Adiponectin suppresses IκB kinase activation induced by tumor necrosis factor-α or high glucose in endothelial cells: role of cAMP and AMP kinase signaling

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Wu X, Mahadev K, Fuchsel L, Ouedraogo R, Xu S, Goldstein BJ. Adiponectin suppresses IκB kinase activation induced by tumor necrosis factor-α or high glucose in endothelial cells: role of cAMP and AMP kinase signaling. Am J Physiol Endocrinol Metab 293: E1836–E1844, 2007. First published October 16, 2007; doi:10.1152/ajpendo.00115.2007.—Adiponectin is a protein secreted from adipocytes that exhibits salutary effects in the vascular endothelium by signaling mechanisms that are not well understood. In obesity-related disease states and type 2 diabetes, circulating substances, including tumor necrosis factor-α (TNFα) and high glucose, activate IκB kinase (IKK)β and reduce the abundance of its substrate, inhibitor of IκB (IκB)α, leading to nuclear translocation of the transcription factor NF-κB and stimulation of an inflammatory signaling cascade closely associated with endothelial dysfunction. The present study demonstrates that the globular domain of adiponectin (gAd) potently suppresses the activation of IKKβ by either TNFα or high glucose in human umbilical vein endothelial cells and ameliorates the associated loss of IκBα protein. Interestingly, activation of AMP kinase was substantially more effective than cAMP signaling in suppressing high glucose-induced IKKβ activity, whereas both pathways were comparably active in suppressing the TNFα-induced increase in IKKβ. Both cAMP/protein kinase A signaling and activation of the AMP kinase pathway played a role in the suppression by gAd of TNFα- and high glucose-mediated IKKβ activation. These findings support an important role for adiponectin in anti-inflammatory signaling in the endothelium and also imply that multiple pathways are involved in the cellular effects of adiponectin.

inflammation; endothelial dysfunction; insulin resistance; NF-κB

ADIPONECTIN IS AN ABUNDANT circulating plasma protein secreted by adipose tissue that exhibits beneficial effects in the vasculature as well as insulin-sensitizing effects in cells responsive to the metabolic effects of insulin (7, 27). Circulating levels of adiponectin are decreased in individuals with obesity and type 2 diabetes, suggesting a potential role for adiponectin in the vascular disease that frequently accompanies these disorders (18). Adiponectin levels have also been prospectively shown to be negatively associated with coronary artery disease in type 1 diabetes, suggesting that it may also protect against the adverse vascular effects of hyperglycemia (4).

Inflammatory signaling in the vascular endothelium, triggered by circulating cytokines such as tumor necrosis factor-α (TNFα) in visceral obesity or by hyperglycemia in diabetes mellitus, has been implicated in pathological endothelial cell activation and early vascular events in atherogenesis (2, 26). The mediator NF-κB generates a programmed nuclear transcription cascade that is a major source of the inflammatory response in endothelial cells (8). NF-κB is regulated through its protein interactions with the inhibitor of IκB (IκB) inhibitory proteins, which, on cellular stimulation, are rapidly phosphorylated on serine, ubiquitinated, and degraded in the proteasome, releasing NF-κB to function as a nuclear transcription factor (1, 25). Cytokines activate NF-κB by inducing IκB phosphorylation via IκB kinase (IKK). IKK is a complex of at least three subunits: α and β, which are kinase enzymes, and γ, which has a regulatory role. IKKβ, in particular, has been shown to play a prominent role in mediating cellular insulin resistance resulting from cytokine stimulation (28). High glucose has also been implicated in inflammatory signaling in endothelial cells via activation of NF-κB (9, 24). Recent work has also shown that high glucose may act upstream of NF-κB at the level of IKKβ activity in bovine aortic endothelial cells, resulting in impaired insulin-stimulated production of nitric oxide (NO) (11).

Prior work has shown that adiponectin has several important signaling effects in the endothelium, including enhancing NO generation, reducing reactive oxygen species (ROS) generation, and blocking inflammatory signaling cascades (7). Adiponectin inhibited TNFα-induced expression of the adhesion molecules VCAM-1, E-selectin, and ICAM-1 on the surface of endothelial cells and reduced TNFα-induced adhesion of mononcytic THP-1 cells to cultured endothelial cells (20). Adiponectin also has been shown to suppress NF-κB activation induced by TNFα without affecting TNFα-mediated activation of several MAP kinases, stress-activated kinases, and Akt (12, 21). Although the cellular mechanisms used by adiponectin signal transduction in the endothelium have not been fully characterized, they appear to involve multiple pathways, in particular, those mediated by 5′-AMP-activated protein kinase (AMP kinase) and cAMP/protein kinase A (PKA) signaling (7).

In the present study, we evaluated whether adiponectin suppressed the activation of the NF-κB cascade as an upstream site involving inhibition of the enzyme activity of IKKβ. Both TNFα- and high glucose-stimulated IKKβ activation and the potential involvement of cAMP and PKA signaling were ex-

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amine using a cultured human umbilical vein endothelial cell (HUVEC) model.

MATERIALS AND METHODS

Materials. The pTReHisA vector and Escherichia coli TOP10 strain
cells were obtained from Invitrogen (Carlsbad, CA). Acticline Etox
column was from Sterogene Bioseparations (Carlsbad, CA). The
Limulus Amebocyte Lysate Pyrogen Plus detection kit was from
BioWhittaker (Walkersville, MD). HUVECs were from Cell Applica-
tions (San Diego, CA), and endothelial basal medium-2 (EBM-2) and
growth factors were obtained from Cambrex BioScience (Walk-
kersville, MD). The AMP kinase inhibitor compound C (6-[(3-
methylpyrrolo[2,3-d]pyrimidin-4-yl)thio]-2-pyridyl) was kindly provided by
Merck Research Laboratories (Rah-
way, NJ). Glutathione S-transferase (GST)-IkBα protein was from
Santa Cruz Biotechnology (Santa Cruz, CA). IKKβ and IkBα rabbit polyclo-
nal antibody and antibody to AMP kinase (α1 + α2) and
PKA(c-a) rabbit polyclonal antibody were from Cell Signaling Tech-
nology (Danvers, MA). [γ-32P]ATP and the cAMP Biotrak enzyme
immunooassay (ELA) system were from GE Healthcare (Piscataway,
NJ). AMP kinase-α1 rabbit monoclonal antibody, SAMS substrate
peptide, PKA assay kit, small interfering RNA (siRNA) SMARTpool
AMP kinase-α1, siRNA SMARTpool PKA, siRNA nonspecific con-
trol pool, siIMPORTER siRNA, and the plasmid DNA transfection
reagent were obtained from Millipore/Upstate (Lake Placid, NY).
Protein A agarose beads were from Pierce (Rockford, IL). Enhanced
chemiluminescence (ECL) reagents were from Perkin-Elmer Life
Sciences (Boston, MA). Horseradish peroxidase-conjugated secondary
antibodies were obtained from GE Healthcare (Piscataway, NJ). Magnesium/ATP cocktail and IKK substrate peptide were from
Upstate Biotechnology (Lake Placid, NY). Bio-Safe Coomassie Stain
Solution was from Bio-Rad (Hercules, CA). 5-Aminoisimadazole-4-
carboxamide-1-β-d-ribofuranoside (AICAR), Rp-adenosine 3',5'-cy-
clic monophosphorothioate (Rp-cAMP), 2',3'-dideoxyadenosine
(ddAdo), and other reagents were obtained from Sigma-Aldrich
(St. Louis, MO).

Recombinant adiponectin protein. The recombinant globular do-
main of human adiponectin was subcloned into the pTReHisA Bacte-
rial expression vector and expressed as an NH2-terminal (his)6-
tagged fusion protein in E. coli TOP10 strain by induction with isopropyl-
β-thigalactopyranoside. The protein was purified under native con-
titions and was applied to an Acticline Etox column (Sterogene
Bioseparations) to remove endotoxin contamination, as we described
previously (30).

Cell culture and treatment. HUVECs before passage 4 were cul-
tured to 80% confluence on six-well plates with growth medium
EBM-2 supplemented with endothelial cell growth factors (Clonetics) and
2% FBS. After a washing with PBS, cells were made quiescent
in human endothelial cell-free medium (SFM) with 5 mmol/l glucose
and 1% BSA, with no growth factor supplement for 3 h. Cells were
incubated at 37°C with the indicated concentration of globular adipon-
ecin for 3 h before treatment with the indicated concentration of TNFα
for 5 min or high glucose for 24 h. Where indicated, cells were also
treated with ddAdo (100 μM) or Rp-cAMP (10 μM) for 20 min
before the addition of globular domain of adiponectin (gAd); forskolin
(2 μM), AICAR (2 mM), or compound C (10 μM) was added during
the last 20 min of incubation before cell lysis.

Immunoprecipitation and I KK kinase assay using GST- IkBα pro-
tein as substrate. Kinase assays were performed as previously de-
scribed (6, 14) using substrate protein according to the reagent
manufacturer’s instructions with minor modifications. Briefly, cells were
lysed with ice-cold deoxyxygenated buffer including 50
mM HEPES, pH 7.4, 150 mM NaCl, 1% (vol/vol) Triton X-100, 5
mM EDTA, 5 mM EGTA, 20 mM Na pyrophosphate, 20 mM NaF, 1
mM MgCl2, 10% (vol/vol) glycerol, 1 mM Na orthovanadate, 1 mM
β-glycerophosphate, 0.5 mM diithiothreitol, and a protease inhibitor
cocktail (Sigma). The cell lysate was sonicated twice for 10 s each on
ice and centrifuged at 13,000 rpm for 5 min at 4°C. The protein
concentration was estimated using Bio-Rad protein dye reagent as
described by the manufacturer.

Aliquots containing 400 μg of total protein from the cell lysate were
immunoprecipitated with 2 μl of IKKβ polyclonal antibody
overnight at 4°C and then incubated with 35 μl of protein A agarose
beads for 2 h at 4°C. The protein A agarose beads, antibody, and IKK
protein complex was washed four times using enzyme dilution buffer
(20 mM MOPS, pH 7.5, 1 mM EDTA, 5% glycerol, 0.1% β-mercapto-
ethanol, 1 mg/ml BSA) for three of the washes and kinase reaction
buffer (8 mM MOPS, pH 7.0, 0.2 mM EDTA) once. The kinase
reaction was performed using IKK protein coupled to protein A beads
with 1 μg of GST-IkBα protein, 10 μCi of [γ-32P]ATP in magne-
sium/ATP cocktail at 30°C for 30 min. The reaction was ended by
addition of 4× Laemmli protein sample buffer, and samples were
boiled at 100°C for 5 min. After centrifugation at 4,000 rpm for 2 min,
supernatant was loaded to 10% polyacrylamide gel for SDS-PAGE.
The gel was fixed in solution with 10% glacial acetic acid and 20%
ethanol, 1 mg/ml BSA) for three of the washes and kinase reaction.

Western blotting. Protein immunoblotting was performed essen-
tially as previously reported (17). Twenty-five to fifty micrograms of
protein were resolved by 10% SDS-PAGE and transferred to nitro-
cellulose membranes. Primary antibody immunoblotting was per-
formed following the manufacturer’s instructions. After incubation
with horseradish peroxidase-conjugated secondary antibodies, pro-
teins were visualized by ECL exposure to X-ray film. Immunoblotting
signals were quantitated using an ImageStation 440CF (Kodak,
Rochester, NY).

Immunoprecipitation and I KK kinase assay using I KK peptide as
substrate. Cell lysate preparation and immunoprecipitation were the
same as above. The kinase reaction was performed using IKK protein
coupled to protein A beads with 200 μM IKK substrate peptide
(KKKERRRRDHRDSDLMSKDEE) and 10 μCi of [γ-32P]ATP
in magnesium/ATP cocktail at 30°C for 30 min. IKK protein
was separated from reaction mixture by centrifugation at 4,000 rpm for 2
min to end the kinase reaction. Aliquots of 20 μl of supernatant were
spotted onto the center of 2×2 cm Whatman P81 paper. The P81
squares were washed three times with 0.75% (vol/vol) phosphoric
acid and once with acetone to eliminate unlabelled binding and then
transferred to a vial containing 5 ml of scintillation cocktail. The
radioactivity was then counted in a β-counter. IKKβ kinase activity
was expressed as the amount of substrate peptide phosphorylation
relative to control.

Measurement of cellular cAMP content. HUVECs were cultured on
24-well plates and were treated when cells reached 80–90% conflu-
cy. An aliquot of 100 μl of cell lysate was used for each cAMP
measurement. Intracellular cAMP content was measured using a
cAMP Biotrak EIA system (GE Healthcare) according to the manu-
facturer’s instructions.

Immunoprecipitation and PKA activity assay. Aliquots containing
400 μg of cell lysate protein were incubated with 2 μg of anti-
PKAc-α polyclonal rabbit antibody overnight at 4°C and then
incubated with 30 μl of protein A agarose beads for 2 h at 4°C. Enzyme
activity was assayed using a PKA assay kit and the instructions
provided by the manufacturer (Upstate, Lake Placid, NY). Briefly,
agarose beads were washed four times with assay dilution buffer
(ADB) (20 mM MOPS, pH 7.2, 125 mM β-glycerophosphate, 25 mM
EGTA, 5 mM sodium orthovanadate, and 5 mM dithiothreitol) and
then incubated with 10 μl of ADB, 5 μl of 20 μM cAMP, 5 μl of
kemptide, 10 μl of inhibitor cocktail (10 μl of the inhibitor peptide
to the negative controls), and 10 μl of [γ-32P]ATP in magnesium/ATP
cocktail for 10 min at 30°C. An aliquot of 25 μl was blotted on the
P81 paper square, which was then washed three times with 0.75%
SAMS peptide (HMRSAMSGLHLVKRR) per milligram protein

1 and PKA. Four pooled selected AMP kinase-duplex oligonucleotides were based on the human cDNAs encoding

MATERIALS AND METHODS. After pretreatment with 3 gAd for 3 h, cells
were treated with TNF at the indicated concentrations for 5 min. Cells were
lysed, and protein samples were immunoprecipitated with IKK antibody
followed by assay using the IKK substrate peptide. dpm, Disintegrations per
minute. Data are expressed as means ± SE. *P < 0.001 vs. control; **P < 0.001 vs. the respective TNFα-stimulated samples.

The radioactivity was then counted in a -counter. PKA kinase
activity was expressed as picomoles of 32P incorporation into the
substrate kemptide (LRRA-SLG) per milligram protein per minute.

Immunoprecipitation and AMP kinase enzyme assay. Immunopre-
cipitation followed by AMP kinase enzyme assay were described as
before (30). Briefly, aliquots containing 200 µg of cell lysate protein
were incubated with 1.0 µg of anti-AMP kinase α1 + α2 polyclonal
rabbit antibody overnight at 4°C and then incubated with 30 µl of
protein A agarose beads for 2 h at 4°C. The agarose was washed four
times with AMP kinase reaction buffer (20 mM HEPES-NaOH, pH
7.2, 0.4 mM dithiothreitol with 300 µmol of AMP) and then
incubated with 20 µl of AMP kinase reaction buffer, 10 µl of
SAMS substrate peptide with final concentration of 80 µM, and 10
µCi of [γ-32P]ATP mixed with 75 mM magnesium chloride and
500 µmol of unlabelled ATP for 15 min at 30°C. Aliquots of 35 µl
were spotted onto the center of a 2-cm square of Whatman P81
paper. The P81 squares were washed three times with 0.75%
(vol/vol) phosphoric acid and once with acetone. The P81 square
was transferred to a vial containing 5 ml of scintillation cocktail.
The radioactivity was then counted in a -counter. PKA kinase
activity was expressed as picomoles of 32P incorporated into the
substrate kemptide (LRRA-SLG) per milligram protein per minute.

siRNA-mediated knockdown of AMP kinase-α1 and PKA. siRNA
duplex oligonucleotides were based on the human cDNAs encoding
AMP kinase-α1 and PKA. Four pooled selected AMP kinase-α1- or
PKA-specific siRNA duplexes, nonsilencing control siRNA, and
siIMPORTER siRNA transfection reagent were used according to the
manufacturer’s instructions (Millipore/Upstate). HUVECs were
plated on six-well plates before transfection and were 50% confluent
manufacturer’s instructions (Millipore/Upstate). HUVECs were
plated on six-well plates before transfection and were 50% confluent

Statistical analysis. Quantitative data are presented as means ± SE
for three to five experiments. Statistical analysis was based on Stu-
dent’s t-test for comparison of two groups. A P value <0.05 was used to
determine statistical significance.

RESULTS

IKKβ substrate peptide phosphorylation. TNFα activates the
proinflammatory IKKβ/NF-κB signaling pathway in various

Fig. 2. Effect of adiponectin (gAd) on TNFα-induced IKKβ activity in human umbilical vein endothelial cells (HUVECs) using IKK substrate peptide. HUVECs were cultured as described in MATERIALS AND METHODS. After pretreatment with 3 gAd for 3 h, cells
were treated with TNF at the indicated concentrations for 5 min. Cells were
lysed, and protein samples were immunoprecipitated with IKKβ antibody
followed by assay using the IKK substrate peptide. dpm, Disintegrations per
minute. Data are expressed as means ± SE. *P < 0.001 vs. control; **P < 0.001 vs. the respective TNFα-stimulated samples.
Cellular IκBα protein mass assay. Since activation of IKKβ leads to the phosphorylation of its cellular substrate protein IκBα followed by its ubiquitination and proteolytic degradation, we also measured the effect of gAd on cellular IκBα protein levels as a functional confirmation of changes in IKKβ activity that we observed using the above kinase assays. The loss of IκBα mass following TNFα stimulation was rapid. A dose-response experiment showed that TNFα concentrations as low as 7.5 ng/ml reduced IκBα mass by 60–80% within a 5-min incubation period (not shown). As shown in Fig. 3, stimulation with TNFα (15 ng/ml) for 5 min reduced IκBα mass in HUVECs by an average of 62%. Prior cell treatment with gAd (3 μg/ml for 3 h) did not affect basal IκBα abundance; however, gAd fully abrogated the reduction in IκBα mass induced by TNFα.

**Fig. 3.** Effect of adiponectin (gAd) on TNFα-induced degradation of IκBα in HUVECs. HUVECs were cultured as described in MATERIALS AND METHODS. Cells were preincubated for 3 h without or with adiponectin (3 μg/ml) and then treated where indicated with 15 ng/ml TNFα for 5 min before snap-freezing of the cells and lysis into the buffer described in MATERIALS AND METHODS. Following immunoprecipitation with anti-IκBα antibody, the protein kinase activity was assayed by phosphorylation of GST-IκBα substrate as described in MATERIALS AND METHODS. For definitions of ddAdo, Rp-cAMP, AICAR, and compound C, see MATERIALS AND METHODS. Data are expressed as means ± SE. *P < 0.01 vs. control; **P < 0.01 vs. gAd and TNFα treated; ***P < 0.04. Rp-cAMP, a specific inhibitor of PKA, similarly diminished the effect of gAd by 42% (P < 0.04).

AMP kinase activation was also implicated in the suppression of IKKβ activity by gAd. Like forskolin, activation of AMP kinase with AICAR completely blocked the ability of subsequent TNFα stimulation to increase IKKβ activity (Fig. 4). Inhibition of AMP kinase with compound C (Merck) also diminished the effect of gAd to suppress TNFα-stimulated IKKβ activity, by 40% (P < 0.04).

**High glucose-induced activation of IKKβ**. High glucose has been shown to initiate an inflammatory signaling cascade in endothelial cells, including the activation of IKKβ (11). Incubation of HUVECs in medium containing 25 mM glucose for 24 h increased IKKβ activity by 1.8-fold (Fig. 5). Prior treatment with gAd (3 μg/ml) completely inhibited the increase in IKKβ activity stimulated by high glucose conditions.

Studies with signaling effectors and inhibitors provided evidence that both cAMP and AMP kinase signaling play a role in the suppression of high glucose-induced IKKβ activation by gAd (Fig. 5). Interestingly, forskolin activation of adenylate cyclase was ineffective in suppressing IKKβ activity induced by high glucose conditions. These findings are in contrast to the data from TNFα-induced IKKβ activation, where forskolin was fully effective in suppressing IKKβ activation by cell treatment with TNFα (Fig. 4). The cAMP signaling pathway was also implicated in the effect of gAd, since treatment with the adenyl cyclase inhibitor ddAdo also partially inhibited

**Fig. 4.** Effect of various inhibitors and effectors on TNFα-induced activation and adiponectin (gAd) suppression of TNFα-induced activation of IKKβ in HUVECs. Data are expressed as means ± SE. *P < 0.01 vs. control; **P < 0.04 vs. gAd and TNFα treated; ***P < 0.01 vs. TNFα treated.

**Fig. 5.** Effect of forskolin, Rp-cAMP, and compound C on the IKKβ activity stimulated by high glucose. Data are expressed as means ± SE. *P < 0.01 vs. TNFα treated. Rp-cAMP, a specific inhibitor of PKA, similarly diminished the effect of gAd by 42% (P < 0.04).
the effect of gAd to suppress IKKβ activation by high glucose by 45% (P = 0.05). Rp-cAMP, the PKA inhibitor, similarly diminished the effect of gAd by 37%. AMP kinase activation with AICAR also completely protected the cells from the high glucose-induced increase in IKKβ activity. AMP kinase also appeared to be involved in gAd signaling, since inhibition of AMP kinase with compound C significantly diminished the effect of gAd to suppress high glucose-stimulated IKKβ activity by 72% (P < 0.04).

gAd increases cellular cAMP levels under TNFα stimulation or in high glucose conditions. To provide further mechanistic evidence for a role of cAMP/PKA signaling in the endothelial action of gAd, we first measured cellular cAMP levels under conditions identical to those used for measuring IKKβ activity. TNFα stimulation had no effect on cAMP levels in the HUVECs (Fig. 6A). However, pretreatment with gAd for 3 h before treatment with TNFα for 5 min significantly increased the cAMP level by 29% (P < 0.05), to a degree similar to the 46% increase observed following treatment with the adenylyl cyclase activator forskolin. gAd appears to signal via adenylyl cyclase, since the increase in cellular cAMP content was abrogated by the cyclase inhibitor ddAdo.

Under high glucose conditions for 24 h, there was no significant increase in cellular cAMP (Fig. 6B). However, addition of gAd for the last 3 h of incubation increased cAMP 2.1-fold under normal glucose conditions and by 54% in 25 mM glucose (both P < 0.05). As with TNFα, the effect of gAd was blocked by ddAdo and mimicked by forskolin treatment.

**HUVEC PKA activity is increased by gAd under TNFα stimulation or in high glucose conditions.** The activation of PKA enzyme activity by gAd was consistent with the observed increases in cAMP described in Fig. 6. Cellular PKA activity was measured in immunoprecipitates using a radiolabeled peptide substrate assay (Fig. 7). In parallel with the cAMP levels shown above, TNFα itself did not alter cellular PKA activity, which was significantly increased 49 and 66% by gAd treatment without and with stimulation by TNFα, respectively (both P < 0.05; Fig. 7A). Treatment with the direct PKA inhibitor Rp-cAMP suppressed gAd stimulation of PKA by 90% under conditions of TNFα stimulation, and the adenylyl cyclase inhibitor ddAdo completely blocked gAd stimulation of PKA.

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The figure legends and the graphs are as follows:

**Fig. 5.** Effect of various inhibitors and effectors on high glucose-induced activation and adiponectin (gAd) suppression of high glucose-induced activation of IKKβ in HUVECs. These studies were performed as described in the legend to Fig. 4, except that in place of TNFα stimulation, HUVECs were incubated in 25 mM glucose medium (HG) for 24 h before the addition of adiponectin (3 μg/ml) for 3 h with or without the reagents shown for 20 min before cell lysis. Following immunoprecipitation with IKKβ antibody, the protein kinase activity was assayed by phosphorylation of GST-IκB substrate as described in MATERIALS AND METHODS. Data are expressed as means ± SE. #P < 0.01 vs. high glucose-treated; **P < 0.01 vs. control; *P < 0.05 vs. gAd and high glucose treated.

**Fig. 6.** Effect of TNFα, high glucose, adiponectin (gAd), ddAdo, and forskolin on cAMP content in HUVECs. A: cells were stimulated with or without gAd for 3 h before 10 ng/ml TNFα for the last 5 min of incubation. Where indicated, cells were treated with ddAdo for 20 min before the addition of gAd; forskolin was added during the last 20 min of incubation. Cells were lysed, and 100 μl of the cleared lysate were used for cAMP assay as described in MATERIALS AND METHODS. Data are expressed as means ± SE. n = 4. *P < 0.05 vs. control.
Under high glucose incubation conditions, PKA activity in HUVECs was also unchanged, but treatment with gAd for 3 h before cell lysis showed 96 and 56% increases in PKA in conditions of normal and high glucose, respectively (both P < 0.05; Fig. 7B). Treatment with the direct AMP kinase inhibitor compound C significantly reduced the gAd stimulation of AMP kinase by 71% under conditions of TNFα stimulation. The effect of gAd on AMP kinase activity was also mimicked by the AMP kinase activator AICAR.

Under high glucose incubation conditions for 24 h, AMP kinase activity in HUVECs was unchanged, but treatment with gAd for 3 h before cell lysis showed an 84% and 2.3-fold increase in AMP kinase in conditions of normal and high glucose, respectively (both P < 0.05; Fig. 8B). The effect of gAd on AMP kinase activity was also mimicked by the AMP kinase activator AICAR.

Fig. 7. Effect of TNFα, high glucose, gAd, and other agents on PKA activity in HUVECs. A: cells were stimulated with or without gAd for 3 h before 10 ng/ml TNFα for the last 5 min of incubation. Where indicated, cells were treated with ddAdo or Rp-cAMP for 20 min before the addition of gAd; forskolin was added during the last 20 min of incubation. B: cells were treated with 25 mM glucose (HG) for 24 h. Before lysis, cells were stimulated where indicated with gAd for 3 h; cells were then treated with ddAdo or Rp-cAMP for 20 min before the addition of 25 mM glucose. Cells were lysed, and PKA activity was assayed as described in MATERIALS AND METHODS. Data are expressed as means ± SE; n = 3. *P < 0.05 vs. control; #P < 0.05 vs. gAd and TNFα or high glucose conditions.

Fig. 8. Effect of TNFα, high glucose, gAd, and other agents on AMP kinase activity in HUVECs. A: cells were stimulated with or without gAd for 3 h before 10 ng/ml TNFα for the last 5 min of incubation. Where indicated, cells were treated with ddAdo or Rp-cAMP for 20 min before the addition of gAd; forskolin was added during the last 20 min of incubation. B: cells were treated with 25 mM glucose (HG) for 24 h. Before lysis, cells were stimulated where indicated with gAd for 3 h; cells were then treated with ddAdo or Rp-cAMP for 20 min before the addition of 25 mM glucose. Cells were lysed, and AMP kinase activity was assayed as described in MATERIALS AND METHODS. Data are expressed as means ± SE; n = 3. *P < 0.05 vs. control; #P < 0.05 vs. gAd and TNFα or high glucose conditions.
Adiponectin suppression of IkB kinase activation

RNAi-mediated knockdown of AMP kinase-α1 and PKA and effect on gAd suppression of IKKβ activity in HUVECs. With the use of siRNA transfection, the cellular mass of AMP kinase-α1 and the α-catalytic subunit of PKA were reduced by 89 and 86%, respectively (Fig. 9). Under these conditions, we tested the effect of reduction of these signaling kinases on the action of gAd in HUVECs treated with TNFα or high glucose (Fig. 10). After transfection with control siRNA, TNFα increased IKKβ activity by 2.4-fold, which was suppressed 27% by treatment with gAd. Following knockdown of PKA, TNFα increased IKKβ by 48%, but gAd suppression of this action of TNFα was completely blocked. After transfection for knockdown of AMP kinase-α1, TNFα stimulated IKKβ activity by 53%, and gAd significantly reduced the effect of TNFα by 30% (P < 0.05).

To test the effect of PKA and AMP kinase-α1 knockdown on gAd signaling in conditions of high glucose, an additional 24 h of treatment was necessary for the high glucose incubation after the 24-h incubation following siRNA transfection. Following the transfection with control siRNA, high glucose increased IKKβ activity by 2.2-fold over basal level; this activity was suppressed 77% by gAd treatment (Fig. 10B). Unfortunately, the prolonged culture conditions required for the high glucose-induced changes in IKKβ led to a significant increase in basal IKKβ activity following knockdown of PKA or AMP kinase-α1, by 43 or 63%, respectively. These effects were not observed after the shorter experimental duration of the TNFα stimulation experiments shown in Fig. 10A. Nevertheless, loss of PKA mass in high glucose conditions fully abrogated the gAd suppression of IKKβ activation, supporting an important role for PKA in this cellular effect of gAd. With loss of AMP kinase-α1 mass, the increase in basal IKKβ activity and poor stimulation by high glucose appear to reflect altered cellular viability, obviating use of this approach to test whether AMP kinase-α1 knockdown affects gAd suppression of IKKβ activation in high glucose conditions.

Discussion

This study provides new evidence that adiponectin suppresses inflammatory signal generation triggered by both TNFα and high glucose at the level of IKKβ enzyme activation in endothelial cells. In addition, we found that both AMP kinase and cAMP signaling play a role in the effects of adiponectin to block the rise in IKKβ activity induced by both TNFα and high glucose. Interestingly, activation of adenylyl cyclase suppressed the activation of IKKβ induced by TNFα but was relatively ineffective in opposing the effects of high glucose to activate IKKβ. Nevertheless, the cAMP pathway and AMP kinase signaling were both implicated in the effect of gAd on IKKβ activity induced by either TNFα or high glucose concentrations. These results are of interest, since they support an important role for adiponectin in anti-inflammatory signaling in the vasculature and also imply that multiple pathways are involved in the cellular effects of adiponectin.

Prior work in the vasculature has shown that adiponectin suppresses the characteristic pleiotropic proinflammatory activation response pattern in endothelial cells that includes stimulation of the NF-κB pathway, upregulation of cell adhesion molecules, and diminished endothelial NO generation and bioavailability (15, 18). We have also found that a major endothelial effect of adiponectin is the suppression of ROS generation in response to treatment with oxidized LDL or high

Fig. 9. siRNA-mediated knockdown of AMP kinase-α1 and PKA. HUVECs were cultured on 6-well plates and transfected with siRNA at 50% confluency. At 24 h posttransfection, aliquots of 25 μg of cell lysate protein were used for immunoblot analysis for AMP kinase-α1 and PKA as described in MATERIALS AND METHODS.

Fig. 10. Effect of knockdown of PKA and AMP kinase-α1 on TNFα- and high-glucose- and adiponectin (gAd)-mediated IKKβ activity in HUVECs. siRNA-mediated knockdown of PKA and AMP kinase-α1 in HUVECs is described in MATERIALS AND METHODS. At 24 h posttransfection, cells were pretreated with gAd for 3 h and then treated with 10 ng/ml TNFα for 5 min (A) and 25 mM glucose for 24 h (B). Cells were lysed, and IKKβ activity was assayed using IKK peptide as substrate. Data are expressed as means ± SE; n = 3. *P < 0.05 vs. control; #P < 0.05 vs. TNFα or high glucose conditions.
glucose conditions (19, 23). However, there is controversy as to whether the NF-κB cascade is activated by ROS, especially at the level of IKKβ, since its enzyme activity has been shown to be oxidatively inhibited by H₂O₂ (14). Thus the mechanism of adiponectin suppression of inflammatory NF-κB signaling has not been well characterized.

The effects of adiponectin in metabolically responsive liver, skeletal muscle, and adipose cells to enhance insulin action are closely integrated with the pleiotropic enzyme AMP kinase (10, 27, 30). AMP kinase is also activated by adiponectin in endothelial cells and has been shown to be involved in adiponectin enhancement of endothelial NO availability (3, 13, 22). In addition, adiponectin signaling to NO generation was shown to be linked, at least in part, to phosphorylation of a-kinase activation (3) and formation of a complex involving endothelial nitric oxide synthase, heat shock protein-90, and Akt (31). Adiponectin effects on angiogenesis were also found to be dependent on adiponectin-stimulated phosphorylation of both AMP kinase and Akt (22). AMP kinase appears to be upstream of Akt, since disruption of AMP kinase activation inhibited adiponectin-induced Akt phosphorylation (22).

In addition to AMP kinase, evidence has been accumulating to support an important role for a cAMP/PKA-linked pathway in adiponectin endothelial signaling. Ouchi et al. (21) initially reported that the inhibitory effect of adiponectin on TNFα signaling in endothelial cells was accompanied by cAMP accumulation and blocked by an inhibitor of either adenylate cyclase or PKA. The inhibitory effect of adiponectin on TNFα-induced IL-8 synthesis in endothelial cells was shown to be associated with increased intracellular cAMP levels and PKA activity and blocked by PKA inhibition (12).

In our studies of adiponectin suppression of ROS generation induced by high glucose in endothelial cells, adiponectin increased cellular cAMP content, and inhibition of PKA blocked the antioxidant effect of adiponectin (23). Increasing endothelial cell cAMP with forskolin or dibutylryl cAMP also suppressed glucose-induced ROS production. In murine peritoneal macrophages, adiponectin was recently shown to increase cAMP and PKA activity and reduce leptin-induced TNFα production by blocking ERK1/2 and p38 MAPK phosphorylation (32). Thus the cAMP/PKA pathway is a major signaling pathway that appears to mediate at least some of the beneficial actions of adiponectin to counter the adverse effects of TNFα or high glucose in endothelial and potentially other vascular or circulating cell types.

Additional work will be necessary to help define the regulation of the upstream mechanisms of inflammatory endothelial signaling via IKK activation, which clearly plays a pivotal role in this process. Recent studies have provided new insight into mechanisms of TNFα-stimulated IKK activation by protein interactions including ubiquitination of receptor interacting protein-1 and polyubiquitin binding by NF-κB essential modulator (5, 16, 29). To date, the mechanism by which high glucose mediates activation of IKK remains poorly understood (11). It will be of interest to determine how common signaling pathways modulated by adiponectin affect the activation of IKKβ by the divergent upstream mediators TNFα and high glucose.

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