Metformin Inhibits Cytokine-Induced Nuclear Factor κB Activation Via AMP-Activated Protein Kinase Activation in Vascular Endothelial Cells

Yoshiyuki Hattori, Kunihiro Suzuki, Sachiko Hattori, Kikuo Kasai

Abstract—AMP-activated protein kinase (AMPK) is tightly regulated by the cellular AMP:ATP ratio and plays a central role in regulation of energy homeostasis and metabolic stress. Metformin has been shown to activate AMPK. We hypothesized that metformin may prevent nuclear factor κB (NF-κB) activation in endothelial cells exposed to inflammatory cytokines. Metformin was observed to activate AMPK, as well as its downstream target, phosphoacetyl coenzyme A carboxylase, in human umbilical vein endothelial cells (HUVECs). Metformin also dose-dependently inhibited tumor necrosis factor (TNF)-α–induced NF-κB activation and TNF-α–induced IκB kinase activity. Furthermore, metformin attenuated the TNF-α–induced gene expression of various proinflammatory and cell adhesion molecules, such as vascular cell adhesion molecule-1, E-selectin, intercellular adhesion molecule-1, and monocyte chemoattractant protein-1, in HUVECs. A pharmacological activator of AMPK, 5-amino-4-imidazole carboxamide riboside (AICAR), dose-dependently inhibited TNF-α- and interleukin-1β–induced NF-κB reporter gene expression. AICAR also suppressed the TNF-α- and interleukin-1β–induced gene expression of vascular cell adhesion molecule-1, E-selectin, intercellular adhesion molecule-1, and monocyte chemoattractant protein-1 in HUVECs. The small interfering RNA for AMPKα1 attenuated metformin or AICAR–induced inhibition of NF-κB activation by TNF-α, suggesting a possible role of AMPK in the regulation of cell inflammation. In light of these findings, we suggest that metformin attenuates the cytokine-induced expression of proinflammatory and adhesion molecule genes by inhibiting NF-κB activation via AMPK activation. Thus, it might be useful to target AMPK signaling in future efforts to prevent atherogenic and inflammatory vascular disease. (***Hypertension*. 2006;47:1183-1188.**)

Key Words: endothelium ■ cell adhesion molecules ■ diabetes mellitus

In the treatment of type 2 diabetes mellitus, metformin is associated with decreased macrovascular morbidity and mortality, independent of improvements in glycemic control, as demonstrated in the UK Prospective Diabetes Study.[1] This suggests that metformin might alter the risk of atherothrombotic disease through mechanisms other than a reduction of blood glucose levels. One clinical trial has shown reduced levels of soluble intercellular adhesion molecule (ICAM)-1 and soluble vascular cell adhesion molecule (VCAM)-1 by treatment with metformin in subjects with impaired glucose tolerance,[2] whereas another trial has shown an association between treatment of diabetic patients with metformin and decreased levels of soluble VCAM-1 and soluble E-selectin, unrelated to changes in glycemic control.[3] Metformin may, thus, have specific effects on endothelial function.

AMP-activated kinase (AMPK) is a highly conserved heterotrimeric kinase that functions as a metabolic switch, thereby coordinating the cellular enzymes involved in carbohydrate and fat metabolism to enable ATP conservation and synthesis.[4] AMPK is activated by conditions that increase the AMP:ATP ratio, such as exercise and metabolic stress. The effects of stress, exercise, and other conditions, which induce hypoxia and ischemia, on AMPK activation have been extensively examined. When the AMP:ATP ratio increases, AMPK is activated by AMPK kinase, and a conformational change is induced by combining with AMP, thereby decreasing the AMP:ATP ratio by switching off ATP-consuming pathways and switching on ATP-generating pathways.[4]

Activated nuclear factor κB (NF-κB) has been identified in situ in human atherosclerotic plaques but is absent or present in only very small amounts in vessels devoid of atherosclerosis.[5] A number of genes of which the products have been implicated in the development of atherosclerosis are regulated by NF-κB. Leukocyte adhesion molecules, such as VCAM-1, ICAM-1, and E-selectin, as well as the chemokines (chemoattractant cytokines), monocyte chemoattractant protein (MCP)-1, and interleukin (IL)-8, recruit circulating mononuclear leukocytes to the arterial intima.[6–8] The induction of other NF-κB–dependent genes, such as tissue factor, might tip the procoagulant/anticoagulant balance of the en-

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dothelium toward coagulation. Still, other products of target genes, including cyclin D1, may induce cell proliferation or stimulate cell survival at sites of atherosclerotic lesions. Therefore, a coordinated induction of NF-κB–dependent genes might promote atherosclerosis.[9]

In the present study, we hypothesized that metformin may prevent NF-κB activation in endothelial cells exposed to inflammatory cytokines. We examined the effects of metformin on NF-κB activation, as well as the expression of NF-κB–mediated genes, such as VCAM-1, ICAM-1, E-selectin, and MCP-1, in vascular endothelial cells. We also examined the effects of 5-aminoimidazole-4-carboxamide-1-β-ribofuranoside (AICAR),[10] an AMPK activator, on NF-κB activation, as well as the expression of NF-κB–mediated genes. We found that metformin inhibits the cytokine-induced expression of proinflammatory and adhesion molecule genes by suppressing NF-κB activity via AMPK activation.

Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics and cultured in EBM medium supplemented with 5% FCS in the standard fashion. The cells in this experiment were used within 3 to 4 passages and were examined to ensure that they demonstrated the specific characteristics of endothelial cells. Mouse SVEC4 cells (axillary lymph node, vascular endothelial; SV40 transformed) were also cultured in DMEM containing 10% FCS and observed to demonstrate the typical cobblestone morphological appearance of endothelial cells.

Western Blot Analysis

HUVECs treated with tumor necrosis factor (TNF)-α in the presence or absence of metformin for various intervals were lysed using cell lysis buffer (Cell Signaling) with 1 mmol/L PMSF. The protein concentration of each sample was measured using a Bio-Rad detergent-compatible protein assay. Subsequently, β-mercaptoethanol was added to a final concentration of 1%, after which each sample was denatured by boiling for 3 minutes. Samples containing 10 μg of protein were resolved by electrophoresis on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad), after which they were incubated with anti-phospho-Thr-172 AMPK polyclonal antibody and anti-phospho-Ser-79 ACC polyclonal antibody (Cell Signaling) and MCP-1 polyclonal antibody (Santa Cruz Biotech). For the IκB experiments, the membranes were incubated with IκB-α antibody or phospho-IκB-α antibody (Cell Signaling). The binding of each of these antibodies was detected using sheep anti-rabbit IgG horseradish peroxidase (1:20 000) and an ECL Plus system (Amersham).

NF-κB Activation

To study NF-κB activation, SVEC4 cells were stably transfected with a *cis*-reporter plasmid containing the luciferase reporter gene linked to 5 repeats of NF-κB binding sites (pNFκB-Luc, Stratagene), as described previously.[11] For this, the pNFκB-Luc plasmid was transfected together with a pSV2neo helper plasmid (Clontech) into SVEC4 cells using a FuGEN 6 transfection reagent (Boehringer Mannheim). The cells were then cultured in the presence of G418 (Clontech) at a concentration of 500 μg/mL, and the medium was replaced every 2 to 3 days. Approximately 3 weeks after transfection, G418-resistant clones were isolated using a cloning cylinder and analyzed individually for expression of luciferase activity. Several clones were also selected for analysis of NF-κB activation. Luciferase activity was measured using a luciferase assay kit (Stratagene). Similarly, the cells were stably transfected with a *cis*-reporter plasmid containing the luciferase reporter gene linked to 7 repeats of AP-1 sites (pAP-a-Luc, Stratagene).[11]

We also measured changes in the levels of NF-κB p50 and p65 in nuclear extracts from HUVECs using a transcription factor assay kit (Active Motif Japan). Nuclear extracts were prepared with a NE-PER nuclear extraction reagent (Pierce), after which p50 and p65 were quantified using Jurkat nuclear extract as the standard.

IκB Kinase Assay

IκB kinase (IKK) activity was examined using an immune complex kinase assay with glutathione *S*-transferase (GST)-IkBα (1-55) as the substrate, as described previously.[12] Briefly, the cells were solubilized in ice-cold buffer and then centrifuged at 15 000*g* for 20 minutes. IKKα and IKKβ were recovered from the cell lysate by immunoprecipitation, after which the immune complexes were incubated with 20 μL of reaction buffer containing 20 mmol/L HEPES/NaOH (pH 7.4), 10 mmol/L $MgCl_2$, 50 μmol/L NaCl, 100 mmol/L Na_3VO_4, 20 mmol/L β-glycerophosphate, 1 mmol/L dithiothreitol, 100 mmol/L ATP, 0.1 μCi [γ-^{32}P]ATP, and 10 μg GST-IkBα (1-55) at 30°C for 20 minutes. After SDS-polyacrylamide gel electrophoresis, the phosphorylation of GST-IkBα was estimated using an imaging plate (Fuji Film).

Small Interfering RNA Transfection

The day before transfection, plates were inoculated with an appropriate number of SVEC4 cells in serum-containing medium to ensure 50% to 70% confluence the following day. AMPKα1 small interfering RNA [(siRNA) Santa Cruz Biotechnology, Inc] mixed with siLentFect (Bio-Rad) was added to the cells at a concentration of 10 nM. Forty-eight hours after transfection, TNF-α–induced NF-κB activity was compared with that of control cells.

Real-Time PCR of HUVEC mRNA

For quantitative measurement of mRNA, 2 μg of total RNA was treated with DNAse I for 15 minutes and subsequently used for cDNA synthesis. Reverse transcription was performed using a SuperScript preamplification system (Gibco BRL) with random oligonucleotide primers. The following primers were used: ICAM-1 forward 5′-CCGGAAGGTGTATGAACTGA-3′, reverse 5′-GGCAGCGTAGGGTAAGGTT-3′; VCAM-1 forward 5′-GGCAGAGTACGCAAACACTT-3′, reverse 5′-GGCTGTAGCTCCCCGTTAG-3′; E-selectin forward 5′-GCCTTGAATCAGACGGAAGC-3′, reverse 5′-TGATGGGTGTTGCGGTTTC-3′; MCP-1 forward 5′-CAAACTGAAGCTCGCACTCTC-3′, reverse 5′-GCTGCAGATTCTTGGGTTGTG-3′; and GAPDH forward 5′-GGAGAAGGCTGGGGCTCAT-3′, reverse 5′-TGATGGCATGGACTGTGGTC-3′. A typical reaction (50 μL) contained 1/50 of reverse transcription–generated cDNA and 200 nM of primer in 1× SYBR Green RealTime Master Mix (Toyobo) buffer. The PCR reactions were carried out in a LineGene system (BioFlux) under the following conditions: 95°C for 5 minutes, 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s.

Statistical Analysis

Data are presented as the mean±SEM. Multiple comparisons were evaluated by ANOVA followed by Fisher's protected least significant difference test. $P<0.01$ was considered statistically significant.

Results

Metformin Activates AMPK in HUVECs

Treatment of HUVECs with metformin resulted in time-dependent activation of AMPK, as monitored by phosphorylation of AMPK and its downstream target, ACC (Figure 1). Thus, metformin activates AMPK in vascular endothelial cells, as reported previously in hepatocytes and skeletal muscle.[13,14]

Metformin Inhibits NF-κB Activation

We initially examined the effect of incubation with TNF-α for 2 hours on NF-κB activation in SVEC4 cells. TNF-α

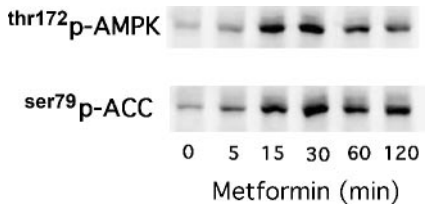

Figure 1. Metformin activates AMPK in vascular endothelial cells. HUVECs were treated with 10 mmol/L of metformin for the indicated time periods before lysis, after which each cell lysate was probed with antibodies specific for phosphorylated forms of AMPK and ACC.

induced a 7-fold increase in NF-κB–mediated reporter gene expression. Metformin dose-dependently suppressed TNF-α–induced activation of NF-κB (Figure 2A). We then examined the effect of siRNA for AMPKα1 on metformin-induced inhibition of NF-κB. The inhibition was partially but significantly attenuated in siRNA-transfected cells (Figure 2A).

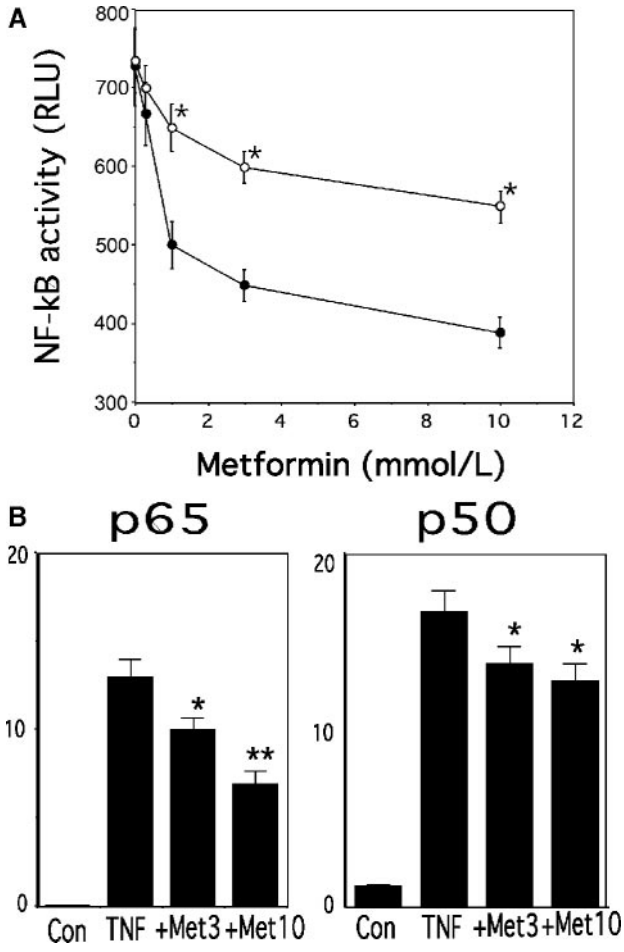

Figure 2. (A) The effects of metformin on TNF-α–induced NF-κB activation and the effects of AMPK siRNA transfection on metformin-induced inhibition of TNF-α–induced NF-κB activation. Metformin dose-dependently suppressed TNF-α–activated NF-κB–mediated reporter (●), and siRNA blunted metformin-induced inhibition of NF-κB activity (○). Results represent the mean±SEM (n=4). *$P<0.01$. (B) HUVECs were stimulated with TNF-α in the presence or absence of metformin (Met3, 3 mmol/L; Met10, 10 mmol/L) for 30 minutes. NF-κB p65 or p50 subunits were quantified within nuclear extracts using a transcription factor assay kit using Jurkat nuclear extract as the standard. Results represent the mean±SEM (n=4). *$P<0.05$, **$P<0.01$.

We also measured p50 and p65 in nuclear extracts from untreated cells and cells treated with TNF-α in the presence or absence of metformin (3 or 10 mmol/L). Both p50 and p65 markedly increased 30 minutes after stimulation with TNF-α from very low levels. This increase was significantly blunted by 25% and 47% for p50 and p65, respectively, in the presence of 10 mmol/L of metformin (Figure 2B).

Similarly, we examined whether TNF-α induces AP-1–mediated reporter gene expression in SVEC4 cells and the effect of metformin on it. AP-1–dependent transactivation increased 1.5-fold relative to unstimulated levels in TNF-α–treated cells, and metformin had no effect at concentrations from 0.1 to 10 mmol/L.

TNFα Stimulates IκB Phosphorylation by Inducing IKK Activity, and Metformin Inhibits TNF-α–Induced IKK Activity and IκB Phosphorylation

We first determined whether TNF-α–induced NF-κB activation occurs through phosphorylation and subsequent degradation of IκB. To determine whether TNF-α causes IκB-α phosphorylation in HUVECs, Western blot analysis using anti-phospho-Ser32 of IκB-α antibody was performed. TNF-α induced IκB phosphorylation in 15 minutes, and decreased levels of phospho-IκB-α were observed at 60 minutes (Figure 3A). The blot was then reprobed with anti-IκB antibody, producing evidence of significant degradation within 15 to 30 minutes. After this, IκB synthesis was reactivated, possibly by NF-κB, by 120 minutes (Figure 3A). Next, the effect of metformin on TNF-α–induced IκB-α degradation was determined 30 minutes after exposure to TNF-α. Metformin partially inhibited TNF-α–induced IκB-α degradation (Figure 3B).

A radiolabeled, phosphorylated, IκB-α–specific band was detected in TNF-α–treated cells, whereas it was undetectable in untreated cells, demonstrating induction of IKK activity by TNF-α (Figure 3C). IKK activity was dose-dependently inhibited by treatment of the cells with metformin (Figure 3C). The remaining half of the immunoprecipitated samples were analyzed by Western blot analysis using anti-IKKa/b antibody, which showed identical expression levels of IKK, suggesting expression of IKK in these cells. Identical amounts of IκB were also detected when an equal volume of kinase reaction mixture was loaded into the SDS-PAGE column, followed by Western blot analysis using anti-IκB antibody (Figure 3C).

Metformin Inhibits Induction of mRNA of VCAM-1, E-Selectin, ICAM-1, MCP-1, and Protein of MCP-1

Incubation for 24 hours with TNF-α substantially induced the gene expression of VCAM-1, E-selectin, and ICAM-1. Metformin dose-dependently inhibited TNF-α–induced gene expression (Figure 4A). We also examined the effect of metformin on TNF-α–induced protein expression of MCP-1. Metformin dose-dependently suppressed the TNF-α–induced protein levels of MCP-1 (Figure 4B).

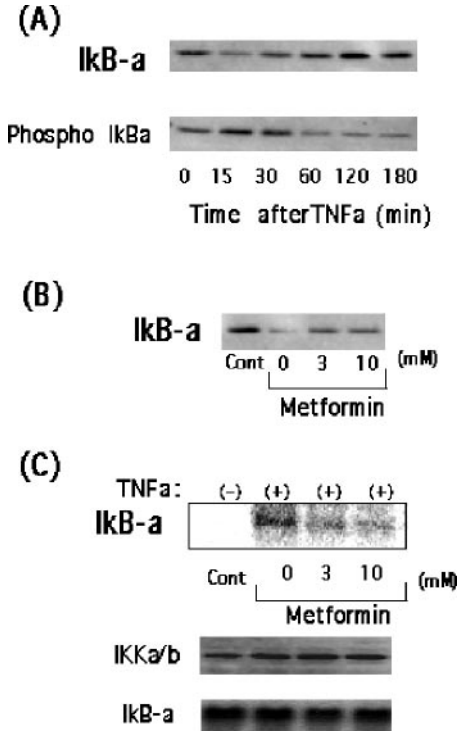

Figure 3. (A) HUVECs were incubated with TNF-α for 0 to 180 minutes. The cells were lysed and subjected to Western blot analysis using anti-IkB-α and anti-phospho-IkB-α antibodies. (B) The effect of metformin on IκB-α degradation in HUVECs. Cells were incubated for 30 minutes with metformin (3 and 10 mmol/L), followed by TNF-α for 30 minutes. Cells were then lysed and subjected to Western blot analysis using anti-IkB-α antibody. (C) The effect of metformin on IKK activity in HUVECs. Cells were incubated for 30 minutes with metformin (3 and 10 mmol/L), followed by TNF-α for 15 minutes. Cells were then lysed and immunoprecipitated with anti-IKKα/β antibody and used for kinase assay using recombinant IkB-α as a substrate. Note that equal band densities for IKKα/β and IkB-α were observed.

AICAR Inhibits NF-κB Activation

A 6-fold and 3.9-fold increase in NF-κB–mediated reporter gene expression was induced by TNF-α and IL-1β, respectively, in SVEC4 cells. Dose-dependent inhibition of NF-κB activation by AICAR was observed when added to cells 1 hour before TNF-α or IL-1β (Figure 5A). One mM of AICAR completely suppressed NF-κB activation. Next, we examined the effect of siRNA for AMPKα1 on AICAR-induced inhibition of NF-κB. TNF-α–induced NF-κB–mediated reporter gene expression was inhibited by AICAR in a dose-dependent manner. This inhibition was partially but significantly attenuated in siRNA-transfected cells (Figure 5B). siRNA inhibited AMPK expression by ≈80% (data not shown); thus, inhibition of NF-κB by AICAR seems to occur through AMPK activation.

AICAR Inhibits Induction of VCAM-1, E-Selectin, ICAM-1, and MCP-1 mRNA

Incubation for 24 hours with TNF-α or IL-1β substantially induced the gene expression of VCAM-1, E-selectin, and ICAM-1. Induction of TNF-α- or IL-1β–induced gene expression was markedly suppressed by cotreatment with an

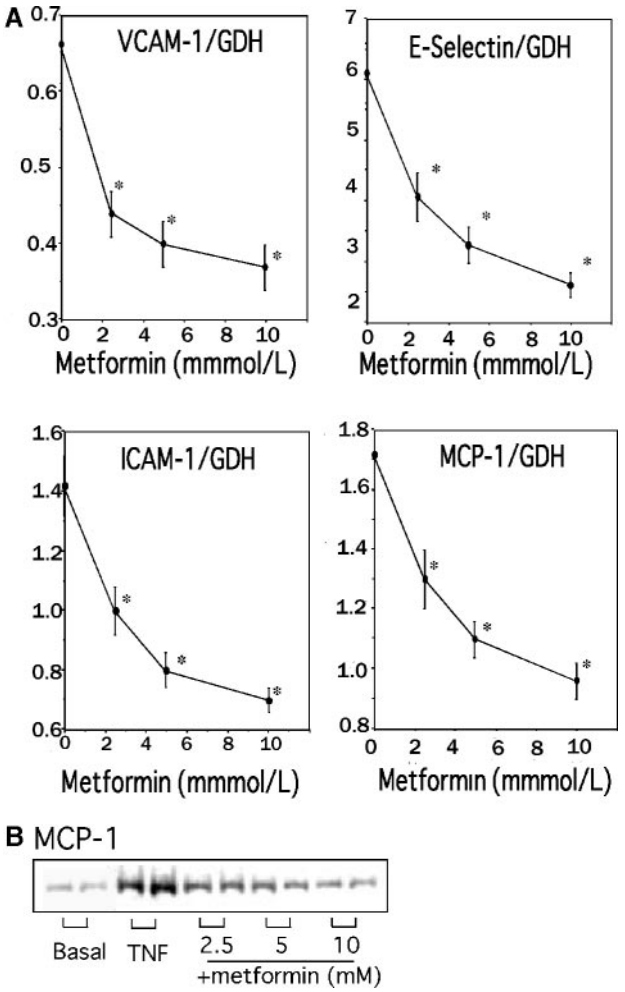

Figure 4. (A) Effects of metformin on TNF-α–induced VCAM-1, E-selectin, ICAM-1, and MCP-1 mRNA expression. Metformin dose-dependently inhibited VCAM-1, E-selectin, ICAM-1, and MCP-1 mRNA levels. Each bar represents the mean±SEM (n=4). *$P<0.01$. (B) Effects of metformin on TNF-α–induced MCP-1 expression. Metformin dose-dependently inhibited MCP-1 protein levels.

NF-κB inhibitor, BAY11–7082, which selectively and irreversibly inhibits cytokine-induced IκB phosphorylation,[15] suggesting that the induction of these genes is NF-κB dependent (data not shown). Coincubation with 1 mmol/L AICAR markedly diminished the TNF-α- or IL-1β–induced gene expression of VCAM-1, E-selectin, ICAM-1, and MCP-1 (Figure 6).

Discussion

In the present study, we demonstrated that metformin inhibits TNF-α–induced NF-κB activation in vascular endothelial cells. Thus, metformin inhibited the NF-κB–dependent gene expression of various inflammatory and cell adhesion molecules, including VCAM-1, E-selectin, ICAM-1, and MCP-1. Metformin is known to activate AMPK, and we confirmed the AMPK activation by metformin in HUVECs. We questioned whether this may be associated with inhibition of cytokine-induced NF-κB activation. Transfection of AMPK siRNA, which caused ≈80% inhibition of AMPK expression,

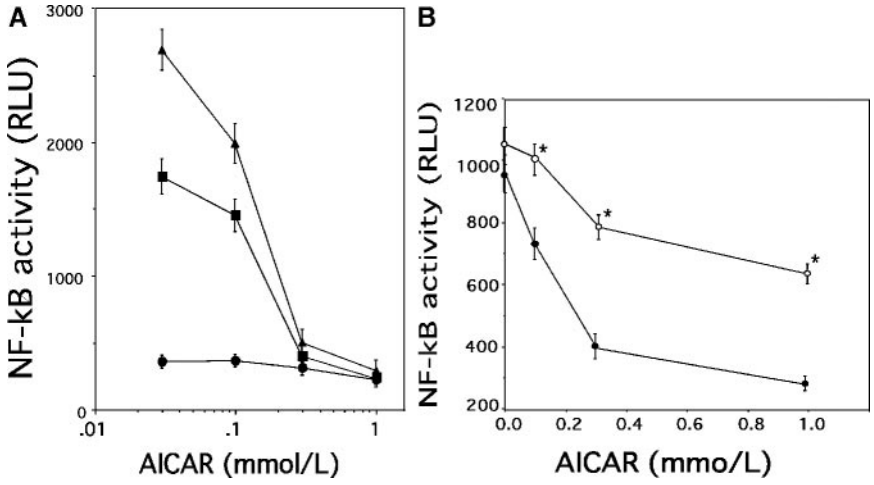

Figure 5. (A) Effects on TNF-α- and IL-1β–induced activation of NF-κB. Dose-dependent suppression of NF-κB activation was observed with AICAR. Results represent the mean±SEM (n=4). Control (no stimulation), ●; TNF-α, ▲; and IL-1β, ■. (B) Effects of AMPK siRNA transfection on AICAR-induced inhibition of TNF-α–induced NF-κB activation. Dose-dependent suppression of TNF-α–induced NF-κB activation was observed with AICAR (●) and siRNA-blunted AICAR-induced inhibition of NF-κB activity (○). Results represent the mean±SEM (n=4). *$P<0.01$.

significantly attenuated metformin-induced inhibition of NF-κB activation by TNF-α in endothelial cells. AICAR, an AMPK activator, was also observed to suppress cytokine-induced NF-κB activation, which was markedly attenuated by transfection of siRNA into endothelial cells. These data suggest that AMPK activation may be responsible for the inhibition of NF-κB activation.

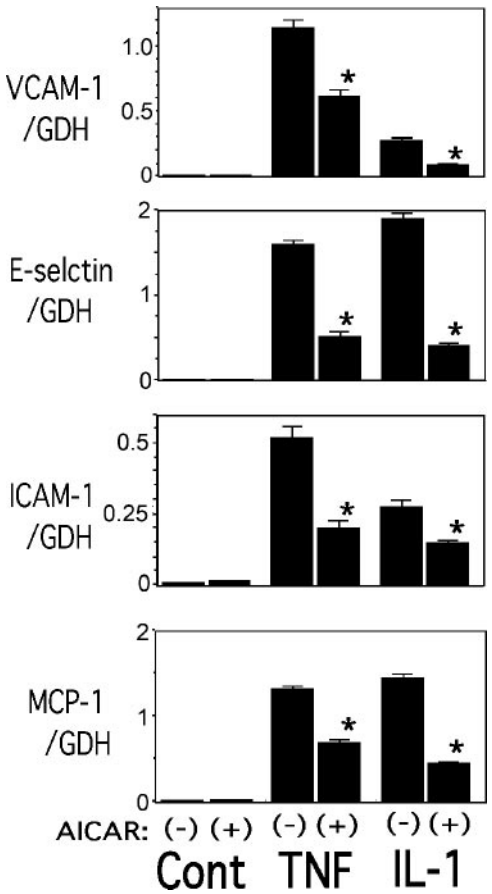

Figure 6. Effects of AICAR on TNF-α- and IL-1β–induced VCAM-1, E-selectin, ICAM-1, and MCP-1 mRNA expression. Coincubation with 1 mmol/L AICAR significantly diminished VCAM-1, E-selectin, ICAM-1, and MCP-1 mRNA levels. Each bar represents mean±SEM (n=4). *$P<0.01$.

We demonstrated that metformin inhibits the expression of proinflammatory and adhesion molecule genes by blocking phosphorylation and subsequent degradation of IκB-α. These data suggest that metformin might suppress TNF-α–induced NF-κB activation before IκB phosphorylation. We further demonstrated stimulation of IκB-α phosphorylation by TNF-α through induction of IKK activity and that metformin inhibits IKK activity and TNF-α–induced IκB-α phosphorylation. Thus, metformin-activated AMPK may suppress NF-κB activation by inhibiting IKK activity in vascular endothelial cells. It has been reported that AICAR attenuates lipopolysaccharide-induced activation of NF-κB via down-regulation of IkB kinase α/β activity in glial cells.[16] This is the same mechanism as we showed in vascular endothelial cells, suggesting that AMPK activation may inhibit cytokine-induced NF-κB activation by suppressing IKK activity.

The UK Prospective Diabetes Study has demonstrated that treatment with metformin decreases macrovascular morbidity and mortality independent of glycemic control.[1] In addition, a clinical trial has shown reduced levels of soluble ICAM-1 and soluble VCAM-1 by metformin in subjects with impaired glucose tolerance,[2] whereas another trial has shown an association between treatment of diabetic patients with metformin and decreased levels of soluble VCAM-1 and soluble E-selectin, unrelated to changes in glycemic control.[3] Metformin may, thus, have specific effects on endothelial function, which suppress atherogenic changes. It has been reported that the plasma levels of metformin in human are estimated to be ≈10 μmol/L after administration of 500 mg of metformin.[17] Although the present study used higher concentration of metformin, significant effects were observed at <1 mM. Thus, the findings in this experiment using HUVECs may have significance for human treatment with this drug. The observation that exercise and adiponectin, as well as treatment with thiazolidinediones and metformin, all of which activate AMPK, reduce inflammation, endothelial dysfunction, and atherosclerotic vascular disease suggests that AMPK might be a useful therapeutic target.[18] It seems that attenuation of NF-κB activation through activation of AMPK might play a role in the vascular protective effects of metformin.

Perspectives

We demonstrated that metformin inhibits the expression of proinflammatory and adhesion molecule genes by blocking NF-κB activation in vascular endothelial cells. Metformin through AMPK activation attenuate phosphorylation and subsequent degradation of IκB-α by inhibiting IKK activity, resulting in suppression of cytokine-induced NF-κB activation. Although experiments on cultured cells do not necessarily represent the in vivo state, the present results suggest that metformin may serve for antiatherogenic drug for diabetic subjects.

Acknowledgments

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