Activation of AMP-Activated Protein Kinase Reduces Hyperglycemia-Induced Mitochondrial Reactive Oxygen Species Production and Promotes Mitochondrial Biogenesis in Human Umbilical Vein Endothelial Cells

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We previously proposed that the production of hyperglycemia-induced mitochondrial reactive oxygen species (mtROS) is a key event in the development of diabetes complications. The association between the pathogenesis of diabetes and its complications and mitochondrial biogenesis has been recently reported. Because metformin has been reported to exert a possible additional benefit in preventing diabetes complications, we investigated the effect of metformin and 5-aminimidazole-4-carboxamide ribonucleoside (AICAR) on mtROS production and mitochondrial biogenesis in cultured human umbilical vein endothelial cells. Treatment with metformin and AICAR inhibited hyperglycemia-induced intracellular and mtROS production, stimulated AMP-activated protein kinase (AMPK) activity, and increased the expression of peroxisome proliferator-activated response-γ coactivator-1α (PGC-1α) and manganese superoxide dismutase (MnSOD) mRNAs. The dominant negative form of AMPKα1 diminished the effects of metformin and AICAR on these events, and an overexpression of PGC-1α completely blocked the hyperglycemia-induced mtROS production. In addition, metformin and AICAR increased the mRNA expression of nuclear respiratory factor-1 and mitochondrial DNA transcription factor A (mtTFA) and stimulated the mitochondrial proliferation. Dominant negative–AMPK also reduced the effects of metformin and AICAR on these observations. These results suggest that metformin normalizes hyperglycemia-induced mtROS production by induction of MnSOD and promotion of mitochondrial biogenesis through the activation of AMPK-PGC-1α pathway. Diabetes 55:120–127, 2006

Diabetic microvascular disease is a leading cause of blindness, terminal renal failure, and nerve damage in developed countries. Diabetic macrovascular disease leads to an increased risk of cardiovascular disease, stroke, and limb amputation. The social and economic consequences of managing diabetes and its complications make it a major public health issue. Prospective clinical studies, such as the Diabetes Control and Complications Trial (1), U.K. Prospective Diabetes Study (2), and our Kumamoto Study (3–5) showed a strong relationship between hyperglycemia and diabetic microvascular complications in both type 1 and type 2 diabetes and suggested the efficacy of intensive blood-glucose control in preventing the initiation and progression of microvascular complications. However, the effect of intensive therapy to prevent diabetes complications may be limited because of difficulty in maintaining blood glucose concentrations close to the normal range. Other approaches are therefore required to prevent a progression of diabetes complications based on the elucidation of the mechanisms controlling diabetes complications.

We previously demonstrated that hyperglycemia-induced production of reactive oxygen species (ROS) to be abrogated by inhibitors of the mitochondrial metabolism or by an overexpression of uncoupling protein (UCP)-1 or manganese superoxide dismutase (MnSOD) in both bovine aortic endothelial cells (6) and human mesangial cells (7). In addition, the normalization of the mitochondrial ROS (mtROS) production by each of these agents prevents the glucose-induced activation of protein kinase C, the formation of advanced glycation end products, and the accumulation of sorbitol in bovine vascular endothelial cells, all of which are known to be involved in development of diabetes complications (6). Since hyperglycemia-induced mtROS production may be a key event in the development of diabetes complications, the investigation of cellular molecules and agents to reduce hyperglycemia-induced mtROS production may contribute to the development of new pharmacological approaches to prevent diabetes complications.

In 1998, the U.K. Prospective Diabetes Study reported intensive glycemic control with metformin, one of the most widely used oral drugs for the treatment of type 2 diabetes, to decrease the risk of diabetes-related end points in overweight patients with type 2 diabetes in
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comparison to sulfonylurea or insulin therapy (8). Given the equivalent HbA1c levels obtained by every therapy, the possible additional benefit to reduce cardiovascular events is not explicable on the basis of glycemic control. Recently, it has been reported that metformin activates AMP-activated protein kinase (AMPK) in both hepatocytes and skeletal muscles (9). On the other hand, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an AMPK activator (10,11), increases the production of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), at least in part, through an AMPK-related mechanism (12,13) in rat epicardial muscle. PGC-1α could bind to and coactivate the transcriptional function of nuclear respiratory factors (NRF-1) on the promoter for mitochondrial DNA replication/transcription and a stimulus for the regulation of mitochondrial biogenesis and function (14). Therefore, metformin may promote mitochondrial biogenesis through an AMPK–PGC-1α–related mechanism, and this chain of events may affect the hyperglycemia-induced mtROS production.

The present study was designed to determine the effect of metformin, AICAR, and the overexpression of either dominant negative AMPKα1 (T172A) or PGC-1α on mtROS production in cultured human umbilical vein endothelial cells (HUVECs). In addition, to clarify the role of AMPK and PGC-1α induction in mtROS production and mitochondrial biogenesis, we measured expression levels of MnSOD, PGC-1α, NRF-1, and mtTFA mRNAs, mitochondrial DNA content, and the number of mitochondria in the cells.

**RESEARCH DESIGN AND METHODS**

**Cells and culture.** HUVECs were isolated from human umbilical cord vein biopsies by collagenase treatment as described previously (15) and used in passages 2–5. The cells were grown in M199 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 μg/ml penicillin, 250 μg/ml amphotericin B, 1 mmol/l glucose, 5 IU/ml heparin, and 50 μg/ml endothelial cell growth supplement at 37°C within humidified 5% CO2/95% air. Cultured cells were identified as endothelial cells based on their morphology and the presence of von Willebrand factor using indirect immunofluorescence microscopy.

**Drug treatment.** Confuent HUVECs were incubated for 16 h in M199 containing 1% fetal bovine serum, and then the medium was changed to fresh M199 (1% FCS) containing either 5.6 or 30 mmol/l glucose. HUVECs were also incubated in M199 containing 1% fetal bovine serum, and then the medium was changed to fresh M199 after 24 h under each condition and then prepared for electron microscopy. In brief, the pellets were fixed in 2.5% glutaraldehyde/osmium tetroxide and then sectioned. Thin sections were obtained and viewed by transmission electron microscopy (Hitachi H-7500) for measurement of glyceraldehyde-3-phosphate dehydrogenase activity. HUVECs were incubated for 24 h under each condition by a QIAamp DNA Mini Kit (Qiagen). The specific primer for mtDNA was designed for Complex II (succinate-ubiquinone oxidoreductase) (Human Complex II: 5'-ATAGTCTAAGGGCTGGATG-3' and 5'-CTGAGTTTCTCTGGCTACT-3'), human MnSOD: 5'-GAGCGTTTGGTCTGGT-3' and 5'-TCGCTGCTTCTG-3'), human NRF-1: 5'-TCTGATGATGATGATGGA-3', human mtTFA: 5'-TTGCCAATGG-3', and the LightCycler System (Roche Molecular Biochemicals) was used.

**Mitochondrial quantification.** HUVECs were cultured at 37°C for 24 h under each condition and then prepared for electron microscopy. In brief, the pellets were fixed in 2.5% glutaraldehyde/osmium tetroxide and then sectioned. Thin sections were obtained and viewed by transmission electron microscopy (Hitachi H-7500). The data were expressed as the mitochondrial density (mitochondrial number per cytoplasmic area). A minimum of 20 cells from each treatment were assessed.

**Measurement of glyceraldehyde-3-phosphate dehydrogenase activity.** HUVECs were incubated for 24 h under each condition. Preparation of the cytosolic fraction and measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was performed as described previously (19).

**Statistical analysis.** The data were expressed as the mean ± SE. A statistical analysis was performed using the unpaired Student’s t test or one-way ANOVA followed by the Newman-Keuls multiple-comparison test. P values <0.05 denoted the presence of a statistically significant difference.

**RESULTS**

**Effect of metformin on ROS production in HUVECs.** To evaluate the effect of metformin and AICAR on the intracellular ROS production in HUVECs, we used fluorescent probe H2DCF-DA. As shown in Fig. 1A, H2DCF-DA–associated fluorescence significantly increased in the HUVECs after incubation with 30 mmol/l glucose (253.5 ± 9.2% of 5.6 mmol/l glucose) when compared with incubation with 5.6 mmol/l glucose. Interestingly, metformin and AICAR, an AMPK activator, reduced this increased ROS production in a dose-dependent manner (2 mmol/l metformin: 61.8 ± 2.5% of 30 mmol/l glucose; 2 mmol/l AICAR: 21.8 ± 2.9% of 30 mmol/l glucose), although the inhibitory effect of metformin on ROS production was weaker than that of AICAR. To elucidate whether these agents ameliorate the hyperglycemia-induced ROS production in the mitochondria, we selected the reduced MitoTracker Red probe. As shown in...
Fig. 1B, the fluorescence of MitoTracker Red significantly increased with 30 mmol/l glucose when compared with 5.6 mmol/l glucose. As we previously reported, this hyperglycemia-induced fluorescence was suppressed by the overexpression of UCP-1 or MnSOD, both of which could abolish mtROS production. Moreover, similar to the results using a H2DCF-DA probe, metformin, and AICAR also decreased this hyperglycemia-induced fluorescence of MitoTracker Red.

**Effect of AMPK activation on mtROS production in HUVECs.** To evaluate whether metformin and AICAR could activate AMPK in HUVECs, we performed a Western blot analysis using anti-AMPK phosphothreonine 172-specific antibody. Paralleled with the effect of metformin and AICAR on mtROS production, threonine-172 phosphorylation within AMPK**1 was increased by either metformin or AICAR in HUVECs (141.2 ± 10.9 or 321.6 ± 20.1% of 30 mmol/l glucose, respectively) (Fig. 2A), and the effect of metformin on AMPK activation was weaker than that of AICAR. In contrast, an overexpression of dominant negative AMPK**1 (T172A) attenuated both the metformin- and AICAR-induced AMPK phosphorylation (Fig. 2A).

To clarify the role of AMPK activation in the mtROS production, we evaluated the effect of the overexpression of dominant negative AMPK**1 (T172A) on the hyperglycemia-induced increase in the fluorescence of MitoTracker Red in HUVECs. As shown in Fig. 2B, overexpression of dominant negative AMPK**1 (T172A) diminished the inhibitory effect of metformin and AICAR on the hyperglycemia-induced increase in the fluorescence of MitoTracker Red.

**Effect of agents that alter the AMPK activity related to the expression of PGC-1α mRNA in HUVECs.** To verify the relationship between AMPK activation and PGC-1α induction in HUVECs, we evaluated the effect of agents that alter AMPK activity on the expression of PGC-1α mRNA using the quantitative real-time RT-PCR. As shown in Fig. 3A, the PGC-1α mRNA was significantly increased by either metformin or AICAR when compared...
with the incubation with 30 mmol/l glucose (172.7 ± 5.6 and 192.9 ± 7.4% of 30 mmol/l glucose, respectively). In contrast, the overexpression of dominant negative AMPKα1 (T172A) suppressed the metformin- or AICAR-induced expression of PGC-1α mRNA (103.4 ± 7.4 and 103.3 ± 7.6% of 30 mmol/l glucose, respectively). As shown in Fig. 3B, similar to the effect of metformin and AICAR, the overexpression of PGC-1α completely abolished the hyperglycemia-induced mtROS production.

**Effect of agents on expression of MnSOD mRNA in HUVECs.** To clarify the mechanism by which metformin and AICAR reduce mtROS, we measured the mRNA expression of MnSOD that could decrease the amount of mtROS using the quantitative real-time RT-PCR. As shown in Fig. 4, the significant increase of MnSOD mRNA was observed by either metformin, AICAR, or by the overexpression of PGC-1α (197.6 ± 12.1, 451.4 ± 29.7, and 618.2 ± 64.6 of 30 mmol/l glucose, respectively). The overexpression of dominant negative AMPKα2 (T172A) suppressed the metformin- or AICAR-induced expression of MnSOD mRNA (110.3 ± 15.0 and 121.4 ± 11.0% of 30 mmol/l glucose, respectively).

**Effect of agents that alter AMPK activity on mitochondrial biogenesis in HUVECs.** To verify the effect of AMPK activation on mitochondrial biogenesis, we measured the mRNA levels of NRF-1 and mtTFA using the quantitative real-time PCR, mitochondrial DNA content by the quantitative real-time PCR, and the mitochondrial number by transmission electron microscopy. As shown in Fig. 5, the mRNA levels of NRF-1 (Fig. 5A) and mtTFA (Fig. 5B) significantly increased by either metformin, AICAR, or by the overexpression of PGC-1α (NRF-1: 155.5 ± 7.8, 144.7 ± 6.9, and 379.0 ± 24.1% of 30 mmol/l glucose, respectively; mtTFA: 175.8 ± 10.2, 187.5 ± 9.8, and 340.2 ± 16.9% of 30 mmol/l glucose, respectively). As expected, the overexpression of dominant negative AMPKα1 (T172A) suppressed the metformin- or AICAR-induced expression of NRF-1 and mtTFA mRNAs (NRF-1: 100.3 ± 6.8 and 99.2 ± 4.2% of 30 mmol/l glucose, respectively; mtTFA: 103.4 ± 9.3 and 103.2 ± 6.4% of 30 mmol/l glucose, respectively).

As shown in Fig. 6, the mitochondrial DNA content significantly increased by either metformin or AICAR or by the overexpression of PGC-1α compared with 30 mmol/l glucose.
glucose (124.3 ± 8.8, 122.2 ± 10.7, and 179.3 ± 7.8% of 30 mmol/l glucose, respectively). The overexpression of dominant negative AMPKα1 (T172A) suppressed the metformin- or AICAR-induced increase in the mitochondrial DNA content (96.2 ± 10.8 and 95.5 ± 10.7% of 30 mmol/l glucose, respectively). In addition, a transmission electron microscopy study demonstrated that an overexpression of PGC-1α resulted in an apparent increase in the mitochondrial number when compared with incubation with 30 mmol/l glucose (Fig. 7A). Although the effect of metformin or AICAR is less dramatic than that of the overexpression of PGC-1α, metformin or AICAR also significantly increased the mitochondrial number in comparison to incubation with 30 mmol/l glucose. Quantitative morphometry performed on 20 cells of each group indicated the mitochondrial number/cytoplasmic area in HUVECs to be 171.1 ± 13.1, 177.5 ± 18.1, or 279.9 ± 11.7% of 30 mmol/l glucose by either metformin, AICAR, or PGC-1, respectively (Fig. 7B). The overexpression of dominant negative AMPKα1 (T172A) suppressed the metformin- or AICAR-induced increase in the mitochondrion number (90.0 ± 12.8 and 90.5 ± 13.8% of 30 mmol/l glucose, respectively).

**DISCUSSION**

The production of ROS is known to be increased in diabetic patients (20), and such an increase may contribute to the development of diabetes complications (21,22). In addition, we previously proposed that hyperglycemia-induced ROS production from the mitochondria electron transport chain was a key event in the development of diabetes complications (6,23). In this study, we first confirmed that metformin, which has been reported to exert a possible additional benefit in the prevention of diabetes complications independently of its antihyperglycemic effect (8,24,25), inhibited the hyperglycemia-induced intracellular ROS production as measured by H2DCF-DA and...
the mtROS production as measured by the MitoTracker Red probe (26). The fluorescence of H$_2$DCF-DA indicates intracellular ROS production (7). In contrast, the reduced MitoTracker Red probe can specifically detect mtROS, since this probe accumulates inside mitochondria and is oxidized predominantly by reactions involving hydrogen peroxide production (18). In agreement with our previous reports (6,7), the fluorescence of both H$_2$DCF-DA and MitoTracker Red was increased with 30 mmol/l glucose when compared with 5.6 mmol/l glucose. These results demonstrate for the first time that metformin can decrease hyperglycemia-induced intracellular and mtROS production. Therefore, metformin could prevent diabetes complications, at least in part, by reducing the hyperglycemia-induced mtROS production.

The molecular basis for the effect of metformin on glucose and lipid homeostasis is still unknown. The activation of AMPK has recently been reported to mediate the beneficial metabolic effects of metformin (9). In addition, PGC-1$\alpha$ has been reported to increase through an AMPK-related mechanism (12,13). Therefore, we investigated the role of AMPK activation and PGC-1$\alpha$ induction in hyperglycemia-induced mtROS production. The present study demonstrated that, similar to metformin, AICAR, which stimulates the AMPK activity (10,11), reduced the hyperglycemia-induced intracellular and mtROS production. As expected, AMPK activation, evaluated by threonine-172 phosphorylation of AMPK, was found to be increased by either metformin or AICAR, and overexpression of dominant negative AMPK$\alpha_1$ (T172A) decreased both metformin- and AICAR-induced AMPK phosphorylation. Furthermore, the overexpression of AMPK$\alpha_1$ (T172A) blocked the inhibitory effect of metformin and AICAR on mtROS production. These observations suggested that the inhibitory effect of metformin on hyperglycemia-induced mtROS production was dependent on AMPK activation. Similarly, both metformin and AICAR increased the expression of PGC-1$\alpha$ mRNA in accordance with the activation of AMPK, and the overexpression of PGC-1$\alpha$ completely abolished the hyperglycemia-induced mtROS production. Therefore, AMPK activation and the resulting induction of PGC-1$\alpha$ could be required to reduce the hyperglycemia-induced mtROS production by metformin.

AMPK$\alpha$ subunit has recently been found to exist as two distinct gene products termed $\alpha_1$ and $\alpha_2$. The $\alpha_1$ subunit is reported to be rather widely expressed, whereas the $\alpha_2$ subunit is particularly highly expressed in skeletal and cardiac muscles and in liver (27). We confirmed that overexpression of dominant negative AMPK$\alpha_1$ did not change the expression of AMPK$\alpha_2$ isoform in HUVECs. However, overexpression of dominant negative AMPK$\alpha_1$ completely decreased the phosphorylation of total AMPK. Since it has been unclear whether these isoforms have distinct functions, further study will be required to examine whether dominant negative AMPK$\alpha_2$ could decrease metformin- and AICAR-induced AMPK phosphorylation in HUVECs.

A question of whether AMPK activation and PGC-1$\alpha$ induction can decrease the mtROS production arises. We herein demonstrated that metformin, AICAR, and an overexpression of PGC-1$\alpha$ could increase the expression of MnSOD mRNA. In addition, an increase of MnSOD mRNA induced by metformin or AICAR was reduced by an overexpression of AMPK$\alpha_1$ (T172A). Since MnSOD has been reported to ameliorate the mtROS production, these results imply that metformin reduces the mtROS production, at least in part, by MnSOD induction, even though it is not clear at present whether the expression level of MnSOD induced by these agents is sufficient to reduce the production of mtROS. In addition, the mechanisms by which metformin and AICAR decrease the mtROS production were investigated. The results demonstrated that metformin and AICAR decreased the mtROS production, at least in part, by MnSOD induction, even though it is not clear at present whether the expression level of MnSOD induced by these agents is sufficient to reduce the production of mtROS. In addition, the mechanisms by
which AMPK or PGC-1α induction increase the expression of MnSOD remain unclear. Forkhead transcription factor FOXO3a has recently been reported to protect quiescent cells from oxidative stress by directly increasing the quantities of MnSOD mRNA (28). Since PGC-1α has been reported to bind and coactivate FOXO1 during insulin-regulated gluconeogenesis in the liver (29), the PGC-1α and FOXO family are thus expected to play an important role in the expression of MnSOD in HUVECs. Further studies are required to clarify the role of MnSOD induction by these agents in mtROS production and the mechanisms by which AMPK or PGC-1α induction can increase the MnSOD gene expression in HUVECs.

We herein also demonstrated that either metformin, AICAR, or an overexpression of PGC-1α increased the expression of NRF-1 and mtTFA mRNA, both of which are regulators of mitochondrial DNA transcription and replication (14). In addition, these agents or an overexpression of PGC-1α could stimulate the mitochondrial proliferation evaluated by mitochondrial DNA content and numbers. Furthermore, NRF-1 and mtTFA mRNA induction and mitochondrial proliferation by metformin or AICAR were blocked by overexpression of a dominant negative form of AMPKα1. These findings suggest that metformin controls mitochondrial biogenesis through AMPK and PGC-1α induction. Mitochondria are the primary source of ATP production, and disruption of mitochondrial respiratory function is regarded as a key factor in the development of pathologic complications in heart (30) and other tissues (31,32) in diabetes. In addition, it was reported that strict blood glucose control prevented or reserved ultrastructural and functional abnormalities of hepatocyte mitochondria in critically ill patients, and intensive insulin therapy on mitochondrial integrity may contribute to the clinical benefits (33). Therefore, the promotion of mitochondrial biogenesis by metformin through AMPK and PGC-1α induction may help to prevent diabetes complications.

It is unclear whether mtROS production is associated with mitochondrial biogenesis. However, hyperglycemia-induced mitochondrial enlargement and dysfunction in neurons have been reported to be blocked by thienyltrifluoroacetone (34), an inhibitor of mtROS production (6). In addition, the formation of megamitochondria or mitochondria giants was induced by chloramphnicol- or hydrazine-induced free radicals and suppressed by free radical scavengers such as α-tocopherol and coenzyme Q10 (35). Furthermore, hydrogen peroxide treatment on the mitochondria isolated from the rat hearts decreased its transcriptional activity (36). Therefore, the promotion of mitochondrial biogenesis may be responsible for normalization of hyperglycemia-induced mtROS production by metformin and AICAR. On the other hand, it is unclear whether mitochondrial proliferation can contribute to reduced mtROS production. Further studies are required to clarify the relationship between the mtROS production and mitochondrial biogenesis.

The effect of metformin in hyperglycemia-induced vascular damage has not yet been clarified. Recent work has demonstrated that hyperglycemia-induced GAPDH inhibition was a consequence of activation of poly (ADP-ribose) polymerase, which was activated by DNA strand breaks produced by mtROS overproduction (19). Inhibition of GAPDH results in activation of four major pathways of hyperglycemic damage: activation of protein kinase C, increased hexosamine pathway flux, increased advanced glycosylation end product formation, and increased polyol pathway. In our experiment, we found that hyperglycemia significantly but slightly decreased GAPDH activity, and metformin normalized the hyperglycemia-induced GAPDH inhibition (90.7 ± 0.99 and 108.7 ± 3.29% of 5.6 mmol/l glucose, respectively). In addition, overexpression of dominant negative AMPKα1 inhibited metformin-induced GAPDH activation (67.6 ± 0.18% of 5.6 mmol/l glucose). Therefore, metformin could prevent the development and/or progression of diabetes complications by inhibition of mtROS production and resulting activation of GAPDH activity. Metformin is one of the oral drugs used for the treatment of type 2 diabetes. Metformin could prevent diabetes complications by decreasing blood glucose level and by reducing mtROS production through AMPK activation and/or PGC-1α induction.

In conclusion, the findings of this study demonstrate that metformin and AICAR normalize hyperglycemia-induced mtROS production, at least in part, though AMPK, PGC-1α, and MnSOD induction. In addition, metformin and AICAR promote mitochondrial biogenesis (Fig. 8). Our findings suggested that a blockade of hyperglycemia-induced mtROS production though AMPK or PGC-1α induction could therefore be useful in the design of new pharmacological approaches to prevent diabetes complications.

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REFERENCES


