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Angiotensin II-induced apoptosis in human endothelial cells is inhibited by adiponectin through restoration of the association between endothelial nitric oxide synthase and heat shock protein 90

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Abstract Adiponectin can protect vessels from injury by promoting the activity of endothelial nitric oxide synthase (eNOS) with increased nitric oxide production. Recently, it was demonstrated that eNOS activity is highly regulated by heat shock protein 90 (HSP90). We tested the hypothesis that adiponectin can prevent endothelial cell injury produced by angiotensin II through promotion of the association between eNOS and HSP90. Cultured human umbilical vein endothelial cells (HUVECs) were treated with angiotensin II (2 μM) to induce apoptosis. In the presence of globular adiponectin, apoptosis was inhibited in a dose–response manner. Angiotensin II-induced apoptosis was also inhibited by treatment with an NO donor and by combined treatment with both angiotensin II type 1 and type 2 receptor blockers. Western blotting and immunoprecipitation of the lysates from the treated cells showed that globular adiponectin could restore the association between eNOS and HSP90 and enhance the phosphorylation of eNOS. In conclusion, angiotensin II-induced human endothelial cell apoptosis can be prevented by adiponectin through promotion and stabilization of the association between eNOS and HSP90.

Keywords: Adiponectin; Angiotensin II; Heat shock protein 90; Endothelial nitric oxide synthase; Apoptosis; Endothelial cell

1. Introduction

Adiponectin is abundantly expressed in adipose tissue. It has been reported that adiponectin is reduced in patients with increased insulin resistance, such as obesity, type 2 diabetes, coronary artery disease and hypertension [1–3]. In addition to the metabolic effects, adiponectin was found to protect vessels from damage through various mechanisms. It was demonstrated that adiponectin could inhibit adhesion of monocyte to endothelial cells [4,5] and transformation of macrophage to foam cell [6]. In one in vivo study, adiponectin-deficient mice exhibited excessive vascular remodeling to acute injury [7]. In another mouse atherosclerosis model, enhanced adiponectin expression could reduce atherosclerotic changes [8]. Recently, it was demonstrated that adiponectin can directly stimulate the production of nitric oxide (NO), an important mediator that regulates vascular tone and function, through AMPK–PI3–kinase–Akt–eNOS-signaling axis [9,10].

It is well known that impairment in endothelial nitric oxide synthase (eNOS) activity plays an important role in the pathogenesis of endothelial cell dysfunction. Recent studies indicate that eNOS is highly regulated by post-translational modifications, such as Akt-induced phosphorylation [11,12] and interaction with several regulatory proteins, such as heat shock protein 90 (HSP90) [13–15]. Binding of HSP90 ensures recruitment of activated Akt to eNOS\HSP90 complex and phosphorylation of eNOS [13]. HSP90 also plays an important role in the balance between NO and superoxide. HSP90 inhibitors, such as radicicol and geldanamycin, can increase eNOS-dependent superoxide anion production by uncoupling eNOS [16,17]. These observations suggest that the association of HSP90 with eNOS is critical in eNOS-associated NO production.

Endothelial cell injury is considered a critical event in the pathogenesis of atherosclerosis, plaque erosion, and thrombus formation [18]. In atherosclerosis lesions, the turnover rates of endothelial cells are accelerated and local endothelial cell apoptosis is implicated in this process [19]. Endothelial cell injury can be induced by high glucose, angiotensin II and oxidized low-density lipoprotein [20–23]. One recent study has shown that high molecular weight adiponectin can prevent human umbilical vein endothelial cells (HUVECs) from apoptosis through AMPK signaling pathway [24].

In the present study, we tested the hypothesis that adiponectin can prevent endothelial cell apoptosis induced by angiotensin II through promotion of the association between eNOS and HSP90.

2. Materials and methods

2.1. Cell culture and cell treatment

Human umbilical vein endothelial cells were cultured as previously described [25]. Cells were seeded at a density of 1×10^5 per 75 cm^2 flask in medium 199 (Gibco), supplemented with 20 mmol/l HEPES, 100 μg/ml endothelial cell growth substance (Collaborative Research Inc), and

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20% fetal calf serum (Gibco). The cultures were maintained at 37 °C with a gas mixture of 5% CO_2–95% air. Subcultures were performed with trypsin-EDTA. All media were supplemented with 5 U/ml heparin, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. Medium was refreshed every third day. Endothelial cells of the third to fifth passages in actively growing condition were used for experiments. HUVECs were treated with media containing angiotensin II (2 μM) for 18 h in the presence or absence of globular adiponectin (2, 5, or 10 μg/ml, Phoenix), sodium nitroprusside (SNP, an NO donor, 30 μM, Sigma), NG-nitro-L-arginine methyl ether (L-NAME, an eNOS inhibitor, 100 μM, Sigma), radicicol (an HSP90 inhibitor, 20 μM, Calbiochem), LY294002 (a phosphatidylinositol 3 ([PI_3]) kinase inhibitor, 20 μM, Sigma), losartan (an angiotensin II type I receptor blocker, 500 nM, MSD) or PD123319 (an angiotensin II type II receptor blocker, 500 nM, Sigma).

2.2. Western blot

Cells were lysed in the lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40 [NP-40], 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride [PMSF], 1 mg/ml aprotinin, and leupeptin, pH 7.4) on ice for 20 min. The protein concentrations of the cell lysates were determined. The cell lysates were subjected to electrophoresis on 8% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) for the detection of eNOS, phospho-eNOS, Akt, and phospho-Akt. The samples were then electrotransferred and immunoblotted with anti-eNOS, anti-phospho-eNOS (Ser1177), anti-Akt or anti-phospho-Akt (Ser473). After blocking, blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Cappel). The protein expression was determined with the enhanced chemiluminescence (ECL) kit (Amersham) and exposed to the filters of Kodak X-Omat films. β-actin was incorporated in the immunoblot as a control for the loading protein amount.

2.3. Immunoprecipitation

Cell lysates were incubated with anti-eNOS polyclonal antibody (StressGen) and protein A/G (Santa Cruz Biotechnology) to immunoprecipitate eNOS and then immunoblotted with anti-HSP90 antibody (StressGen) to determine the amount of HSP90 associated with eNOS. Immunoglobulin G heavy chain was also immunoblotted as a control for the loading protein amount.

2.4. Detection of apoptosis

Apoptosis of the treated HUVECs was detected by the ELISA method of cell death detection (Roche Applied Science) and caspase-3 activation. This cell death detection assay is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively. It allows specific determination of mono- and oligonucleosomes in the cell lysates. Also, we determined the capase-3 activity by subjecting the cell lysates to Western blotting with anti-caspase-3 polyclonal antibody (Santa Cruz Biotech).

2.5. Statistical analysis

Data were expressed as means ± S.E.M. Changes in apoptosis of different conditions were compared by unpaired Student's *t* test when the analysis of variance (ANOVA) showed significant difference. A *P* value <0.05 was considered statistically significant.

3. Results

3.1. Effects of adiponectin on angiotensin II-induced apoptosis in HUVECs

HUVECs were treated with angiotensin II (2 μM) for 18 h with or without 1 h pre-treatment with globular adiponectin (2, 5, or 10 μg/ml). Apoptosis was then measured. As shown in Fig. 1A, angiotensin II-induced apoptosis was inhibited by adiponectin in a dose–response manner. The caspase-3 cleavage product was present in the angiotensin II-treated HUVECs, which was diminished by the pretreatment with globular adiponectin in a dose–response manner (Fig. 1B).

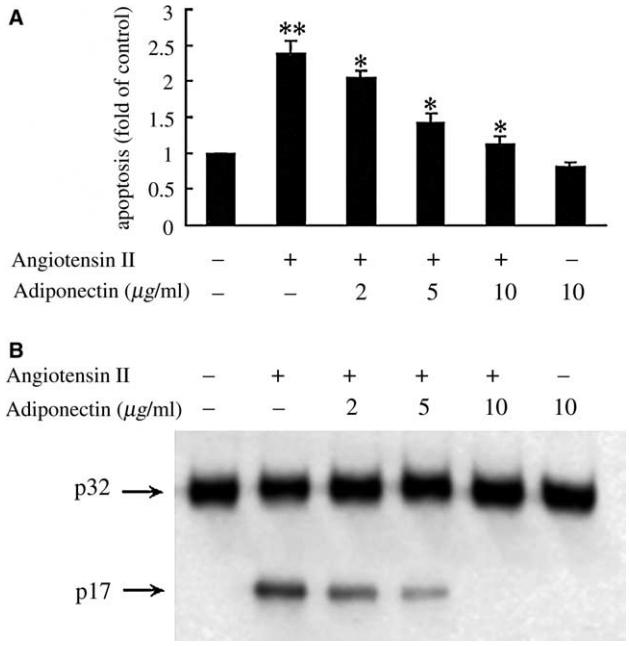

Fig. 1. Effects of adiponectin on angiotensin II-induced apoptosis. HUVECs were exposed to angiotensin II (2 μM) for 18 h with or without pre-treatment with various concentrations of globular adiponectin (2, 5 or 10 μg/ml) for 1 h. (A) Globular adiponectin prevents angiotensin II-induced apoptosis in a dose–response manner (*$P < 0.05$ compared with control and previous condition; **$P < 0.05$ compared with control). (B) Lysates from the treated HUVECs were immunoblotted with polyclonal antibody against caspase-3. Data are presented as means ± S.E.M. ($n = 3$).

3.2. The role of eNOS in the preventive effect of adiponectin on angiotensin II-induced apoptosis in HUVECs

As shown in Fig. 2A, angiotensin II-induced apoptosis in HUVECs was inhibited by pre-treatment with globular adiponectin (10 μg/ml), combined angiotensin II receptor blockers (angiotensin II type I receptor blocker, Losartan, plus angiotensin II type II receptor blocker, PD123319), or SNP for 1 h. Pre-treatment of HUVECs with L-NAME abolished the anti-apoptotic effect of adiponectin and combined angiotensin II receptor blockers. Western blotting of caspase-3 (Fig. 2B) showed that adiponectin and SNP could prevent the production of caspase-3 cleavage product, while treatment with L-NAME reversed this preventive effect.

3.3. Effect of adiponectin on the phosphorylation of eNOS and Akt in angiotensin II-treated HUVECs

HUVECs were pre-treated with globular adiponectin or combined angiotensin II blockers for 1 h followed by exposure to angiotensin II for 18 h. The lysates of the treated cells were then immunoblotted with anti-eNOS, anti-Akt, anti-phospho-eNOS or anti-phospho-Akt antibody. As shown in Fig. 3, the phosphorylation of eNOS and Akt was strongly expressed in control experiment. eNOS phosphorylation was markedly inhibited by angiotensin II treatment and this effect could be prevented by pre-treatment of the cells with either globular adiponectin or combined angiotensin II receptor blockers. The expression of phospho-eNOS and phospho-Akt was similar in cells treated with adiponectin and in control experiment. LY294002, an inhibitor of PI_3 kinase, could inhibit the phosphorylation of both Akt and eNOS. Of note, the protein

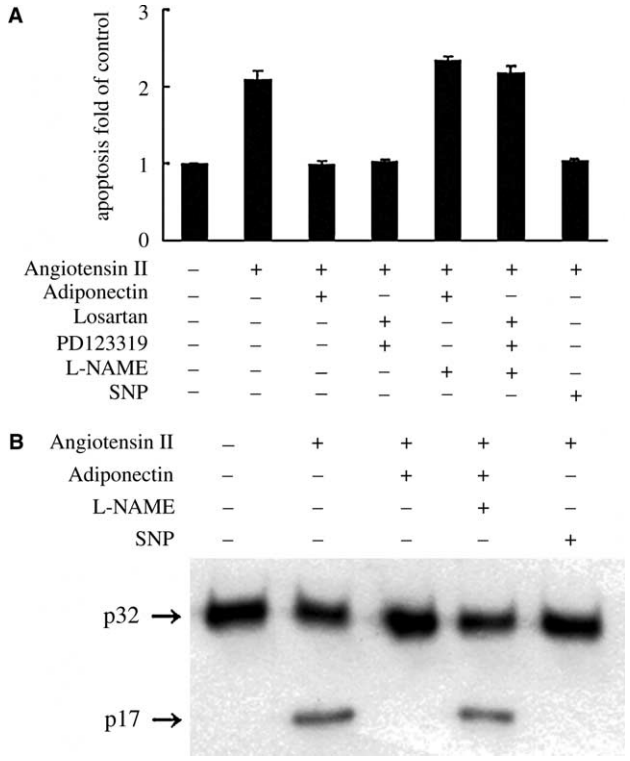

Fig. 2. Effects of L-NAME (100 μM), combined angiotensin-II receptor blockers (Losartan, 500 nM and PD123319, 500 nM), and sodium nitroprusside (SNP, 30 μM) on angiotensin II-induced apoptosis in HUVECs in the absence or presence of globular adiponectin (10 μg/ml). (A) Angiotensin II-induced apoptosis in HUVECs is prevented by globular adiponectin, combined angiotensin-II receptors blockers and SNP, respectively. The preventive effect of adiponectin and combined angiotensin-II receptor blockers on angiotensin II-induced apoptosis is blocked by L-NAME. (B) Lysates from treated HUVECs were immunoblotted with polyclonal antibody against caspase-3. The presence of caspase-3 is prevented by treatment with globular adiponectin and SNP, respectively. This preventive effect is abolished by L-NAME. Data are presented as means ± S.E.M. ($n = 3$).

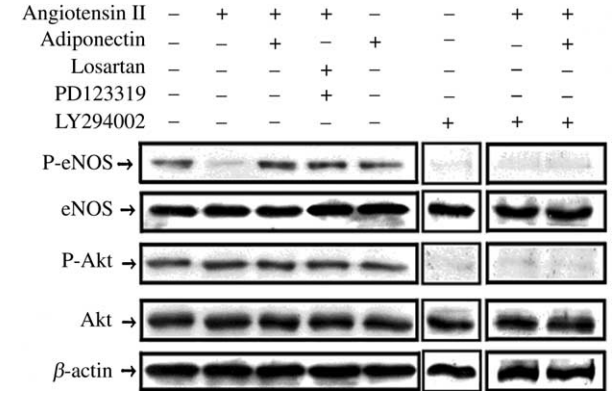

Fig. 3. The effects of adiponectin and combined angiotensin II receptor blockers on the expression and the phosphorylation of eNOS and Akt in angiotensin-II treated HUVECs. HUVECs were exposed to angiotensin II for 18 h with or without pre-treatment with globular adiponectin or combined angiotensin II receptor blockers (Losartan and PD123319). Cell lysates were immunoblotted with anti-eNOS, anti-Akt, anti-phospho-eNOS and anti-phospho-Akt polyclonal antibodies. LY294002, a PI3 kinase inhibitor, could inhibit the phosphorylation of Akt and eNOS.

amounts of eNOS, Akt and phospho-Akt were not significantly altered in all conditions.

3.4. *Effects of adiponectin on the association between eNOS and HSP90*

After pre-treatment with adiponectin or radicicol for 1 h, HUVECs were subjected to angiotensin II exposure for 18 h. The protein levels of eNOS, phospho-eNOS and HSP90 in the cell lysates were measured. Proteins from the same cell lysates were subjected to Western blotting and immunoprecipitation for eNOS and HSP90. Our results showed that the association between eNOS and HSP90 (Fig. 4A) and the phosphorylation of eNOS (Fig. 4B) were significantly inhibited by angiotensin II. Pre-treatment of the cells with globular adiponectin could restore the eNOS\HSP90 association and the phosphorylation of eNOS while radicicol, an HSP90 inhibitor, reversed the effects of adiponectin on eNOS and HSP90. Fig. 4C

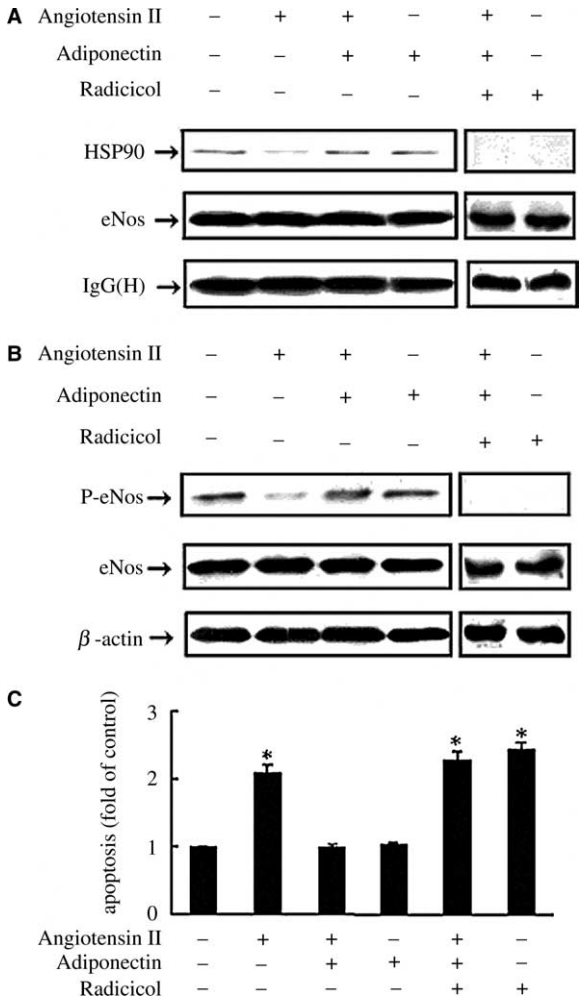

Fig. 4. The effects of adiponectin and radicicol on the association of eNOS\HSP90 and the phosphorylation of eNOS in angiotensin-II treated HUVECs. HUVECs were pre-treated with globular adiponectin or radicicol before exposing to angiotensin II. (A) Lysates from treated HUVECs were immunoprecipitated with polyclonal antibody against eNOS. Immunoprecipitates were then Western blotted for eNOS and HSP90. (B) Cell lysates were immunoblotted with anti-phospho-eNOS and anti-eNOS polyclonal antibodies. (C) The protective effect of adiponectin was abolished by radicicol. *, significant increase of apoptosis.

demonstrated that the protective effect of adiponectin on angiotensin II-induced apoptosis was abolished by pre-treatment with radicicol.

4. Discussion

In the present study, we demonstrated that globular adiponectin can prevent angiotensin II-induced endothelial cell apoptosis through the promotion of the association between eNOS and HSP90. Kobayashi et al. recently showed that the high molecular weight form of adiponectin rather than trimer or hexamer form could prevent HUVECs from apoptosis induced by fasting [24]. The basic building block of adiponectin is a tightly associated trimer, which is formed by association between three monomers at the globular domains. Four to six trimers associate through their collagenous domains to form higher-order structures or oligomers. Without the collagenous domain, the globular domain of adionectin still trimerizes but does not associate into higher-order structures [26]. It is revealed that different recombinant adiponectin products might produce different pharmacological effects. Studies on the bioactivity of full length adiponectin comparing to that of the globular domain alone have yielded divergent results [27,28]. It is possible that adiponectin exists as variable protein complexes that exert different effects in various tissues [26].

We showed that the phosphorylation of eNOS was strongly upregulated at baseline in cells cultured in complete medium (Fig. 3) and was markedly inhibited by LY294002, an inhibitor of the PI_3 kinase-dependent-phosphorylation of Akt. This indicated that the PI_3 kinase-dependent phosphorylation of Akt plays an important role in the phosphorylation of eNOS in HUVECs. A number of studies have explored the intracellular signaling pathways of endothelial cells that regulate adiponectin-stimulated NO generation [9,10]. These studies have pointed to a pivotal role of AMPK–PI3–kinase–Akt dependent pathway in the phosphorylation of eNOS [9,10]. Yet, in the present study, we showed that Akt and phospho-Akt strongly expressed at baseline and not changed through the experiments (Fig. 3). There were several possible explanations. First, in previous studies, endothelial cells were cultured with serum-free or low serum medium for several hours before putting the cells into experiment. This treatment would markedly suppress the phosphorylation of Akt and eNOS as compared with that in full medium (data not shown). In contrast, in the present study, we used a medium containing 20% fetal calf serum in the experiments, which would induce a prominent Akt phosphorylation and rendered a near saturation for the phosphorylation of Akt at baseline. This would make the effect of adiponectin on Akt phosphorylation masked. Second, we did not measure AMPK protein expression in this study. It is possible that eNOS was phosphorylated directly by AMPK [9,10]. But, this possibility might be excluded from a recent study which showed that, in HUVECs, AMPK functions upstream of Akt [10]. Of note, eNOS can be phosphorylated on many residues. The phosphorylation of eNOS was detected by antibodies against serine 1177, a most frequently studied phosphorylation site of eNOS [10,13–15]. Whether there are other phosphorylated residues of eNOS involved in the process needs further investigation.

The association between eNOS and HSP90 has recently been demonstrated to be critical in the regulation of eNOS function [13–17]. If not stimulated, eNOS is associated with caveolin and remains in a membrane-bound, inactive state [29,30]. When stimulated with vascular endothelial growth factor (VEGF), the eNOS–caveolin complex is disrupted by Ca(2+)/calmodulin and the association between eNOS and HSP90 is promoted [13]. The eNOS-bound HSP90 can then recruit VEGF-activated Akt to the complex to induce phosphorylation of eNOS [13–15]. The binding of HSP90 to eNOS ensures the transition from the early Ca2+-dependent to the late phosphorylation-dependent activation of the eNOS [13]. Failure of this binding can cause eNOS uncoupling and increase eNOS-dependent superoxide anion production [16]. One of the novel findings of the present study is that angiotensin II can inhibit the formation of eNOS\HSP90 complex through an action on the angiotensin II receptor (Fig. 4A). In one in vitro study, Marrero et al. [31] have demonstrated that angiotensin II receptor may directly bind eNOS to inhibit its activity. Although the molecular pathway of angiotensin II action is not completely clear, our results lead to the postulation that stabilization of the eNOS/HSP90 complex produced by adiponectin could interfere with the binding to angiotensin II receptor and prevent the angiotensin II-induced de-activation of eNOS.

Angiotensin II importantly contributes to the pathobiology of atherosclerosis and vascular disease not only via its role in hypertension but also via its direct effects on vascular cell injury, growth and migration [21,32,33]. Studies on the effect of angiotensin II on apoptosis and NO production of endothelial cells have revealed inconsistent results. Ohashi et al. [34] showed that the apoptosis of porcine retinal endothelial cells induced by serum starvation was inhibited by angiotensin II. Cai et al. [35] showed that angiotensin II increased NO production in bovine and mouse aortic endothelials cells. Bayraktutan [36,37] also showed that angiotensin II increased eNOS activity and NO production in rat coronary and aortic endothelials. One study has demonstrated that angiotensin II can induce apoptosis of HUVECs via activation of the caspase cascade and this effect can be completely abrogated by NO [21]. The study also showed that angiotensin II-induced apoptosis in HUVECs was affected by the complex interplay between type 1 and type 2 receptors of angiotensin II, as demonstrated by a reduction in apoptosis when blockers of both types of receptors were simultaneously added [21]. These observations might imply that the effects of angiotensin II on the induction of apoptosis and NO production in endothelial cells may be markedly affected by the origin of the cells and also the difference in species of experimental animals.

In this study, we showed that adiponectin can prevent detrimental effect of angiotensin II by maintaining the stability of eNOS\HSP90 complex. These observations have provided a possible molecular mechanism for adiponectin in the protecting effects against vascular injury and remodeling [7,8]. Our findings also proposed a new pathway for angiotensin–NO interaction which holds an important impact on several cardiovascular diseases [22].

In conclusion, we have delineated a possible signaling pathway of adiponectin in preventing angiotensin II-induced apoptosis in human endothelial cells. In this mechanism, eNOS\HSP90 uncoupling induced by angiotensin II is reversed and the activation of caspase-3 and subsequent apoptosis is

prohibited by the action of adiponectin. These findings may contribute to the possible mechanism for adiponectin in direct protection against vascular injury.

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