Adiponectin protects palmitic acid induced endothelial inflammation and insulin resistance via regulating ROS/IKKβ pathways

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Endothelial inflammation and insulin resistance (IR) has been closely associated with endothelial dysfunction. Adiponectin (APN), an adipocyte-secreted hormone from adipose tissues, showed cardioprotective effects. Here, the protective effect of APN on palmitic acid (PA)-induced endothelial inflammation and IR was investigated. Cultured human umbilical vein endothelial cells (HUVECs) were treated with PA without or without APN pretreatment. The expression of inflammatory cytokines TNF-α, IL-6, adhesion molecule ICAM-1 were determined by western blotting, ELISA, and real-time PCR. The protein expression and protein-protein interaction were determined by western blotting and immunoprecipitation. The intracellular reactive oxygen species (ROS) and nitric oxide (NO) production were monitored with fluorescence probes. PA-induced secretion of TNF-α, IL-6, and expression of ICAM-1 at protein and mRNA levels, which was significantly inhibited by APN. PA treatment caused increase of ROS generation, NOX2, p-IKKα, p-IκBα, p-p65 expression, and p-IκBα-IKKβ interaction, which were all partly reversed by APN. ROS scavenger N-acetylcysteine (NAC) and NF-κB inhibitor PDTC showed similar effect on PA-induced secretion of TNF-α, IL-6, and expression of ICAM-1. Furthermore, APN and NAC pretreatment restored PA-induced increase of p-IRS-1(S307), decrease of p-IRS-1(Tyr). In addition, insulin-triggered expression of p-IRS-1(Tyr), p-PI3K, p-AKT, p-eNOS and NO generation were inhibited by PA, which were also restored by both APN and NAC. These results suggested that APN ameliorated endothelial inflammation and IR through ROS/IKKβ pathway. This study shed new insights into the mechanisms of APN’s cardiovascular protective effect.

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1. Introduction

Endothelial cells are sensitive to various nociceptive stimuli, including reactive oxygen species (ROS), endotoxin, and pro-inflammatory cytokines [1,2]. Endothelial inflammation has been implicated in a panel of cardiovascular and related diseases such as diabetes mellitus [3,4], and atherosclerosis [5]. Various exogenous and endogenous stimulus promotes endothelial inflammation by increasing the expression of inflammatory factors such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) [6,7]. Insulin resistance (IR), a hallmark of metabolic disorders including type 2 diabetes, is frequently associated with endothelial dysfunction [8]. Endothelial IR is the result of a prolonged and excessive inflammatory process in the vascular wall, which often begins with inflammatory changes in the endothelium. Inflammation impairs insulin-signaling PI3K/Akt and the downstream activation of nitric oxide synthase (eNOS) to reduce nitric oxide (NO) release. Reduced eNOS phosphorylation and decreased NO availability were considered as important characteristics of IR in endothelial cells [2,9]. It has been well established that oxidative stress resulted from overproduction of ROS played a key role in endothelial IR [10].

Adiponectin (APN) is an adipocyte-derived hormone with immunomodulatory, anti-inflammatory, cardio-protective, insulin-sensitizing, and anti-angiogenic effects [11,12]. Suppression of inflammatory cytokines including IL-6 and TNF-α by APN has been reported. APN inhibited lipopolysaccharide (LPS) stimulated TNF-α production mediated by transcriptional and post-transcriptional mechanisms in RAW 264.7 macrophages [13]. In a rat cerebral ischemia-reperfusion injury model, APN significantly inhibited expression of TNF-α, interleukin (IL)-1beta, and IL-8 [14]. APN inhibited LPS-induced IL-6 production in 3T3-L1 adipocytes [15] but failed to do so in LPS treated periodontal ligament

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(PDL) and gingival fibroblasts [16]. Interestingly, APN could also induce IL-6 production in adult mouse cardiac fibroblasts [17]. Furthermore, the inhibitory effect of APN on TNF-α-induced ICAM-1 in endothelial cells has been reported [18].

Palmitic acid (PA), a major component of dietary saturated fat, consists of almost 20% of the total serum free fatty acids (FFA). High levels of palmitic acid was widely used to investigate FFA-induced endothelial inflammation and IR [19,20]. Herein, the protective effect and mechanisms of APN on endothelial inflammation and IR were investigated with a PA-induced endothelial model.

2. Materials and methods

2.1. Materials

PA, 3-(4,5)-dimethylthiaiazol(-z)-yl-3,5-di-phenyltetrazolium romide (MTT), N-acetyl cysteine (NAC), 5-(6)-carboxy-2',7'-dichlor odihydrofluorescein diacetate (DCFH2-DA), and ammonium pyroclidinedithiocarbamate (PDTC) were purchased from Sigma (St. Louis, MO, USA). Adiponectin was purchased from RayBiotech (Norcross, GA, USA, catalogue number: 228-10508-3). Primary antibodies for NOX1, NOX2, NOX4 were purchased from Santa Cruz Biotechnology (Beverly, MA, USA). Adiponectin was purchased from RayBiotech (Shiga, Japan). ELISA kits were purchased from Sangon Biotech (Shanghai, China). Primers and other materials for real-time PCR (RT-PCR) was obtained from Beyotime Institute of Biotechnology (Haimen, China).

2.2. Cell culture

HUVECs (Gibco, Life Technologies Corp. C-003-5C) from newborn were cultured in Ham’s F-12K (Kaighn’s) medium (Gibco, Life Technologies Corp.21127030) with endothelial cell growth supplement from bovine neural tissue (ECGS) from Sigma in a humidified atmosphere of 5% CO2. The cellular fluorescence was also observed by a fluorescence microscope.

2.3. MTT assay

Cells cultured in 96-well microplate were exposed to PA (1–500 μM) for 24 h and the cell viability was determined as our previous report [21].

2.4. RT-PCR

Confluent cells in six-well plates (5.0 × 10⁶/well) with or without APN (5 μg/mL) pretreatment for 1 h were incubated with PA for 24 h. The RNA was extracted with TRIzol Reagent. About 2 μg of total RNA was reverse-transcribed into cDNA using First Strand cDNA Synthesis Kit (Toyobo, Japan). RT-PCR was performed using SYBR Green PCR reagents (Applied Biosystems). The specific primers were as listed in Table 1.

2.5. Measurement of intracellular ROS production

Approximately 5.0 × 10⁶/well cells were seeded in six-well plate overnight. APN (5 μg/mL) or NAC (5 mM) were pretreated for 1 h followed by PA treatment for another 2 h. Then the cells were incubated with DCFH2-DA (10 μM) in the dark at 37 °C for 40 min. Cellular fluorescence for 10⁶ cells per sample was analyzed using flow cytometry (Becton Dickinson FACS CantoTM, USA) with wavelength of 488/525 nm.

The cellular fluorescence was also observed by a fluorescence microscope.

2.6. ELISA assay for TNF-α and IL-6

Cells were seeded in 24-well plates at a density of 2 × 10⁵ cells/well and pretreated with APN (5 μg/mL), NAC (5 mM) and PDTC (10 μM) for 1 h, and then incubated with PA (200 μM) for another 24 h. The medium was collected and centrifuged (3000 rpm, 4 °C). The levels of TNF-α and IL-6 in the supernatant were assayed with commercial ELISA Kits following manufacturer’s instructions.

2.7. Detection of NO production

Cells were pretreated with APN (5 μg/mL) or NAC (5 mM) for 1 h and then treated with PA (200 μM) for 2 h. Cells were then loaded with DAF-FM DA (5 μM) at 37 °C for 30 min in the dark. After rinsed with PBS, cells were stimulated with insulin (100 nM) for 5 min, and then fixed in 2% paraformaldehyde (v/v) at 4 °C for 5 min. Fixed cells were examined using a fluorescent microscope.

2.8. Western blotting

Total proteins were extracted after various treatment. After determination of protein content by BCA Protein Assay Kit (Lafayette, CO, USA), 30 μg of protein was subjected to 5–12% SDS-PAGE and transferred onto PVDF membrane (Bio-Rad Laboratories, Inc) followed by blocking with 5% non-fat milk in TBST (20 mM Tris-HCl, 500 mM NaCl, and 0.1% Tween 20). The membranes were incubated with corresponding primary antibodies (1:2000) overnight at 4 °C. The protein–antibody complexes were detected by ECL Advanced Western Blot detection Kit. The intensity of the bands was quantitated with QuantityOne software (Bio-Rad).

2.9. Immunoprecipitation

Proteins were extracted from treated cells. After determination of the protein concentrations, cell extract was incubated with anti-IKKβ antibody (2 μg) for 2 h at 4 °C followed by incubation with 20 μL of proteinA/G plus-agarose beads overnight with constant shaking. Then the beads were washed 3 times with ice-cold radio
immunoprecipitation assay (RIPA) buffer and bound protein was extracted by adding 40 μL 2 × SDS sample buffer and boiling for 5 min. The complexes were subjected to SDS-PAGE and followed by Western blotting.

2.10. Statistical analysis

Data were expressed as the means ± SD from at least three separate experiments. The differences between groups were analyzed using Prism 5.0 (Graph Pad Software Inc, San Diego, CA) with one-way analysis of variance followed by SNK test. p < 0.05 is considered statistically significant.

3. Results

3.1. APN inhibited PA-induced expression of ICAM-1, TNF-α, and IL-6 in HUVECs

The cytotoxic effect of PA on endothelial cells was examined firstly. PA (1–200 μM) showed no cytotoxic effect on endothelial viability (Fig. 1A). APN alone at 5 μg/ml showed no effect on the mRNA expression of TNF-α (Fig. 1B), and IL-6 (Fig. 1C), and protein expression of ICAM-1 (Fig. 1D). Furthermore, PA significantly increased the mRNA expression of ICAM-1, TNF-α, and IL-6 and the protein expression of ICAM-1, which was effectively inhibited by APN pretreatment (Figs. 2A–D).

3.2. APN reduced PA-induced ROS production and regulated NOx2 in HUVECs

Compared with control, PA (200 μM) treatment induced approximately four folds of ROS increase, which was dramatically abolished by APN or NAC pretreatment (Fig. 3A) as determined by flow cytometry. Similar effect was observed in fluorescence microscope (Fig. 3B). In PA-stimulated cells, the protein expression of NOx2 and NOx4 was dramatically increased, whereas the expression of NOx1 remained unchanged. Increased NOx2 but not NOx4 was inhibited APN pretreatment (Fig. 3C).

3.3. APN inhibited PA-induced ROS/NF-κB pathway in HUVECs

PA stimulation resulted in increased phosphorylation of IKKβ, IκBα, and NF-κB p65, which could be effectively blocked by APN or NAC pretreatment (Figs. 4A–C). Furthermore, co-immunoprecipitation results showed PA stimulation increased interaction between p-IKKβ and p-IκBα, which was significantly decreased by both APN and NAC as well (Fig. 4D).

Fig. 1. The cytotoxicity of PA on endothelial cells and the effect of APN on endothelial inflammation. HUVECs (1.0 × 10⁴ cells/well) in 96-well plates were exposed to PA (1–500 μM) for 24 h and the cell viability was examined with MTT assay (A). Cells were treated with APN (5 μg/mL) for 24 h and the expression of ICAM-1 was detected by western blotting (D), and the production of TNF-α and IL-6 was detected by RT-PCR (B and C). Cont, control group; APN, adiponectin; PA, palmitic acid; N.S, no significance.
3.4. APN suppressed inflammation via ROS and NF-κB in HUVECs

PA-induced ICAM-1 expression was significantly inhibited by APN, NAC, and PDTC, a NF-κB inhibitor at both protein (Fig. 5A) and mRNA (Fig. 5B) levels. Furthermore, PA induced mRNA expression of TNF-α and IL-6 was inhibited by APN, NAC, and PDTC (Figs. 5C and E). Similar inhibitory effect was observed on PA induced secretion of TNF-α and IL-6 in ELISA assay (Figs. 5D and F).

3.5. APN modified IRS-1 phosphorylation in HUVECs

As shown in Figs. 6A and B, exposure of endothelial cells to PA led to enhanced serine phosphorylation of IRS-1 (S307) and decreased tyrosine phosphorylation of IRS-1(Tyr), both of which were partly restored by APN and NAC. Furthermore, insulin treatment triggered tyrosine phosphorylation of IRS-1(Tyr), which was inhibited by PA. APN or NAC pretreatment restored the inhibitory effect of PA (Fig. 6C).

3.6. APN restored PI3K/Akt/eNOS phosphorylation and NO production in the presence of PA in HUVECs

Insulin stimulation induced protein expression of phosphorylation of PI3K, Akt, and eNOS, which was significantly inhibited by PA. This inhibitory effect of PA was partly restored by APN and NAC (Fig. 7A). Furthermore, intensive green fluorescence was observed in insulin treated endothelial cells revealing the increased generation of NO, which was suppressed by PA. However, this inhibitory effect of PA on insulin-mediated NO release was partly restored by APN and NAC as well (Fig. 7B).

4. Discussion

APN, a pleiotropic adipocytokine, shows potent anti-inflammatory effect by suppression of the production of the potent pro-inflammatory cytokines [22,23]. It also exerts insulin-sensitizing effects through binding to its receptors, leading to activation of AMPK, PPAR-α, and presumably other yet-unknown signaling pathways [24,25]. However, its effect on endothelial inflammation and IR remains elusive. In this study, using a PA-induced endothelial model, we found that: (1) APN inhibited PA-induced endothelial inflammation and IR. (2) The inactivation of the ROS/IKKβ pathway account for this inhibitory effect of APN.

It is well established that TNF-α acts as a pro-inflammatory cytokine while IL-6 demonstrates both pro- and anti-inflammatory properties [26,27]. PA-induced TNF-α and IL-6 expression was reported in HUVECs [28–30], microvascular endothelial cells [31,32], human arterial endothelial cells [33]. ICAM-1, a transmembrane protein, plays important roles in stabilizing endothelial and leukocyte interactions and facilitating leukocyte endothelial transmigration [34]. Increased expression of ICAM-1 in response to PA was found in Human microvascular endothelial cells [32], HUVECs [28], mouse endothelial cells [35], endothelial cells from human adipose tissue microvasculature [36]. Similar to these reports, increased expression of ICAM-1, TNF-α, and IL-6 at both mRNA and
Fig. 3. APN reduced PA-induced ROS production via NOX2 in endothelial cells. Cells were treated with PA (200 μM) for 2 h after pretreatment with APN or NAC for 1 h. The ROS generation was measured by flow cytometer (A) and fluorescence microscope (B). Cells were treated with PA (200 μM) for 30 min after pretreatment with APN or NAC for 1 h. The NOXs expression was determined by Western blotting (C). Cont, control group; APN, adiponectin; PA, palmitic acid; NAC, N-acetylcysteine.

Fig. 4. APN inhibited IKKβ/NF-κB activation in PA-treated HUVECs. Cells were treated with PA for 30 min after pretreatment with APN and NAC for 1 h. The expression of p-IKKβ, p-IκBα, and p-p65 was determined by western blotting (A–C). The interaction of IKKβ and IκBα was determined by co-immunoprecipitation (D). Cont, control group; APN, adiponectin; PA, palmitic acid; NAC, N-acetylcysteine.
protein levels were observed in present study. Though the inhibitory effect of APN on LPS-induced TNF-\(\alpha\) production in RAW 264.7 macrophages [13], IL-6 production in 3T3-L1 adipocytes [15] and on TNF-\(\alpha\)-induced ICAM-1 in endothelial cells [18] has been reported, its effect on PA-induced ICAM-1, TNF-\(\alpha\), and IL-6 expression in HUVECs remains unclear. Here, we showed that APN inhibited PA-induced ICAM-1, TNF-\(\alpha\), and IL-6 expressions at both mRNA and protein levels suggesting that APN was one potent anti-inflammatory adipokine in endothelial cells.

PA-induced endothelial expression of ICAM-1, TNF-\(\alpha\), and IL-6 was mediated by activation of NF-\(\kappa\)B [19,29,30,32,37,38], ROS formation [30,38], regulation of AMPK and sirtuin 1 activities [39], increase in the ratio of IL-1/IL-1Ra secretion in monocytes [40], regulation of ATP receptors P2X7 and P2X4 [28] etc. Here, we found that PA treatment induced significant ROS formation in endothelial cells. The ROS scavenger NAC inhibited PA-induced ROS formation and expression of ICAM-1, TNF-\(\alpha\), and IL-6 suggesting that the inhibitory effect of APN was mediated by ROS. NADPH oxidase is one of the major sources for ROS in endothelial cells [41,42] and NOX1, NOX2, and NOX4 are three important NADPH oxidases expressed in endothelial cells [43]. Earlier studies showed that PA induced endothelial ROS through protein kinase C-dependent activation of NADPH oxidase [44,45], uncoupling of the mitochondrial respiratory chain [46]. Furthermore, both NOX4 and NOX2 have been implicated in PA-induced endothelial ROS formation [47,48]. Our results showed that PA had no effect on NOX1 expression but dramatically increased both NOX2 and NOX4 expression. Thus, both NOXs might be involved. However, inhibitory effect of APN was only observed on NOX2 suggesting the regulation of NOX2 may of importance in this process. Similar inhibitory effect by APN was observed in ethanol treated macrophages [49]. However, our previous report showed that APN inhibited coronary NOX4 expression isolated from apolipoprotein E knockout mice [50]. This inconsistent might result from the differences of in vitro and in vivo studies. In consistent with previous reports, we found PA induced phosphorylation of IKK\(\beta\), and p65 in endothelial cells

**Fig. 5.** APN suppressed inflammatory cytokines expression via blocking ROS/NF-\(\kappa\)B signal pathway in PA-treated HUVECs. Cells were treated with PA for 30 min after pretreatment with APN (5 \(\mu\)g/mL), NAC (5 mM) and PDTC (10 \(\mu\)M) for 1 h. The ICAM-1 expression was examined by western blotting (A). Cells were treated with PA for 24 h after pretreatment with APN (5 \(\mu\)g/mL), NAC (5 mM) and PDTC (10 \(\mu\)M) for 1 h. The expression and secretion of ICAM-1, TNF-\(\alpha\), and IL-6 were determined by RT-PCR (B, C, and E) and ELISA (D and F), respectively. Cont, control group; APN, adiponectin; PA, palmitic acid; NAC, N-acetylcysteine.
IκBα is an inhibitory protein of NF-κB pathway, which sequestered the NF-κB p65 by forming an inactive complex in the cytoplasm. Phosphorylation of IκBα by IKKβ disassociated IκBα and thus released p65 which activated NF-κB pathway [52]. Here, enhanced phosphorylation of IκBα was induced by PA as well. The inhibitory effect of APN and NAC on phosphorylation of IKKβ, IκBα, and p65 provided evidence that APN was a potent inhibitor for endothelial NF-κB pathway mediated by ROS. The inhibitory effect of PDTC on PA-induced cytokine expression further confirmed the key roles of NF-κB in APN’s effect. Collectively, these results suggested that APN inhibited PA-induced cytokines expressions by inhibiting NF-κB pathway through down-regulating ROS.

Consistent with previous reports [29,30,38,51], increased IRS-1 serine phosphorylation (S307) and decreased IRS-1 tyrosine phosphorylation were observed by PA treatment. The reversed effect of APN and NAC suggested the active roles of ROS in this process. The inhibitory effect of PA on insulin induced IRS-1 tyrosine phosphorylation suggested that the insulin signaling was interfered. The inhibitory effect of APN and NAC revealed that APN restored insulin sensitivity mediated by ROS. Activation of PI3K by phosphorylated IRS-1 in response to insulin is a characteristic of IR. The activated PI3K then phosphorylates and activates the downstream Akt and eNOS, leading to NO release in endothelial cells [30,38]. The inhibited effect of PA on insulin-induced phosphorylation of PI3K/AKT/eNOS suggesting the impaired insulin signaling. This was further confirmed by the decreased NO release. The inhibitory effect of APN and NAC revealed that PA improved IR possible through ROS. As IKKβ can directly phosphorylate IRS-1 serine (S307) leading to an increase in inflammatory cytokine expression and can suppress IRS-1 tyrosine phosphorylation blocking PI3K/AKT/eNOS pathway to induce IR, it serves as a key mediator bridging inflammation and IR. Thus, the inhibitory effect of APN on PA-induced endothelial inflammation and IR was mediated, at least in part, by IKKβ.

In summary, as depicted in Fig. 8, present study showed that APN attenuated PA-induced endothelial inflammation and IR via ROS/IKKβ pathways. These results provided new insights to the roles and mechanisms of APN in improving endothelial dysfunction.
Fig. 7. APN restored insulin-mediated PI3K/Akt/eNOS pathway and NO production in the presence of PA. Cells were treated with PA for 30 min after pretreatment with APN or NAC for 1 h. Then cells were stimulated with insulin (100 nM) for 20 min. The phosphorylation of PI3K, Akt, and eNOS was determined by western blotting (A) and the intracellular NO production was detected with a fluorescence microscopy (B). Cont, control group; APN, adiponectin; PA, palmitic acid; NAC, N-acetylcysteine.

Fig. 8. APN inhibited PA-induced endothelial inflammation and IR by regulating ROS/IKKβ.
Disclosures

We declare that none of the authors has any kind of conflict of interest related to the present work.

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