of ROS generation in endothelial cells (13,14) that play a central role in hyperglycemia-induced endothelial cell apoptosis and vascular complications (15,16,17). Overproduction of intracellular ROS by mitochondria also occurs during the development of hyperglycemia-induced vascular complications (12,18,19). Altered mitochondrial dynamics due to mitochondrial fission were recently linked with endothelial dysfunction in diabetes (20,21). However, the mechanisms
regulating production of cytosolic and mitochondrial ROS and their individual functions in regulating mitochondrial dynamics and apoptosis remain to be elucidated.

Adenosine monophosphate-activated protein kinase (AMPK) is an intracellular energy and stress sensor (22) and is an emerging target for preventing diabetic complications (23), as exhibited by the most common anti-hyperglycemic drugs, rosiglitazone (24) and metformin (25). AMPK prevents apoptosis of endothelial cells (26,27,28) by inhibiting ROS generation by NADPH oxidase (24,29) and mitochondria (30). Additionally, AMPK dephosphorylation is associated with diabetes (22,31,32). It has been reported that C-peptide inhibits high glucose-induced mitochondrial superoxide generation in renal microvascular endothelial cells (7). We recently demonstrated a key role for C-peptide in preventing high glucose-induced ROS generation and apoptosis of endothelial cells through inhibition of transglutaminase (17). However, the mechanism underlying C-peptide-mediated inhibition of intracellular ROS production and subsequent apoptosis remains unclear. Thus, we hypothesized that the potential protective role of C-peptide could be attributed to activation of AMPK, which results in reduced hyperglycemia-induced production of intracellular ROS and altered mitochondrial dynamics that suppress apoptosis of endothelial cells.

In this study, we sought to elucidate the mechanism by which C-peptide protects against hyperglycemia-induced ROS production and subsequent endothelial cell damage. We examined the beneficial effect of C-peptide through AMPKα activation and subsequent protection against hyperglycemia-induced production of intracellular ROS, dysregulation of mitochondrial dynamics, mitochondrial membrane potential (ΔΨm) collapse, and apoptosis of endothelial cells. These studies were confirmed in vivo in streptozotocin-induced diabetic mice using C-peptide supplement therapy delivered
through osmotic pumps. Thus, our study implicates C-peptide replacement therapy as a potentially significant approach for preventing diabetic complications.
RESERCH DESIGN AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins as previously described (17). Cells were maintained in M199 culture media supplemented with 20% FBS, 3 ng/ml bFGF, 5 U/ml heparin, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells between passages 3 and 7 were incubated overnight in low-serum medium (M199 supplemented as described above except with 5% FBS, 1 ng/ml bFGF, and 25 mM HEPES).

Measurement of intracellular and mitochondrial ROS generation and intracellular NO production

Intracellular ROS generation was measured using H$_2$DCFDA staining (Molecular Probes, Eugene, OR) as previously described (17). Cells on 2% gelatin-coated coverslips were treated with 33 mM D-glucose for 48 h and stained with 10 µM H$_2$DCFDA for 10 minutes (min) in serum-free, phenol red-free media. For measurement of mitochondrial ROS, cells were incubated with 5 µM MitoSox Red (Molecular Probes) for 30 min in low-serum media. Cells were stained with 2 µM DAF-FM diacetate (Molecular Probes) for 30 minutes in low-serum medium for measuring intracellular NO production was measured using DAF-FM staining (Molecular Probes). Labeled cells were immediately analyzed by confocal microscopy (Olympus, Fluoview-300, Japan). Single-cell fluorescence intensities were determined for 30 cells per experiment. The levels of intracellular and mitochondrial ROS, and intracellular NO were determined by comparing fold change in fluorescence intensity of treated cells versus control cells.
Mitochondrial staining and analysis of mitochondrial fission

Cells were incubated with 33 mM D-glucose for 48 h and entire mitochondria were visualized in live cells by staining with 200 nM Mito Tracker Red CMXRos (Molecular Probes) for 30 min at 37°C. Cells with a predominantly intact network of tubular mitochondria were identified as normal. Cells with disrupted and predominantly spherical mitochondria were identified as having mitochondrial fission. Ninety cells from three independent experiments were used to calculate the percentage of cells undergoing mitochondrial fission.

Measurement of mitochondrial membrane potential ($\Delta \Psi_m$)

$\Delta \Psi_m$ was monitored by confocal microscopy as described previously (33). Cells were incubated with 33 mM D-glucose for 48 h and stained with 2 μM of JC-1 (Molecular Probes) for 30 min at 37°C. Fluorescence intensities were determined at the single-cell level using confocal microscopy. Data are expressed as a normalized ratio of the fluorescence intensity of the monomeric form (green) to the J-aggregate form (red).

Measurement of apoptosis

Cell death was assessed by DiOC$_6$/PI double-staining using confocal microscopy as described previously (34). Briefly, cells were incubated with 33 mM D-glucose for 72 h and stained with 50 nM DiOC$_6$ and 10 μg/ml PI for 20 min. Stained cells were analyzed by confocal microscopy and differentiated as viable cells (DiOC$_6^{\text{bright}}$/PI-negative), early apoptotic cells (DiOC$_6^{\text{dim}}$/PI-negative), and late apoptotic cells (DiOC$_6^{\text{dim}}$/PI-positive).
Total cell death (defined as the sum of early and late apoptotic cells) and frequency distributions were expressed as percentages of the total cell number.

**Transfection of small interfering RNA (siRNA)**

To suppress AMPKα expression, HUVECs were transfected with AMPKα1/2-specific siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) according to manufacturer’s instructions. Briefly, subconfluent cells were transfected overnight with the AMPKα siRNA duplex, which was synthesized to target the coding sequence of human AMPKα1/2 mRNA. A non-coding siRNA (Dharmacon, Lafayette, CO) was used as a control. Transfection was performed using siLentFect™ Lipid Reagent (Bio-Rad Laboratories, Hercules, CA) for 24 h.

**Generation of diabetes mouse model and C-peptide replacement therapy using osmotic pumps**

Six-week old male C57BL/6 mice were obtained from KOTECH (Pyungtaek, Korea). Experiments were performed in accordance with the guidelines of the Kangwon Institutional Animal Care and Use Ethics Committee. Diabetic mice were generated by a single intraperitoneal injection of streptozotocin (150 mg/kg body weight) as described previously (17). Sufficient hyperglycemia was observed 2 days post injection, as determined by measuring blood glucose and glucosuria. Mice with non-fasting blood glucose levels greater than 16 mM, polyuria, and glucosuria were considered diabetic. One week after the streptozotocin injection, an Alzet mini-osmotic pump 2004 (DURECT, Cupertino, CA) containing C-peptide (American Peptide Company, Inc., Sunnyvale, CA) in PBS with a delivery rate of 35 pmol/min/kg was subcutaneously implanted into each
diabetic mouse in one group. The other diabetic and control mice received sham operations. During the continuous subcutaneous C-peptide perfusion, serum C-peptide levels were measured using a C-peptide Enzyme Immunoassay Kit (RayBiotech, Norcross, GA). During the whole course of these experiments, the vital statistics of each mouse were recorded.

**Measurement of ROS generation and mitochondrial fission in aortic endothelium of mice**

Aorta from control (n=8), diabetic (n=8), and C-peptide-supplemented diabetic (n=7) mice were dissected and cut longitudinally to open the endothelium for immediate staining. For intracellular ROS measurements, aortic segments were quickly transferred to serum-free M199 media and incubated at 37°C with H2DCFDA for 10 min. For assessment of mitochondrial fission, endothelial layers from each group were stained at 37°C for 30 min with 200 nM MitoTracker Red CMXRos. Stained aortic segments were mounted on glass slides and quickly observed by confocal microscopy.

**Western blot analysis**

Proteins were extracted from HUVECs and aortic segments of mice with lysis buffer containing 50 mM HEPES (pH 7.5), 1mM EDTA, 150 mM sodium chloride, 1% Triton X-100, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.1 mM phenylmethanesulfonylfluoride, 25 mM β-glycerophosphate, and 2 mM sodium orthovanadate. Protein extracts were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Protein expression was analyzed with monoclonal antibodies against phospho-AMPKα, AMPKα, dynamin-related protein 1 (Drp-1) (Cell Signaling Technology, Beverly, MA), and
fission-1 protein (Fis-1) (Santa Cruz Biotechnology). Protein bands were visualized with a chemiluminescence reagent (Pierce, Rockford, IL).

**Statistical analyses**

Data processing was performed using Origin 6.1 (OriginLab, Northampton, MA) and expressed as the mean ± S.D. of at least three independent experiments. Statistical significance was determined using analysis of variance. A value of \( p < 0.05 \) was considered statistically significant.
RESULTS

High glucose-induced generation of cytosolic ROS facilitates mitochondrial ROS production

In HUVECs, high glucose (33 mM) significantly increased the levels of intracellular and mitochondrial ROS (Figure 1A and B). However, osmotic control L-glucose had no effect on the ROS levels (Figure 1B). To understand the effect of cytosolic ROS on mitochondrial ROS levels, we inhibited PKC and NADPH oxidase that mediate high glucose-induced cytosolic ROS generation in endothelial cells. The PKC inhibitors, GF109203X and Ro-31-8220, attenuated high glucose-induced generation of intracellular and mitochondrial ROS in a dose-dependent manner (Figure 1C). The well-established NADPH oxidase inhibitors, apocynin and diphenyliodonium (DPI), also prevented intracellular and mitochondrial ROS production in a dose-dependent manner (Figure 1D). These results suggest that PKC-dependent activation of NADPH oxidase increases cytosolic ROS, which then promotes mitochondrial ROS generation under hyperglycemic conditions.

High glucose-induced mitochondrial ROS generation regulates increased intracellular ROS

The superoxide dismutase 2 mimetic Mito-TEMPO inhibited high glucose-induced mitochondrial ROS levels in a dose-dependent manner (Figure 1E). Interestingly, high glucose-induced intracellular ROS was also inhibited by mito-TEMPO in a dose-dependent manner at a rate similar to mitochondrial ROS inhibition (Figure 1E). The role of mitochondrial ROS in the regulation of intracellular ROS was further investigated
using Mdivi-1, a potent inhibitor of Drp-1-mediated mitochondrial fission (35). Mitochondrial fission is believed to be an important cause of hyperglycemia-induced endothelial damage due to overproduction of mitochondrial ROS (20). Mdivi-1 inhibited high glucose-induced production of mitochondrial and intracellular ROS in a dose-dependent manner (Figure 1F). Thus, mitochondrial ROS regulates production of intracellular ROS in response to high glucose. Blocking the production of cytosolic ROS inhibits mitochondrial ROS generation, demonstrating a potential inter-regulation between cytosolic and mitochondrial ROS in response to high glucose in endothelial cells.

**Both cytosolic and mitochondrial ROS mediate high glucose-induced mitochondrial fission and ΔΨₘ collapse**

High glucose treatment significantly induced mitochondrial fission in HUVECs as assessed by MitoTracker Red staining. Control cells showed an intact mitochondrial network. Cells bearing predominantly fragmented and spherical mitochondria were considered to have undergone mitochondrial fission (Figure 2A and B). High glucose-induced mitochondrial fission was prevented by PKC inhibitors GF109203X and Ro-31-8220 and NADPH oxidase inhibitors apocynin and DPI (Figure 2C). These inhibitors also blocked cytosolic ROS generation in response to high glucose in endothelial cells (Figure 1C and D). However, L-glucose had no effect on mitochondrial fission (Figure 2B). High glucose-induced mitochondrial fission was also normalized using mito-TEMPO and Mdivi-1 (Figure 2D). Thus, high glucose-induced mitochondrial fission is mediated by both cytosolic and mitochondrial ROS in endothelial cells.

High glucose stimulated ΔΨₘ collapse in endothelial cells as assessed by JC-1 staining (Figure 2E), whereas L-glucose had no effect (Figure 2F). The PKC inhibitors,
GF109203X and Ro-31-8220, and the NADPH oxidase inhibitors, apocynin and DPI, rescued high glucose-induced $\Delta \Psi_m$ collapse (Figure 2F). Furthermore, high glucose-induced $\Delta \Psi_m$ collapse was normalized by mito-TEMPO and Mdivi-1 (Figure 2G). These results demonstrate that high glucose-induced $\Delta \Psi_m$ collapse is mediated by cytosolic and mitochondrial ROS in endothelial cells.

**C-peptide inhibits high glucose-induced ROS generation, mitochondrial fission, and $\Delta \Psi_m$ collapse**

C-peptide inhibited high glucose-induced generation of intracellular and mitochondrial ROS in a dose-dependent manner, with maximal effect at 1 nM (Figure 3A). As intracellular and mitochondrial ROS both mediate high glucose-induced mitochondrial fission and $\Delta \Psi_m$ collapse (Figure 2), we studied the effects of C-peptide on mitochondrial fission and $\Delta \Psi_m$ collapse in response to high glucose. C-peptide prevented high glucose-stimulated mitochondrial fission (Figure 3B). Consistently, high glucose-induced expression of fission proteins Drp-1 and Fis-1 was inhibited by C-peptide (Figure 3C-E). Additionally, C-peptide rescued high glucose-induced $\Delta \Psi_m$ collapse (Figure 3F). However, heat-inactivated C-peptide had no inhibitory effect on high glucose-induced generation of intracellular and mitochondrial ROS (Figure 3A), mitochondrial fission (Figure 3B), and $\Delta \Psi_m$ collapse (Figure 3F). Thus, C-peptide prevents high glucose-induced mitochondrial fission and $\Delta \Psi_m$ collapse by inhibiting ROS generation.

**Essential roles of AMPKα in C-peptide-mediated prevention of high glucose-induced ROS generation, mitochondrial fission, and $\Delta \Psi_m$ collapse**
C-peptide stimulated phosphorylation of AMPKα and reversed high glucose-induced dephosphorylation of AMPKα (Figure 4A and B). However, heat-inactivated C-peptide had no significant effect on AMPKα phosphorylation (data not shown). AMPKα1/2-specific siRNA suppressed AMPKα expression in a dose-dependent manner (Figure 4C). AMPKα knockdown reversed C-peptide-mediated inhibition of high glucose-induced intracellular and mitochondrial ROS production (Figure 4D). Furthermore, the AMPK inhibitor, compound C, dose-dependently reversed C-peptide-mediated prevention of high glucose-stimulated ROS generation (Figure 4E). AMPK functions as a physiological suppressor of NADPH oxidase and ROS production in endothelial cells (29,30). Thus, it is likely that AMPKα-mediated inhibition of NADPH oxidase is an important mechanism by which C-peptide prevents production of intracellular and mitochondrial ROS in response to high glucose.

We then used AMPKα1/2 siRNA and compound C to investigate the contribution of AMPK to the altered mitochondrial dynamics stimulated by high glucose. AMPKα1/2 siRNA reversed C-peptide-mediated inhibition of mitochondrial fission in the presence of high glucose (Figure 4F). Similarly, compound C prevented C-peptide-mediated inhibition of high glucose-induced mitochondrial fission (Figure 4G). The essential role of AMPKα in C-peptide-mediated prevention of ΔΨm collapse induced by high glucose was also found using AMPKα1/2 siRNA and compound C (Figure 4H and I). Taken together, C-peptide inhibits high glucose-induced generation of intracellular and mitochondrial ROS through activation of AMPKα, resulting in prevention of mitochondrial fission and ΔΨm collapse in endothelial cells.
Additionally, we further investigated whether NO production by C-peptide acts an upstream of AMPK. C-peptide significantly elevated the level of intracellular NO at 0.5 h, with maximal effect at 2 h, (Figure 4J) and the elevated level returned back to the basal level at 12 h (data not shown). L-NAME prevented C-peptide-induced NO production but Compound C had no effect (Figure 4J). C-peptide activation of AMPKα was blocked by L-NAME and the NO donor SNAP reversed HG-induced AMPKα dephosphorylation at 48 h (Figure 4K and L).

**AICAR and metformin mimic C-peptide-mediated prevention of high glucose-induced ROS generation, mitochondrial fission, and ΔΨ<sub>m</sub> collapse through AMPKα activation**

AICAR and metformin each inhibited high glucose-induced generation of intracellular and mitochondrial ROS in a dose-dependent manner (Figure 5A and B). AICAR and metformin also rescued high glucose-mediated inhibition of AMPKα as demonstrated by its phosphorylation at Thr172 (Figure 5C and D). High glucose-stimulated mitochondrial fission was significantly inhibited by AICAR and metformin (Figure 5E). Consistently, these AMPK activators inhibited high glucose-induced expression of Drp-1 and Fis-1 (data not shown). ΔΨ<sub>m</sub> collapse induced by high glucose was significantly reversed by AICAR and metformin (Figure 5F). Additionally, the ROS scavenger Trolox significantly prevented high glucose-stimulated mitochondrial fission and ΔΨ<sub>m</sub> collapse (Figure 5E and F). Thus, AICAR and metformin mimic C-peptide by preventing high glucose-induced mitochondrial fission and ΔΨ<sub>m</sub> collapse through AMPKα-mediated inhibition of intracellular and mitochondrial ROS generation.
C-peptide prevents endothelial cell apoptosis by AMPKα-mediated inhibition of ROS generation in hyperglycemia

We used the 3,3′-dihexyloxacarbocyanine iodide (DiOC6) / propidium iodide (PI) double-staining assay (34) to exam whether C-peptide prevents high glucose-induced apoptosis by activating AMPK signaling. C-peptide prevented high glucose-induced apoptosis of endothelial cells (Figure 6A). D-glucose induced the endothelial cell apoptosis in a dose-dependent manner, which was inhibited by C-peptide (Supplementary Figure 1). However, apoptotic cell death was neither activated by L-glucose, nor inhibited by heat-inactivated C-peptide (Figure 6A). Endothelial cell apoptosis was significantly inhibited by AMPK activators AICAR and metformin, the NADPH oxidase inhibitor apocynin, and the mitochondrial ROS scavenger mito-TEMPO (Figure 6A). Thus, C-peptide prevents high glucose-induced endothelial cell apoptosis through activation of AMPK and inhibition of ROS generation. Moreover, inhibition of high glucose-induced apoptosis by Mdivi-1 (Figure 6A) provides further evidence that Drp-1-dependent mitochondrial fission is important for high glucose-induced endothelial cell apoptosis.

The key role of AMPKα in C-peptide-mediated protection of endothelial cell death was further elucidated using AMPKα 1/2 siRNA and compound C (Figure 6B and C). Transfection of AMPKα 1/2 siRNA or treatment with compound C significantly increased apoptosis (Figure 6B and C). Moreover, C-peptide-mediated inhibition of high glucose-stimulated endothelial cell apoptosis was reversed by AMPKα 1/2 siRNA or compound C (Figure 6B and C). Further, compound C reversed the C-peptide inhibition of dose-dependent high glucose-induced apoptosis (Supplementary Figure 1). Thus, C-
peptide protects endothelial cells from apoptosis by AMPKα-mediated inhibition of ROS generation and prevention of mitochondrial fission in hyperglycemia.

**C-peptide promotes AMPKα activation and prevents ROS generation and mitochondrial fission in the aortas of streptozotocin diabetic mice**

The role of AMPKα in C-peptide-mediated prevention of hyperglycemia-induced ROS generation and mitochondrial fission was further investigated in the aortas of streptozotocin-induced diabetic mice. Five-week diabetic mice showed loss of body weight, increased rate of food and water consumption, and severe hyperglycemia (30.41 mmol/L) with glucosuria, compared with nondiabetic controls (Supplementary Table 1). However, these parameters were not improved in diabetic mice supplemented with C-peptide. Serum C-peptide levels significantly decreased in untreated diabetic mice (P < 0.001) and were fully restored to the normal physiologic range (1.54 nmol/L) in diabetic mice supplemented with C-peptide using osmotic pumps (Supplementary Table 1).

Phosphorylation of AMPKα was decreased in the aortas of diabetic mice and was normalized by C-peptide replacement therapy (Figure 7A and B). C-peptide supplementation also inhibited hyperglycemia-induced generation of intracellular ROS in aortic endothelial cells of diabetic mice (Figure 7C and D). Furthermore, we assessed the preventive effect of C-peptide on mitochondrial fission. Hyperglycemia induced fragmentation and disorganization of mitochondria in the aortas of diabetic mice. C-peptide supplementation prevented hyperglycemia-stimulated mitochondrial fission (Figure 7E). Consistently, we recently reported that C-peptide prevents hyperglycemia-induced apoptosis in the aortas of diabetic mice (17). Thus, consistent with our *in vitro* findings, C-peptide-mediated protection against hyperglycemia-induced apoptosis is due
to AMPKα-dependent prevention of ROS generation and mitochondrial fission in aortic endothelium.
DISCUSSION

The development of vascular complications is associated with C-peptide deficiency in DM. We recently reported that C-peptide protects against ROS-mediated endothelial cell apoptosis; however, the mechanism by which C-peptide exerts ROS inhibition is not understood. In this paper, we elucidated a new mechanism by which C-peptide prevents hyperglycemia-induced vasculopathy. As shown in Figure 8, C-peptide inhibits high glucose-induced generation of intracellular and mitochondrial ROS through activation of AMPKα, which results in suppression of mitochondrial fission and ΔΨm collapse and protects against endothelial cell apoptosis in response to hyperglycemia.

We targeted PKC-dependent NADPH oxidase and mitochondria, because they are reported to be the two main sources of ROS generation in response to hyperglycemia in endothelial cells (12,14). Cytosolic NADPH oxidase and mitochondria played essential roles in amplifying intracellular ROS production in hyperglycemia. Inhibitors of the PKC-dependent NADPH oxidase pathway prevented high glucose-induced mitochondrial ROS generation, suggesting that cytosolic ROS facilitates mitochondrial ROS generation. Similarly, mitochondrial and intracellular ROS generation was inhibited by mito-TEMPO and Mdivi-1. Our results demonstrated the inter-regulation between cytosolic and mitochondrial ROS generation in response to high glucose in endothelial cells. Consistent with our findings, there was a recent report (36) of a vicious cycle in which mitochondrial superoxide stimulates cytosolic NADPH oxidase in a feed-forward fashion in response to angiotensin II in endothelial cells. Thus, it is likely that hyperglycemia triggers a positive feedback loop of intracellular ROS amplification in endothelial cells,
in which cytosolic ROS production by PKC-dependent NADPH oxidase potentiates mitochondrial ROS increase and release, resulting in a further increase in cytosolic ROS.

We further investigated the potential link between ROS-dependent mitochondrial fission and apoptosis of endothelial cells in hyperglycemia. We found a prominent increase in mitochondrial fission upon treatment with high glucose in HUVECs. Blocking cytosolic ROS generation, inhibiting mitochondrial fission with Mdivi-1, or enhancing mitochondrial anti-oxidant activity with mito-TEMPO prevented mitochondrial fission. These results demonstrate that both cytosolic and mitochondrial ROS can enhance mitochondrial fission. We also found that ROS production and its downstream mitochondrial fission contributed to $\Delta \Psi_m$ collapse, which is considered to be an early stage of apoptosis (37). High glucose-induced $\Delta \Psi_m$ collapse was successfully prevented by blocking cytosolic and mitochondrial ROS or by inhibiting mitochondrial fission. Thus, intracellular ROS-mediated mitochondrial fission induces $\Delta \Psi_m$ collapse, which triggers apoptosis of endothelial cells during hyperglycemia (Figure 8).

Our studies identify C-peptide-mediated activation of AMPK$\alpha$ as a novel mechanism that protects against hyperglycemia-induced vascular damage. AMPK$\alpha$ is considered to be an emerging therapeutic target for preventing diabetes complications (38,39,40). AMPK activation depends on phosphorylation of the $\alpha$ catalytic subunit at threonine 172 and AMP binding to the $\gamma$ subunit, whereas ATP promotes dephosphorylation of AMPK (41). Previous studies have shown that AMPK$\alpha$ is dephosphorylated and has diminished activity in diabetes (31,32,42). In this paper, we demonstrated that C-peptide activates phosphorylation of AMPK$\alpha$. Further, we showed that high glucose-stimulated dephosphorylation of AMPK$\alpha$ was reversed by C-peptide in HUVECs. AMPK$\alpha$ siRNA or the AMPK inhibitor, compound C, also inhibited C-
peptide-mediated prevention of high glucose-induced apoptosis of endothelial cells by inhibiting ROS generation, mitochondrial fission, and $\Delta \Psi_{m}$ collapse. Additionally, the beneficial role of C-peptide against intracellular ROS amplification and mitochondrial fission through AMPK$\alpha$ activation was elucidated in the aortas of streptozotocin-induced diabetic mice supplemented with C-peptide. Furthermore, AICAR and metformin mimicked the preventive effect of C-peptide on high glucose-induced ROS generation, AMPK dephosphorylation, mitochondrial fission, $\Delta \Psi_{m}$ collapse, and endothelial cell apoptosis. Thus, it is likely that AMPK$\alpha$ is essential for C-peptide-mediated prevention of hyperglycemia-induced vascular complications.

It would be interesting to elucidate the mechanism by which C-peptide activates AMPK$\alpha$. It is reported that C-peptide stimulates endothelial NO synthase (43). NO can act as an endogenous activator of AMPK in vascular endothelial cells (44). In the present study, C-peptide elevated the level of intracellular NO. Compound C had no significant effect on the C-peptide-induced formation of NO, whereas L-NAME inhibited the NO formation, indicating that AMPK is not involved in the C-peptide-stimulated NO production. C-peptide activated AMPK$\alpha$ at 48 h and the activation was significantly inhibited by L-NAME. Additionally, SNAP recovered high glucose-induced AMPK$\alpha$ dephosphorylation. Thus, it is possible to propose that NO production can contribute to the C-peptide stimulation of AMPK$\alpha$ phosphorylation in endothelial cells; however, it is necessary to elucidate the mechanism by which early NO formation can induce the late activation of AMPK$\alpha$ in response to C-peptide.

The major upstream kinase activating AMPK is the tumor suppressor kinase LBK1 which is essential for the AMPK activation by AICAR (45). In our study, AICAR
mimicked C-peptide by preventing high glucose-induced ROS generation, mitochondrial fission, ΔΨ_m collapse, and cell death, suggesting that C-peptide activation of AMPKα might involve LKB1. Additionally, protein phosphatase 2C might be involved in the C-peptide activation of AMPK, since increase in intracellular AMP levels promotes AMPKα phosphorylation by inhibiting its dephosphorylation by the protein phosphatase 2C (46). However, Ca^{2+}/Calmodulin-dependent protein kinase kinase β is unlikely involved in the C-peptide activation of AMPK because C-peptide has no effect on the level of intracellular Ca^{2+} in endothelial cells (17).

In conclusion, our data indicate that cross-talk between PKC-dependent NADPH oxidase and mitochondrial ROS generation results in a positive feedback loop that amplifies intracellular ROS production in endothelial cells. Amplification of intracellular ROS generation mediates mitochondrial fission, which then leads to ΔΨ_m collapse and apoptosis of endothelial cells in diabetes. Importantly, our data support C-peptide supplementation as a new therapeutic strategy for preventing diabetic vascular complications. C-peptide-mediated activation of AMPKα inhibited hyperglycemia-induced intracellular ROS production, mitochondrial fission, and endothelial cell apoptosis. Supplementation with C-peptide may offer increased benefit beyond the limits of currently available pharmacological agents, such as AICAR and metformin. Thus, C-peptide supplementation should be tested in combination with insulin therapy as a new strategy for preventing vascular complications in type 1 DM and late-stage type 2 DM.
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Author contributions: M.P.B. researched data and wrote the manuscript. Y.-C.L. researched data. Y.-M.K. contributed to discussion. K.-S.H. designed and supervised experiments and edited the manuscript.

Dr. Kwon-Soo Ha is the guarantor of this work and, as such had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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The authors have declared that no potential conflicts of interest exist.
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FIGURE LEGENDS

**Figure 1.** Inter-regulation of high glucose-induced generation of intracellular and mitochondrial ROS. HUVECs were incubated for 48 hours (h) with high glucose (HG, 33 mM D-glucose) in the presence of inhibitors at the indicated concentrations. Intracellular and mitochondrial ROS levels were determined by confocal microscopy as described in the Methods. (A,B) HG induced increases in intracellular and mitochondrial ROS. (A) Measurement of intracellular and mitochondrial ROS levels using H$_2$DCFDA (green, left) and MitoSox Red (red, right). MitoSox Red is colocalized with MitoTracker Green; scale bar, 20 µm. (B) HG induced generation of intracellular and mitochondrial ROS, but L-glucose had no effect; ***p<0.001. (C-F) Dose-dependent inhibition of HG-induced generation of intracellular and mitochondrial ROS by PKC inhibitors GF109203X and Ro-31-2880 (C), NADPH oxidase inhibitors apocynin and diphenylene iodonium (DPI) (D), mito-TEMPO (E), and Mdivi-1 (F). *p<0.05, **p<0.01, ***p<0.001 compared with HG. Data are expressed as the mean ± standard deviation (S.D.) from three independent experiments.

**Figure 2.** Essential roles of cytosolic and mitochondrial ROS in HG-induced mitochondrial fission and $\Delta $$\Psi$$_{m}$ collapse. HUVECs were incubated with HG in the presence of inhibitors for 48 h. Mitochondrial fission and $\Delta $$\Psi$$_{m}$ were determined by confocal microscopy as described in the Methods. (A) Mitochondrial fission was represented by predominantly fragmented mitochondria in HG-exposed cells in comparison with control cells (left); scale bar, 20 µm. (Right) magnified images; scale bar, 5 µm. (B) The percentage of cells undergoing HG-induced mitochondrial fission.
There is no effect with L-glucose. (C) Inhibition of HG-induced mitochondrial fission by PKC inhibitors GF109203X (GF) and Ro-31-2880 (Ro) and NADPH oxidase inhibitors apocynin (Apo) and DPI. (D) Inhibition of HG-induced mitochondrial fission by mito-TEMPO and Mdivi-1. (E) Determination of HG-induced ΔΨ
m collapse; scale bar 20 µm. (F) Inhibition of HG-induced ΔΨ
m collapse by PKC inhibitors GF109203X (GF) and Ro-31-2880 (Ro) and NADPH oxidase inhibitors apocynin (Apo) and DPI. However, L-glucose had no effect on ΔΨ
m collapse. (G) Inhibition of HG-induced ΔΨ
m collapse by mito-TEMPO and Mdivi-1. ***p<0.001, **p<0.01. Data are expressed as the mean ± S.D. of three independent experiments.

Figure 3. C-peptide inhibits HG-induced ROS generation, mitochondrial fission, and ΔΨ
m collapse. HUVECs were incubated with HG for 48 h in the presence of the indicated concentrations (A) or 1 nM (B-F) of C-peptide (CP) or heat-inactivated C-peptide (HI-CP). ROS generation, mitochondrial fission, expression of Drp-1 and Fis-1, and ΔΨ
m were determined as described in the Methods. (A) C-peptide inhibited HG-induced generation of intracellular and mitochondrial ROS in a dose-dependent manner. *p<0.05, **p<0.01, ***p<0.001 compared with HG. (B) C-peptide inhibited HG-induced mitochondrial fission. (C-E) C-peptide inhibited HG-induced expression of Drp-1 (D) and Fis-1 (E). (C) Western blot analysis of Drp-1 and Fis-1 expression. Expression levels were normalized to β-actin. (F) C-peptide inhibited HG-induced ΔΨ
m collapse. However, HI-CP had no inhibitory effect on HG-induced generation of intracellular and mitochondrial ROS (A), mitochondrial fission (B), and ΔΨ
m collapse (F). *p<0.05, **p<0.01, ***p<0.001. Data are expressed as the mean ± S.D. of three independent experiments.
**Figure 4.** Essential roles of AMPKα in C-peptide-mediated inhibition of HG-induced ROS generation, mitochondrial fission, and ∆Ψ_m collapse. HUVECs were transfected with the indicated concentrations of AMPKα1/2 siRNA or treated with the indicated concentrations of compound C and incubated with HG for 48 h. (A,B) C-peptide (CP) activated AMPKα and rescued HG-induced AMPKα inhibition. (A) A representative immunoblot. (B) Quantification of AMPKα phosphorylation normalized to total AMPKα. (C) siRNA concentration-dependent suppression of AMPKα expression. (D,E) AMPKα siRNA (D) or compound C (E) inhibited C-peptide-mediated prevention of HG-induced intracellular and mitochondrial ROS production in a dose-dependent manner. (F,G) Inhibition of HG-induced mitochondrial fission by AMPKα siRNA (F) or compound C (G). (H,I) Inhibition of HG-induced ∆Ψ_m collapse by AMPKα siRNA (H) or compound C (I). (J) Time-course changes in the levels of nitric oxide by C-peptide. HUVECs were treated for the indicated times with 1 nM C-peptide (CP) in the presence of control, 2 mM L-NAME, or 1 µM compound C. Nitric oxide (NO) production was measured by confocal microscopy using DAF-FM diacetate staining. (K,L) Possible role of C-peptide-induced NO production in AMPKα phosphorylation. HUVECs were treated with 1 nM C-peptide (CP), 1 nM C-peptide with 2 mM the NO synthase inhibitor L-NAME, high glucose (HG), or high glucose with the nitric oxide donor 1 nM SANP for 48 h. Cell lysates were subjected to western blot analysis to estimate AMPKα phosphorylation. (K) A representative immunoblot. (L) Quantification of AMPKα phosphorylation normalized to total AMPKα. *p<0.05, **p<0.01, and ***p<0.001. Data are expressed as the mean ± S.D. of three independent experiments.
Figure 5. AICAR and metformin inhibit HG-induced ROS generation, AMPKα dephosphorylation, mitochondrial fission, and ∆Ψm collapse. HUVECs were incubated with HG for 48 h in the presence of the indicated concentrations of AICAR and metformin (Met); 0.1 mM AICAR and 2 mM metformin were used for C-F. (A,B) Inhibition of HG-induced intracellular and mitochondrial ROS generation by AICAR (A) and metformin (B) in a dose-dependent manner. **p<0.01 and ***p<0.001 compared with HG. (C,D) AICAR and metformin rescued HG-induced inhibition of AMPKα. (C) A representative immunoblot. (D) Quantification of AMPKα phosphorylation normalized to total AMPKα. (E) Inhibition of HG-induced mitochondrial fission by AICAR, metformin, and Trolox. (F) Inhibition of HG-induced ∆Ψm collapse by AICAR, metformin, and Trolox. **p<0.01 and ***p<0.001. Data are expressed as the mean ± S.D. of three independent experiments.

Figure 6. Prevention of HG-induced apoptosis by various inhibitors and reversal of C-peptide effect by AMPK siRNA and compound C. HUVECs were incubated with HG for 72 h in the presence of inhibitors (A,C) or AMPKα siRNA (B). Early and late apoptotic cell death rates were determined as described in the Methods. (A) HG-induced apoptotic cell death was prevented by 1 nM C-peptide, 0.1 mM AICAR, 2 mM metformin, 10 µM apocynin, 10 µM mito-TEMPO, and 10 µM Mdivi-1. However, apoptotic cell death was neither activated by L-glucose, nor inhibited by heat-inactivated C-peptide (HI-CP). (B,C) AMPKα siRNA (B) and compound C (C) reversed C-peptide prevention of HG-induced apoptosis. **p < 0.01. Data are expressed as mean ± S.D. of three independent experiments.
**Figure 7.** C-peptide replacement therapy inhibits hyperglycemia-induced AMPKα dephosphorylation, intracellular ROS generation, and mitochondrial fission in diabetic mice. Streptozotocin-induced diabetic mice were subcutaneously implanted for 4 weeks with Alzet mini-osmotic pumps containing C-peptide. Aorta from control (n=8), diabetic (n=8), and diabetic + C-peptide (CP) (n=7) groups of mice were dissected and cut longitudinally to open the endothelium. AMPKα phosphorylation, intracellular ROS generation, and mitochondrial fission were immediately measured as described in the Methods. (A,B) Dephosphorylation of AMPKα at Thr172 was induced by hyperglycemia in diabetic mice and was normalized in C-peptide-supplemented diabetic mice (n=7). (A) A representative immunoblot. (B) Densitometric quantification of AMPKα activation normalized to total AMPKα. (C,D) Generation of intracellular ROS was increased by hyperglycemia in the aortic endothelium of diabetic mice (n=8) and was normalized in C-peptide-supplemented diabetic mice (n=7). (C) Measurement of intracellular ROS levels using H2DCFDA; bar, 20 µm. (D) Quantification of intracellular ROS compared with control. (E) Mitochondrial fission was increased by hyperglycemia in diabetic mice (n=8), and was normalized in C-peptide supplemented mice (n=7); bar, 5 µm. ***p < 0.001.

**Figure 8.** Schematic model depicting the role of C-peptide in the regulation of hyperglycemia-induced vasculopathy through an AMPKα-dependent mechanism.
Figure 3

A

B

C

D

E

F

Intracellular ROS
Mitochondrial ROS

ROS generation (fold)

Mitochondrial Fission (% Cells)

Drp-1
Fis-1
β-actin

Drp-1 expression (fold)

Fis-1 expression (fold)

Δψm (fold)

521x709mm (72 x 72 DPI)
Figure 4

A

B

C

D

E

F

G

594x720mm (72 x 72 DPI)
Figure 4

H

\[
\Delta \lambda_\text{agg} \text{ (fold)}
\]

\text{HG} - - + - +

CP (1 nM) + + + - +

Ctrl siRNA (100 nM) + + - + +

AMPK\alpha siRNA (100 nM) - - - + +

I

\[
\Delta \lambda_\text{agg} \text{ (fold)}
\]

\text{HG} - - + - +

CP (1 nM) + + + - +

Compound C (1 \mu M) + + + + +

J

\[
\text{NO production (fold)}
\]

\text{Incubation time (h)}

Control CP CP + compound C CP + L-NAME

K

\[
p-\text{AMPK} \alpha
\]

\text{AMPK} \alpha

L

\[
p-\text{AMPK} / \text{total AMPK} \text{ (fold)}
\]
Figure 5

A

B

C

D

E

F

579x701mm (72 x 72 DPI)
Figure 6

A

Cell death rate (%)

Control  CP  L-Glucose  Vehicle  Hi-CP  CP  AICAR  Metformin  Apocynin  into-TEMPO  Metr-2

early apoptosis  late apoptosis / necrosis  total cell death

**  **

B

Cell death rate (%)

HG  CP (1 nM)  Ctrl siRNA (100 nM)  AMPKα siRNA (100 nM)

early apoptosis  late apoptosis / necrosis  total cell death

**  **

C

Cell death rate (%)

HG  CP (1 nM)  Compound C (1 μM)

early apoptosis  late apoptosis / necrosis  total cell death

**  **

603x581mm (72 x 72 DPI)
Figure 7

A

B

C

D

E

583x763mm (72 x 72 DPI)
Figure 8

C-peptide

AMPKα

Cytosolic ROS

High Glucose

Mitochondrial ROS

Mitochondrial Fission

ΔΨm collapse

Endothelial apoptosis

Diabetic Vasculopathy
SUPPLEMENTAL MATERIALS

**Supplemental Table 1.** Clinical data from age-matched non-diabetic control, diabetic, and C-peptide-supplemented diabetic mice.

<table>
<thead>
<tr>
<th>Observations</th>
<th>Control</th>
<th>Diabetes</th>
<th>Diabetes + C-peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice (n)</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>25.00±0.82</td>
<td>20.05±0.92***</td>
<td>19.00±2.25***</td>
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<tr>
<td>Food consumption (g/day)</td>
<td>2.73±0.31</td>
<td>5.13±0.42**</td>
<td>5.76±0.40***</td>
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<tr>
<td>Water intake (ml/day)</td>
<td>5.53±0.50</td>
<td>23.00±1.00***</td>
<td>22.70±2.54***</td>
</tr>
<tr>
<td>Non-fasting blood sugar (mM)</td>
<td>8.75±0.95</td>
<td>30.41±2.61***</td>
<td>28.68±3.18***</td>
</tr>
<tr>
<td>(5 weeks after diabetes induction)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Serum C-peptide (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(at 1 week of diabetes induction)†</td>
<td>1.39±0.57</td>
<td>0.13±0.12***</td>
<td>0.11±0.06***</td>
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<tr>
<td>Serum C-peptide (nM)</td>
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<tr>
<td>(4 weeks after C-peptide delivery)</td>
<td>1.20±0.91</td>
<td>&lt;0.10***</td>
<td>1.54±0.85</td>
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</table>

†C-peptide deficiency was assessed in mice before C-peptide replacement therapy at 1 week of diabetes induction.

Data are means ± S.D., **p<0.01, ***p<0.001 compared with control mice.
Supplemental Figure 1. C-peptide prevents dose-dependent endothelial cell death induced by high glucose and compound C reverses the C-peptide effects. HUVECs were incubated with various concentrations of D-glucose for 72 h in the presence of 1 nM C-peptide or both 1 nM C-peptide and 1 µM compound C. Early and late apoptotic cell death rates were determined by DiOC6/PI double staining. Glucose treatment induced dose-dependent apoptotic cell death, which was prevented by C-peptide (CP), and the AMPK inhibitor compound C reversed the C-peptide prevention. Data are expressed as mean ± S.D. of three independent experiments. ***p<0.01, ***p<0.001.

<table>
<thead>
<tr>
<th>Compound C (1 µM)</th>
<th>CP (1 nM)</th>
<th>D-glucose (mM)</th>
<th>Cell death rate (%)</th>
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<tr>
<td></td>
<td></td>
<td>5.5</td>
<td>early apoptosis</td>
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<tr>
<td></td>
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<td>5.5</td>
<td>late apoptosis/necrosis</td>
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<tr>
<td></td>
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<td>5.5</td>
<td>total cell death</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>15</td>
<td></td>
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<tr>
<td>+</td>
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Diabetes