

Adiponectin inhibits palmitate-induced apoptosis through suppression of reactive oxygen species in endothelial cells: involvement of cAMP/protein kinase A and AMP-activated protein kinase

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Abstract

The present study examined whether adiponectin can inhibit palmitate-induced apoptosis, and also the associated mechanisms and signal transduction pathways in human umbilical vein endothelial cells. Cells treated with 500 μ M palmitate for 48 h increased reactive oxygen species (ROS) generation and induced apoptosis. Treatment with antioxidant *N*-acetyl-L-cysteine (1 mM) and globular adiponectin (5 μ g/ml) inhibited palmitate-induced ROS generation and apoptosis. The AMP-activated protein kinase (AMPK) activator 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR; 1 mM), and cAMP activators forskolin (10 μ M) and cholera toxin (200 ng/ml) also displayed the same effects. The inhibitory effects of adiponectin on ROS generation and apoptosis were reversed by the AMPK

inhibitor compound C (40 μ M), cAMP inhibitor SQ22536 (50 μ M), and protein kinase A (PKA) inhibitor H-89 (10 μ M). The inhibitory effect of forskolin on palmitate-induced apoptosis was reversed by compound C, whereas the inhibitory effect of AICAR was not reversed by SQ22536 and H-89. AICAR and forskolin could not inhibit palmitate-induced apoptosis in cells treated with dominant-negative AMPK. Forskolin increased phosphorylated AMPK at both Thr-172 and Ser-485/491. These results suggest that adiponectin inhibits palmitate-induced apoptosis by suppression of ROS generation via both the cAMP/PKA and AMPK pathways. Interaction between cAMP/PKA and AMPK pathways may be involved.

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Introduction

Adiponectin is an abundant adipocyte-derived plasma protein that exerts beneficial effects on insulin sensitivity and vascular function (Matsuzawa 2005). Plasma adiponectin levels are decreased in obese/diabetic rodents and humans (Haluzik *et al.* 2004), and adiponectin knockout mice fed a high-fat diet exhibit severe insulin resistance (Maeda *et al.* 2002). Low adiponectin levels are also associated with vascular dysfunction (Tan *et al.* 2004), and adiponectin treatments are beneficial for atherosclerosis and endothelial cell dysfunction (Ouchi *et al.* 2001, Arita *et al.* 2002). The anti-atherosclerotic effect of adiponectin involves suppression of the proliferation of smooth muscle cells (Arita *et al.* 2002), inhibition of macrophage transformation to foam cells (Ouchi *et al.* 2001), and reduced inflammatory responses in endothelial cells (Kougias *et al.* 2005). The beneficial effects of adiponectin on endothelial cell function are mediated by an increase in

nitric oxide synthesis and anti-apoptotic effect (Chen *et al.* 2003, Goldstein *et al.* 2008).

Apoptosis is a programmed cell death pathway that is essential for tissue development and homeostasis, and is involved in down-regulating cell growth (Fulda & Debatin 2006). Substantial *in vivo* and *in vitro* evidence indicate that apoptosis plays an important role in the pathophysiology of vascular dysfunction induced by various causes. Increased endothelial cell apoptosis can lead to vascular leak and exposure of a thrombogenic subendothelial matrix (Winn & Harlan 2005). Furthermore, as apoptotic endothelial cells become proadhesive for procoagulants, they can promote coagulation (Winn & Harlan 2005).

Elevated plasma-free fatty acid level is a common feature of both poorly controlled type 1 and type 2 diabetes (Shulman 2000, Boden & Shulman 2002), and is associated with obesity and metabolic syndrome (Ruderman & Saha 2006). One of the most common dietary fatty acids is palmitate (de Vries *et al.* 1997, Kong & Rabkin 2000),

a 16-carbon saturated fatty acid that induces apoptosis in many cell types including endothelial cells (Kim *et al.* 2008c).

The present study examined whether adiponectin inhibits palmitate-induced apoptosis in human umbilical vein endothelial cells (HUVECs). Furthermore, the mechanisms and signal transduction pathways in the adiponectin-mediated inhibition of apoptosis by palmitate were examined.

Materials and Methods

Materials

Globular adiponectin was purchased from Phoenix Pharmaceuticals (Phoenix, AZ, USA). Antibodies for the phosphorylated AMP-activated kinase (pAMPK), AMPK, phosphorylated extracellular-regulated kinase (pERK), ERK, phosphorylated-p38 (p-p38), p38, phosphorylated N-Jun N-terminal kinase (pJNK), and JNK were obtained from Cell Signaling Technology (Danvers, MA, USA).

The cAMP activators cholera toxin (CTx) and forskolin, and cAMP inhibitor SQ22536 were purchased from Biomol International (Plymouth Meeting, PA, USA), and palmitic acid, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide thiazolyl blue (MTT), and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich. The AMPK activator AICAR was purchased from Toronto Research Chemicals (North York, ON, Canada), and the AMPK inhibitor compound C, JNK inhibitor, p38 inhibitor SB203580, and ERK inhibitor PD98059 were from Calbiochem (San Diego, CA, USA). Protein kinase A (PKA) inhibitor H-89 was from Stressgen (Victoria, BC, Canada), collagenase type II was from Worthington Biochemical Corporation (Freehold, NJ, USA), and enhanced chemiluminescence (ECL) reagent was from Amersham Biosciences.

HUVECs culture

HUVECs were isolated from human umbilical cords collected from normal deliveries at Yeungnam University. A signed consent was obtained, and the study was approved by the Institutional Review Board of Yeungnam University Medical Center. Vein in cord was washed with prewarmed PBS using a 50 ml syringe and then filled with Hanks' balanced salt solution (HBSS) containing 0.2% collagenase type II. After 15 min, HBSS was collected into a tube and centrifuged at 700 *g* for 5 min. Isolated cells were planted in 100 mm diameter culture dishes coated with 0.1% gelatin (Sigma-Aldrich), and cultured in M199 containing 10% fetal bovine serum and 1% antibiotics. The cells were grown at 37 °C in a humidified atmosphere with 5% CO₂. The cells planted on the culture dish were regarded as passage 0, and the cells from passages 3 to 4 were used.

Cell viability

The MTT assay was conducted as described previously (Kim *et al.* 2008b). Briefly, the MTT was dissolved in PBS at a concentration of 5 mg/ml and sterilized by passage through a 0.22 µm filter. Cells were seeded in wells of a 24-well plate containing 250 µl of the culture medium, and 25 µl MTT stock solution were then added to each well. After incubation for 4 h at 36.5 °C, 300 µl of a dimethyl sulfoxide solution were added to all of the wells and mixed thoroughly to lyse the cells and dissolve the dark blue crystals. After 5 min, 100 µl of the lysis solution were transferred to a well of a 96-well plate, and the absorbance was read on a microplate reader at a wavelength of 550 nm.

Cell apoptosis

Cell apoptosis was assessed by caspase-3 activity and annexin V staining. Caspase-3 activity was measured using a Caspase-3 Colorimetric Assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. This method is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp *p*-nitroanilide by caspase-3, resulting in the release of the *p*-nitroaniline moiety. The production of *p*-nitroaniline was measured at 405 nm using a plate reader. Annexin V staining was measured using a flow cytometer according to the manufacturer's instructions (BD Bioscience, San Jose, CA, USA). Briefly, the cells in a 6-well plate were digested with 0.25% trypsin and then collected by centrifugation. The cells were washed twice with cold PBS and mixed with a 1× binding buffer. The cells (1×10⁵ cells/100 µl binding buffer) were transferred to a tube and then 5 µl annexin V-FITC containing 0.01 M HEPES pH 7.4, 0.14 M NaCl, and 2.5 mM CaCl were added. The mixture was incubated for 15 min at room temperature in the dark. After the addition of 400 µl binding buffer, the level of annexin V-FITC conjugation was detected using the FL1 setting of the FACSCalibur (BD Bioscience).

Reactive oxygen species generation

The generation of reactive oxygen species (ROS) was measured using flow cytometry and live-cell microscopy. For flow cytometry, the cells (3×10⁵ cells/well) were incubated in 10 µM carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFHDA; Molecular Probes, Eugene, OR, USA) at 37 °C for 20 min, harvested by trypsinization, and washed twice with cold PBS. Cells conjugated with carboxy-H₂DCFHDA-FITC (excitation, 494; emission, 524 nm) were detected by using the FL1 setting of the FACSCalibur (BD Bioscience). For live-cell microscopy, the cells were washed two times with PBS and were incubated in sodium acetate-free medium containing 10 µM peroxide-sensitive fluorescent dye H₂DCFHDA (Molecular Probes) for 1 h in the dark. After the incubation medium was aspirated, the cells were washed with PBS and

placed in medium without sodium acetate. Intracellular fluorescence was monitored using a temperature-regulated (37 °C), live-cell microscope (Leika, Wetzlar, Germany). Cells treated with 1 mM hydrogen peroxide (H₂O₂) for 1 h were used as a positive control.

cAMP assays

The cells (2 × 10⁵ cells/well) were seeded in wells of a 24-well plate. After experimental treatment, the incubation medium was aspirated. The cells were lysed by 200 µl 0.1 M HCl containing 0.1% Triton X-100, and the lysis solution was transferred into microcentrifuge tubes. After centrifugation at 1300 g for 5 min at room temperature, cellular cAMP concentrations in the supernatants were determined with a direct enzyme immunoassay kit (Amersham Biosciences) as described by the manufacturer.

Western blotting

The cells were harvested by trypsinization, washed in PBS, and resuspended in a lysis buffer (Invitrogen) containing 1% NP40, 150 mM NaCl, 5 mM MgCl, 10 mM HEPES buffer, leupeptin, and pepstatin A. Protein concentration was determined by the Bradford method (Bio-Rad Laboratories). A 30 µg sample of the total protein per lane was separated by 10% SDS-PAGE. The separated proteins were then transferred to a 0.45 µm polyvinylidene fluoride membrane (Gelman Sciences, Ann Arbor, MI, USA). After blocking with a solution containing 5% skim milk/10 mM Tris-HCl, pH 7.4/150 mM NaCl/0.1% Tween 20, the membrane was incubated overnight at 4 °C with a 1:1000 dilution of the primary antibody. Specific antibody binding was detected using a 1:2000 dilution of sheep anti-rabbit IgG HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature and visualized using an ECL detection reagent (Amersham Biosciences).

Transfection of dominant-negative AMPK $\alpha 1$ and $\alpha 2$

Plasmids encoding c-Myc-tagged forms of dominant-negative AMPK (DN-AMPK) $\alpha 1$ and $\alpha 2$ mixture were provided by Dr J Ha (Department of Molecular Biology, Kyung Hee University College of Medicine, Seoul, South Korea). Subconfluent HUVECs were incubated with adenoviruses expressing β -galactosidase or DN-AMPK (Ad-DN-AMPK) at a concentration of 100 plaque-forming units per cell for 1 h at 37 °C in DMEM without serum as described previously (Lee *et al.* 2003, 2005, Kim *et al.* 2008a).

Statistical analysis

The results are expressed as the mean \pm s.e.m. The difference among groups was analyzed by ANOVA with a *post-hoc* analysis by a Duncan's multiple test. A *P* value < 0.05 was considered significant.

Results

Palmitate induces apoptosis through ROS

Palmitate at 250 and 500 µM decreased cell viability by 12 and 35% respectively over 48 h (Fig. 1A). Palmitate treatment for 48 h increased cell apoptosis in a dose-dependent manner as measured by caspase-3 activity (1.3- and 2.5-fold of the control at 250 and 500 µM palmitate respectively; Fig. 1B). Annexin V staining also revealed that palmitate induced apoptosis in HUVECs (Fig. 1C).

Next, we tested whether ROS was involved in palmitate-induced apoptosis. Treatment of cells with 500 µM palmitate or 1 mM H₂O₂ increased ROS generation as determined by live-cell microscopy and flow cytometry analysis (Fig. 1D and E). Pretreatment of cells with 1 mM of antioxidant NAC significantly blocked palmitate-induced cell apoptosis as measured by caspase-3 activity and annexin V staining (Fig. 2A and B), and blocked ROS generation by palmitate (Fig. 2C and D). These results suggest that palmitate induces apoptosis through the ROS generation in HUVECs.

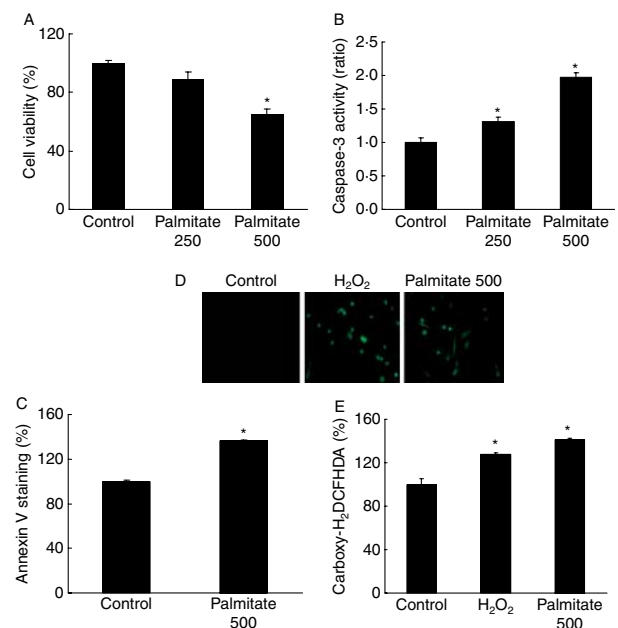


Figure 1 The effects of palmitate on cell survival, apoptosis, and ROS generation in human umbilical vein endothelial cells. Cells were treated with 250 or 500 µM palmitate for 48 h, and cell survival was determined by a MTT assay (A). Cells were treated with 250 or 500 µM palmitate for 48 h, and cell apoptosis was determined by caspase-3 activity (B) and annexin V staining (C). Cells were treated with 500 µM palmitate for 18 h, and ROS was detected using H₂DCFHDA under microscope. Cells treated with 1 mM H₂O₂ were used as a positive control (D). Cells were treated with 500 µM palmitate for 6 h, and ROS was detected using carboxy-H₂DCFHDA with flow cytometry analysis. Cells treated with 1 mM H₂O₂ were used as a positive control (E). The results are reported as the mean \pm s.e.m. of three to five separate experiments. In each experiment, at least three samples were used. **P* < 0.05 versus control.

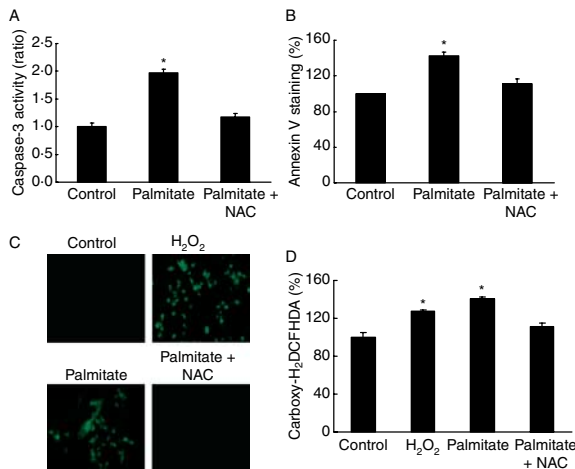


Figure 2 The effects of antioxidant *N*-acetyl-L-cysteine (NAC) on palmitate-induced ROS generation and apoptosis in human umbilical vein endothelial cells. Cells were pretreated with 1 mM NAC for 1 h before exposure to 500 μ M palmitate for 48 h, and then apoptosis was determined by caspase-3 activity (A) and annexin-V staining (B). Cells were pretreated with NAC for 30 min before exposure to 500 μ M palmitate for 18 h, and ROS was detected using H₂DCFHDA under microscope. Cells treated with 1 mM H₂O₂ were used as a positive control (C). Cells were pretreated with NAC for 30 min before exposure to 500 μ M palmitate for 6 h, and ROS was detected using carboxy-H₂DCFHDA with flow cytometry analysis. Cells treated with 1 mM H₂O₂ were used as a positive control (D). The results are reported as the mean \pm S.E.M. of three to five separate experiments. In each experiment, at least three samples were used. * P <0.05 versus control.

Adiponectin inhibits palmitate-induced apoptosis through suppression of ROS

The effect of adiponectin on palmitate-induced ROS generation and apoptosis was determined in HUVECs. Adiponectin exists in the circulation as a full-length protein and a putative proteolytic cleavage fragment consisting of the globular C-terminal domain that is pharmacologically active (Han *et al.* 2007). In this study, globular adiponectin was used at a concentration of 5 μ g/ml (Motoshima *et al.* 2004). Treatment of cells with 500 μ M palmitate increased apoptosis by 2-fold, and 5 μ g/ml globular adiponectin blocked palmitate-induced apoptosis as determined by caspase-3 activity (Fig. 3A). Apoptosis determined by annexin V staining also showed the same result (Fig. 3B). Pretreatment of cells with 5 μ g/ml adiponectin significantly inhibited the production of ROS as determined using live-cell microscopy (Fig. 3C) and flow cytometry analysis (Fig. 3D). These results suggest that adiponectin inhibits palmitate-induced apoptosis through the suppression of ROS generation.

AMPK is involved in adiponectin-mediated suppression of ROS and apoptosis

The mechanisms of the suppressive effects of adiponectin on palmitate-induced apoptosis were investigated. First, the involvement of AMPK pathway in the adiponectin-mediated

effect was determined, since adiponectin is known to activate this pathway. Treatment of cells with adiponectin for 1 h increased pAMPK (Fig. 4A) suggesting that adiponectin increases the activity of AMPK in HUVECs. Adiponectin and the AMPK activator AICAR (1 mM) inhibited palmitate-induced apoptosis, and the suppressive effect of adiponectin on apoptosis was blocked by the AMPK inhibitor compound C (40 μ M) as determined by caspase-3 activity and annexin V staining (Fig. 4B and C). The suppressive effect of adiponectin on ROS generation was also blocked by compound C as determined by live-cell microscopy and flow cytometry analysis (Fig. 4D and E). These results indicate that adiponectin can suppress palmitate-induced ROS generation and apoptosis through the AMPK pathway.

cAMP/PKA is involved in adiponectin-mediated suppression of ROS and apoptosis

Adiponectin is also known to activate cAMP/PKA pathway, and the involvement of this pathway on the inhibitory effect of adiponectin on palmitate-induced apoptosis was investigated. First, the activation of intracellular cAMP by adiponectin was determined. cAMP levels were increased

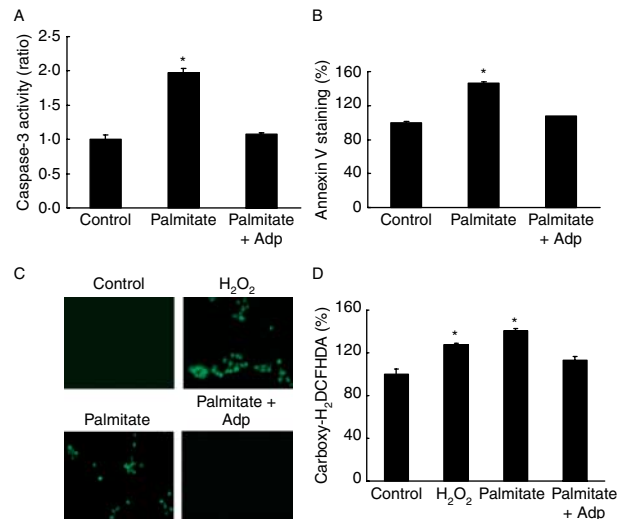


Figure 3 The effects of adiponectin on palmitate-induced ROS generation and apoptosis in human umbilical vein endothelial cells. Cells were pretreated with 5 μ g/ml adiponectin (adp) before exposure to 500 μ M palmitate for 48 h, and apoptosis was determined by caspase-3 activity (A) and annexin V staining (B). Cells were pretreated with 5 μ g/ml adiponectin for 30 min before exposure to 500 μ M palmitate for 18 h, and ROS was detected using H₂DCFHDA under microscope. Cells treated with 1 mM H₂O₂ were used as a positive control (C). Cells were pretreated with 5 μ g/ml adiponectin for 30 min before exposure to 500 μ M palmitate for 6 h, and ROS was detected using carboxy-H₂DCFHDA with flow cytometry analysis. Cells treated with 1 mM H₂O₂ were used as a positive control (D). The results are reported as the mean \pm S.E.M. of three to five separate experiments. In each experiment, at least three samples were used. * P <0.05 versus control.

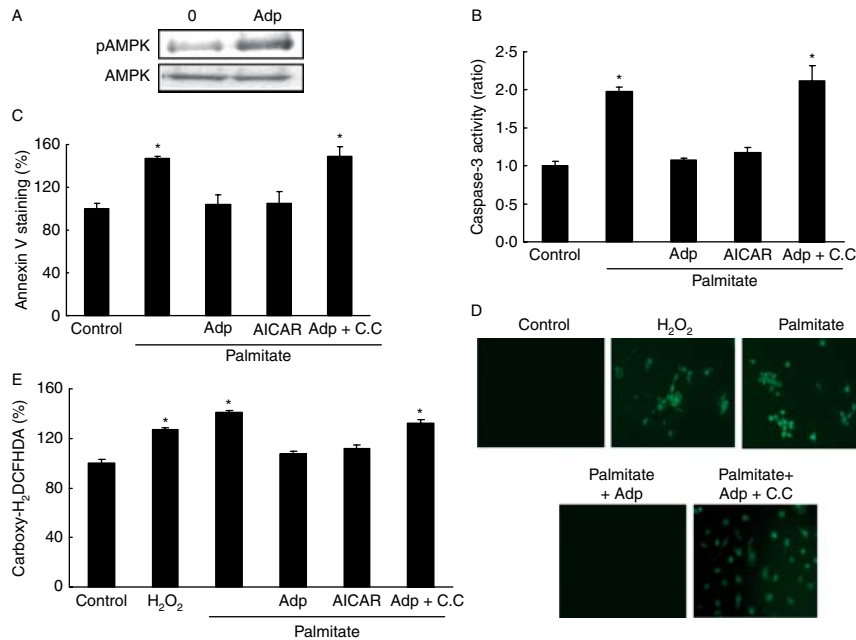


Figure 4 Involvement of AMPK pathway in the inhibitory effects of adiponectin on palmitate-induced ROS generation and apoptosis in human umbilical vein endothelial cells. Phosphorylation of AMPK at Thr-172 was measured in cells treated with 5 µg/ml adiponectin (adp) for 1 h by western blotting (A). Cells were treated with AMPK inhibitor compound C (C.C; 40 µM) for 1 h, and then with adiponectin (5 µg/ml) or AMPK activator AICAR (1 mM) for 1 h before exposure to 500 µM palmitate for 48 h. Cell apoptosis was determined by caspase-3 activity (B) and annexin V staining (C). Cells were treated with AMPK inhibitor compound C (C.C; 40 µM) for 30 min, and then with adiponectin (5 µg/ml) for 30 min before exposure to 500 µM palmitate for 18 h. ROS was detected using H₂DCFHDA under microscope. Cells treated with 1 mM H₂O₂ were used as a positive control (D). ROS was detected using carboxy-H₂DCFHDA with flow cytometry analysis under the same experimental conditions as in (D) except for the incubation time of palmitate (6 h). Cells treated with 1 mM H₂O₂ were used as a positive control (E). The results are reported as the mean ± S.E.M. of three to five separate experiments. In each experiment, at least three samples were used. **P* < 0.05 versus control.

by adiponectin in a time-dependent manner. The level of cAMP increased at 12 and 24 h by adiponectin was comparable to that of cAMP activated by CTx (200 ng/ml) and forskolin (10 µM; Fig. 5A). These results support the view that adiponectin functions as a cAMP activator. Treatment with 200 ng/ml CTx and 10 µM forskolin completely blocked palmitate-induced apoptosis as measured by caspase-3 activity and annexin V staining (Fig. 5B and C). The cAMP inhibitor SQ22536 (50 µM) and PKA inhibitor H-89 (10 µM) reversed the suppressive effect of adiponectin on palmitate-induced apoptosis (Fig. 5B and C). Treatment of cells with adiponectin and forskolin reduced ROS generation by palmitate as assessed using live-cell microscopy and flow cytometry analysis (Fig. 5C). H-89 reversed the suppressive effect of adiponectin on palmitate-induced ROS generation (Fig. 5D). These results support the suggestion that cAMP/PKA pathway is involved in the suppressive effect of adiponectin on palmitate-induced ROS generation and apoptosis.

Crosstalk between cAMP/PKA and AMPK pathways

Crosstalk between cAMP/PKA and AMPK pathways was investigated, since both of these pathways mediate the inhibitory effect of adiponectin on palmitate-induced apoptosis. Pretreatment of cells with the AMPK inhibitor compound C blocked the inhibitory effect of forskolin on apoptosis as measured by caspase-3 activity and annexin V staining. In contrast, the cAMP inhibitor SQ22536 or PKA inhibitor H-89 did not block the inhibitory effect of AICAR on apoptosis (Fig. 6A and B). Furthermore, cells transfected with Ad-DN-AMPK blocked the inhibitory effect of both AICAR and forskolin on palmitate-induced apoptosis (Fig. 6C). Forskolin increased AMPK phosphorylation at Thr-172 and Ser-485/491. Forskolin-mediated activation of pAMPK at both the sites was blocked by the cAMP inhibitor SQ22536 or PKA inhibitor H-89 (Fig. 6D). These results suggest the possibility of crosstalk between cAMP/PKA and AMPK pathways.

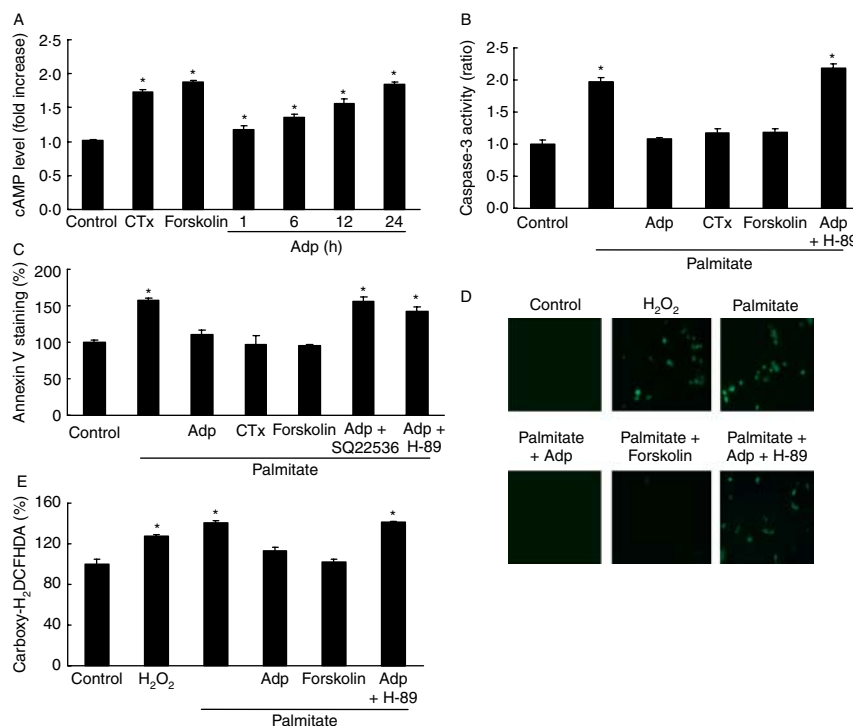


Figure 5 Involvement of cAMP/PKA pathway in the inhibitory effects of adiponectin on palmitate-induced ROS generation and apoptosis in human umbilical vein endothelial cells. Cells were treated with the cAMP activators cholera toxin (CTx, 200 ng/ml) and forskolin (10 μ M) for 1 h, and adiponectin (adp, 5 μ g/ml) for the indicated times. cAMP levels were determined by ELISA (A). Cells were treated with the cAMP inhibitor SQ22536 (50 μ M) or the PKA inhibitor H-89 (10 μ M) for 1 h and then with adiponectin (5 μ g/ml), cholera toxin (200 ng/ml), or forskolin (10 μ M) for 1 h before exposure to 500 μ M palmitate for 48 h. Cell apoptosis was determined by caspase-3 activity (B) and annexin V staining (C). Cells were treated with the PKA inhibitor H-89 (10 μ M) for 30 min and then with adiponectin (5 μ g/ml) or forskolin (10 μ M) before exposure to 500 μ M palmitate for 18 h. ROS was detected using H₂DCFHDA under microscope. Cells treated with 1 mM H₂O₂ were used as a positive control (D). ROS was detected using carboxy-H₂DCFHDA with flow cytometry analysis under same experimental conditions as in (D) except for the incubation time of palmitate (6 h). Cells treated with 1 mM H₂O₂ were used as a positive control (E). The results are reported as the mean \pm S.E.M. of three to five separate experiments. In each experiment, at least three samples were used. * P <0.05 versus control.

Discussion

The present study demonstrates that adiponectin inhibits palmitate-induced apoptosis through the suppression of ROS generation. This suggests that adiponectin acts as an endogenous antioxidant and an anti-apoptotic agent against palmitate in endothelial cells. Both AMPK and cAMP/PKA are involved as signaling pathways.

Palmitate induces apoptosis in diverse cell types such as endothelial cells (Yamaguchi *et al.* 2002), cardiomyocytes (Sparagna *et al.* 2000), pancreatic β -cells (Kwon *et al.* 2004), testicular Leydig cells (Lu *et al.* 2003), human granulosa cells (Mu *et al.* 2001), bovine retinal pericytes (Cacicedo *et al.* 2005), skeletal muscle myotubes (Turpin *et al.* 2006), and human osteoblast cells (Kim *et al.* 2008b).

Although the mechanism by which palmitate induces apoptosis is not completely understood, overproduction of ROS has been suggested as one of the possible causes in several cell types including endothelial cells (Yamagishi *et al.* 2002, Yao *et al.* 2005). In this study, we also showed that palmitate induced apoptosis through ROS generation in HUVECs. Despite recent studies concerning the role of ROS as a signaling molecule (Avshalumov *et al.* 2007), the imbalance between detoxifying antioxidant system and ROS generation leads to divergent pathological disorders (Stocker & Kearney 2004, Schulze & Lee 2005, Takimoto & Kass 2007). Accumulating evidence also indicates that ROS mediates endothelial cell apoptosis to many different stresses such as bacterial toxin, cytokine, and neuronal hormone (Deshpande *et al.* 2000, Suematsu *et al.* 2002, Sylte *et al.* 2004, Kuckelburg *et al.* 2008).

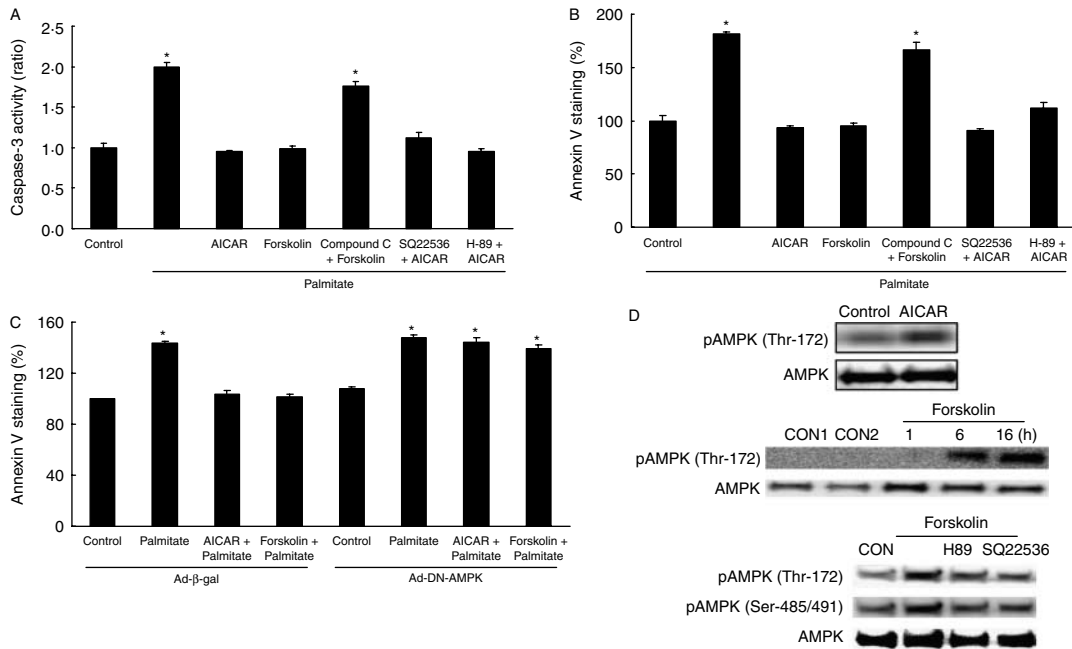


Figure 6 Crosstalk between AMPK and cAMP/PKA pathways in human umbilical vein endothelial cells. Cells were pretreated with compound C (40 μ M), SQ22536 (50 μ M), or H-89 (10 μ M) for 1 h before exposure to forskolin (10 μ M) or AICAR (1 mM). After 1 h, cells were treated with 500 μ M palmitate for 48 h. Apoptosis was determined by caspase-3 activity (A) and annexin V staining (B). Cells were transfected with 100 pfu/cell of adenovirus containing dominant-negative AMPK (Ad-DN-AMPK) or β -galactosidase (Ad- β -gal), and then pretreated with AICAR (1 mM) or forskolin (10 μ M) for 1 h before exposure to 500 μ M palmitate for 48 h (C). AICAR (1 mM) was treated in untransfected cells for 1 h, and forskolin (10 μ M) was treated for the indicated time. Phosphorylated AMPK at Thr-172 and Ser-485/491 was determined by western blotting (D). The results are reported as the mean \pm S.E.M. of three to five separate experiments. In each experiment, at least three samples were used. * $P < 0.05$ versus control.

The suppression of ROS generation by adiponectin is related to various beneficial effects. Adiponectin suppresses the ROS generation induced by hyperglycemia (Ouedraogo *et al.* 2006), oxidative low-density lipoprotein (Motoshima *et al.* 2004), 1-methyl-4-phenylpyridinium ion (Jung *et al.* 2006), acetaldehyde (Jung *et al.* 2006), and angiotensin II (Fujita *et al.* 2008), which leads to an anti-proliferative effect in endothelial cells (Motoshima *et al.* 2004), anti-apoptosis in a neuroblastoma cell line (Jung *et al.* 2006), and inhibits cardiac fibrosis (Fujita *et al.* 2008). Presently, adiponectin also suppressed the ROS production induced by palmitate, which resulted in increased endothelial cell survival. Endothelial cell apoptosis can induce vascular leakage and increase thrombogenesis, leading to vascular dysfunction (Winn & Harlan 2005). Therefore, an anti-apoptotic effect of adiponectin against palmitate could be one of the mechanisms of vascular protection.

The signal transduction pathway mediating the antioxidant effect of adiponectin is a topic of intense research, and AMPK and cAMP pathways have been reported as possible candidates. AMPK is a serine/threonine protein kinase, which was first identified as an energy sensor that responds to reduced energy state such as glucose deprivation, hypoxia, and ischemia (Hardie 2003). Once activated, it switches off

the ATP-consuming anabolic pathway and switches on the ATP-generating catabolic pathway (Hardie *et al.* 1998). Since its discovery, investigations into the effects of AMPK have shown that it also serves as an anti-inflammatory agent (Cacicedo *et al.* 2004, Ayasolla *et al.* 2005) and inhibits cell proliferation (Motoshima *et al.* 2006). Although AMPK increases oxidative stresses that lead to apoptosis in pancreatic β -cells (Cai *et al.* 2007, Kim *et al.* 2007), it plays an opposite role in endothelial cells. Activation of AMPK by the AMPK activator AICAR suppresses ROS generation (Kukidome *et al.* 2006). Moreover, AMPK functions as a signaling pathway of plasma hormones including adiponectin that activates AMPK in endothelial cells (Ouedraogo *et al.* 2006), cardiac fibroblast (Fujita *et al.* 2008), hepatic satellite cells (Adachi & Brenner 2008), skeletal muscle (Yamauchi *et al.* 2002), and vascular smooth muscle cells (Son *et al.* 2008). In the current study, adiponectin also increased AMPK activity in HUVECs, and the antioxidant and anti-apoptotic effects of adiponectin were reversed by AMPK inhibition. These results indicate that AMPK plays a role as a signaling molecule in the adiponectin-mediated beneficial effects.

Recently, besides AMPK, the cAMP signaling pathway was also involved in a variety of adiponectin-mediated beneficial effects. cAMP is a ubiquitous second messenger that mediates

the action of multiple hormones and neurotransmitters (Zaccolo *et al.* 2005), and PKA is considered the most important effector of cAMP (Taylor *et al.* 1992). Intracellular cAMP levels decrease under an oxygen free radical-generating system (Raynaud *et al.* 1997), and cAMP analogs display anti-apoptotic and antioxidant effects (Kim *et al.* 2002, Fujita *et al.* 2006). A substantial body of evidence suggests that adiponectin functions as an activator of cAMP in macrophages (Zhao *et al.* 2005) and endothelial cells (Ouedraogo *et al.* 2006, Chen *et al.* 2008). Moreover, adiponectin-mediated activation of cAMP exerts antioxidative effects as well as anti-inflammatory and anti-atherosclerotic effects (Ouchi *et al.* 2000, Zhao *et al.* 2005, Ouedraogo *et al.* 2006, Chen *et al.* 2008). In the current study, adiponectin activated cAMP level in HUVECs, and the beneficial effect of adiponectin was reversed by the inhibitors of cAMP and PKA suggesting that the cAMP/PKA pathway also mediates the antioxidant and anti-apoptotic effects of adiponectin.

Interestingly, we observed that both the AMPK and cAMP/PKA pathways were effective as adiponectin mediators. This result suggests the possibility of an interaction between these two pathways, and cAMP seems to activate AMPK. The involvement of multiple pathways mediating the effect of adiponectin has also been documented previously. Both the pathways are comparably active in the attenuated activation of I κ B kinase by tumor necrosis factor- α , whereas AMPK is substantially more effective than cAMP in suppressing high glucose-induced I κ B kinase activation in endothelial cells (Wu *et al.* 2007). Contrary to these results, the suppressive effect of adiponectin on high glucose-induced ROS generation can be effectively mediated through the cAMP/PKA-dependent pathway than via AMPK (Ouedraogo *et al.* 2006). However, these studies did not show the relation between these two pathways, although an interaction between cAMP/PKA and AMPK had previously been documented. Activation of cAMP by isoproterenol or forskolin or a cAMP analog increases AMPK activation in adipocytes, and the inhibition of AMPK significantly blocks cAMP-mediated lipolysis by isoproterenol (Yin *et al.* 2003). These results are consistent with our study showing that cAMP is an activator of AMPK activity. However, reduced AMPK activity by cAMP has also been documented. The activation of cAMP attenuates phosphorylation of Thr-172 leading to reduced AMPK activation in pancreatic INS-1 cells by phosphorylation of Ser-485/491 (Hurley *et al.* 2006). However, in our study, cAMP activation increased both the phosphorylation of Ser-485/491 and phosphorylation of Thr-172 suggesting that different effects of cAMP activation on the modulation of AMPK activity depend on the experimental setting and tissues. The mechanisms or signaling pathways responsible for connecting cAMP/PKA and AMPK pathways are currently unknown and require further study.

In conclusion, adiponectin suppresses palmitate-induced ROS generation and apoptosis. Even though AMPK pathway is involved directly, cAMP/PKA pathway also seems to contribute in the anti-apoptotic effect of adiponectin.

The suppression of cAMP-mediated antioxidant and anti-apoptotic effects by AMPK inhibition suggests a possible connection between these two pathways. Further studies are needed to ascertain the crosstalk between these pathways that could simplify adiponectin-mediated multiple and complicated signaling pathways.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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