Adiponectin suppresses IkB 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 IkB 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 IkB kinase (IKK) and reduce the abundance of its substrate, inhibitor of κB (IkB)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 IkBα 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 transcriptional 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 κB (IkB) inhibitory proteins, which, on cellular stimulation, are rapidly phosphorylated on serine, ubiquitinated, and degraded in the proteosome, releasing NF-κB to function as a nuclear transcription factor (1, 25). Cytokines activate NF-κB by inducing IkB phosphorylation via IkB 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 monocytic THP-1 cells to cultured endothelial cells (20). Adiponectin has also been shown by two groups 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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MATERIALS AND METHODS

Materials. The pTREHisA vector and Escherichia coli TOP10 strain cells were obtained from Invitrogen (Carlsbad, CA). Acticlean 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 Applications (San Diego, CA), and endothelial basal medium-2 (EBM-2) and growth factors were obtained from Cambrex BioScience (Walkersville, MD). The AMP kinase inhibitor compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine) was kindly provided by Merck Research Laboratories (Rahway, NJ). Glutathione S-transferase (GST)-IkB protein was from Santa Cruz Biotechnology (Santa Cruz, CA). IKKβ and IkBα rabbit polyclonal antibody and antibody to AMP kinase (α1 + α2) and PKA(α-c) rabbit polyclonal antibody were from Cell Signaling Technology (Danvers, MA). [γ-32P]ATP and the cAMP Biotrak enzyme immunoassay (EIA) 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 control 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-Aminoisoxazole-4-carboxamide-1-β-d-ribofuranoside (AICAR), Rp-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMP), 2′,3′-dideoxyadenosine (ddAdo), and other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Recombinant adiponectin protein. The recombinant globular domain of human adiponectin was subcloned into the pTREHisA bacterial expression vector and expressed as an NH2-terminal (his)–tagged fusion protein in E. coli TOP10 strain by induction with isopropyl-β-thiogalactopyranoside. The protein was purified under native conditions and was applied to an Acticlean Etox column (Sterogene Bioseparations) to remove endotoxin contamination, as we described previously (30).

Cell culture and treatment. HUVECs before passage 4 were cultured 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 serum-free medium (SFM) with 5 mmol/l glucose and 1% BSA, with no growth factor supplement for 3 h. Cells were treated at 37°C with 2% FBS and the indicated concentration of globular adiponectin 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 IKK kinase assay using GST-IkBα protein as substrate. Kinase assays were performed as previously described (6, 14) using substrate protein according to the reagent manufacturer’s instructions with minor modifications. Briefly, cells were lysed with ice-cold deoxygenated 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 dithiothreitol, 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-mercaptoethanol, 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 magnesium/ATP cocktail at 30°C for 30 min. The reaction was ended by addition of 4× Laemml 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% methanol, stained in Bio-Safe Coomassie Stain solution, and dried on Whatman 3MM paper at 60°C for 2 h. The dried gel was covered by X-ray film in a dark room and exposed at −70°C for 1–2 days. Radioactive signal was quantified on an ImageStation 440CF (Kodak, Rochester, NY).

Immunoprecipitation and IKK kinase assay using IKK 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 (KKKKERLLDDRHDSGLDSMKDEE) 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 unlabeled 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.

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

Measurement of cellular cAMP content. HUVECs were cultured on 24-well plates and were treated when cells reached 80–90% confluency. 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 manufacturer’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/ADB 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

AMP kinase-/H9251/duplex oligonucleotides were based on the human cDNAs encoding (HUVECs) using IKK substrate peptide. HUVECs were cultured as described (vol/vol) phosphoric acid and once with acetone. The P81 square

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Fig. 1. Effect of adiponectin (globular domain 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 μg/ml gAd for 3 h, cells were treated with TNFα 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.

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REPORT

Results

IKKβ substrate peptide phosphorylation. TNFα activates the proinflammatory IKKβ/NF-κB signaling pathway in various cell types and contributes to insulin resistance and endothelial dysfunction. In the present work, we focused on the stimulation of IKKβ enzyme activity by TNFα or high glucose in cultured HUVECs and the potential effect of the recombinant gAd to suppress this IKKβ activation. IKKβ activity was measured by two independent assay methods. Initially, cell lysates were absorbed by a specific IKKβ antibody followed by a kinase assay using [32P]ATP and a specific peptide substrate (Fig. 1). Treatment with gAd (3 μg/ml for 3 h) did not significantly affect basal IKKβ activity, whereas treatment with 5 or 15 ng/ml TNFα for 5 min increased IKKβ activity 2.0- and 2.5-fold, respectively (P < 0.001). Prior cell treatment with gAd for 3 h completely inhibited the subsequent activation of IKKβ by stimulation with 5 ng/ml TNFα and significantly reduced the effect of 15 ng/ml TNFα by 44% (P < 0.001).

Phosphorylation of IκBα fusion protein by IKKβ. These results were confirmed with an independent assay method for IKKβ that used a GST fusion protein containing IκBα as a phosphorylation substrate for IKKβ kinase activity (Fig. 2). In these studies, 5 min of treatment with TNFα (7.5 ng/ml) significantly increased GST-IκB phosphorylation in the HUVEC lysates by 1.6-fold (P < 0.05). As in the assay using the peptide substrate, prior treatment with 3 μg/ml gAd for 3 h completely suppressed the TNFα-induced activation of GST-IκB phosphorylation by IKKβ.

Statistical analyses. Quantitative data are presented as means ± SE for three to five experiments. Statistical analysis was based on Student’s t-test for comparison of two groups. A P value <0.05 was used to determine statistical significance.
Cellular IkBα protein mass assay. Since activation of IKKβ leads to the phosphorylation of its cellular substrate protein IkBα followed by its ubiquitination and proteolytic degradation, we also measured the effect of gAd on cellular IkBα protein levels as a functional confirmation of changes in IKKβ activity that we observed using the above kinase assays. The loss of IkBα mass following TNFα stimulation was rapid. A dose-response experiment showed that TNFα concentrations as low as 7.5 ng/ml reduced IkBα 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 IkBα mass in HUVECs by an average of 62%. Prior cell treatment with gAd (3 μg/ml for 3 h) did not affect basal IkBα abundance; however, gAd fully abrogated the reduction in IkBα mass induced by TNFα.

Signaling mechanisms of IKKβ suppression by adiponectin. Adiponectin effects in endothelial cells have been reported to involve several signaling pathways, including AMP kinase and cAMP/PKA. To test the potential involvement of these pathways in adiponectin suppression of TNFα-induced IKKβ activation, we incubated cells with appropriate pharmacological inhibitors before treatment with gAd and TNFα (Fig. 4). In these studies, TNFα alone (10 ng/ml for 5 min) increased IKKβ activity 2.4-fold (P < 0.001). This response was completely suppressed by gAd (P = 0.08 vs. control). Interestingly, activation of adenyl cyclase with forskolin completely suppressed the activation of IKKβ by TNFα, indicating that cAMP is a potential effector of the action of gAd on IKKβ activation. Treatment with ddAdo, an inhibitor of adenyl cyclase, also partially inhibited the effect of gAd to suppress IKKβ activation by TNFα, by 35% (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...
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.
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). The effect of gAd on PKA activity was mimicked by adenylyl cyclase activation with forskolin and abrogated by inhibition of adenylyl cyclase with ddAdo or inhibition of PKA with Rp-cAMP.

AMP kinase activity is increased in HUVECs by gAd under TNF\( \alpha \) stimulation or in high glucose conditions. We also measured the activity of cellular AMP kinase \( \alpha 1 + \alpha 2 \) isoforms using a radiolabeled peptide substrate assay in immuno-precipitates following gAd stimulation in cells also treated by TNF\( \alpha \) or high glucose (Fig. 8). TNF\( \alpha \) itself did not alter cellular AMP kinase activity, which was significantly increased 48 and 58% by gAd treatment without and with stimulation by TNF\( \alpha \), respectively (both \( P < 0.05 \); Fig. 8A). Treatment with the direct AMP kinase inhibitor compound C significantly reduced the gAd stimulation of AMP kinase by 71% under conditions of TNF\( \alpha \) 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 significantly reduced by 71% under conditions of TNF\( \alpha \) stimulation. The effect of gAd on AMP kinase activity was also mimicked by the AMP kinase activator AICAR.

![Fig. 7. Effect of TNF\( \alpha \), 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 TNF\( \alpha \) 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 TNF\( \alpha \) or high glucose conditions.

![Fig. 8. Effect of TNF\( \alpha \), high glucose, gAd, and other agents on AMP kinase \( \alpha 1 + \alpha 2 \) activity in HUVECs. A: cells were stimulated with or without gAd for 3 h before 10 ng/ml TNF\( \alpha \) 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 \( \alpha 1 + \alpha 2 \) 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 TNF\( \alpha \) or high glucose conditions.
TNFα on AMP kinase activity was also mimicked by the AMP kinase activator AICAR and completely abrogated by inhibition with compound C.

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 adenylate 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 glucose to activate IKKβ.

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-, 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 made quiescent 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.
Recent studies have provided new insight into the effects of adiponectin in metabolic diseases. In this process, adiponectin signaling to NO generation was shown to be linked at least in part, to phosphatidylinositol 3'-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 dibutyryl 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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