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Stimulated HSP90 binding to eNOS and activation of the PI3–Akt pathway contribute to globular adiponectin-induced NO production: Vasorelaxation in response to globular adiponectin

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Abstract

The present study examined potential interactions between endothelial NO synthase (eNOS), heat shock protein (HSP)90, and Akt in vascular endothelial cells stimulated with globular adiponectin to produce nitric oxide (NO). Globular adiponectin-induced eNOS phosphorylation was accompanied by eNOS–HSP90–Akt complex formation, resulting in a dose-dependent increase in NO release. Globular adiponectin stimulated binding of HSP90 to eNOS, and inhibition of HSP90 significantly suppressed globular adiponectin-stimulated NO release. Globular adiponectin also caused Akt phosphorylation, and inhibition of PI3 kinase significantly suppressed globular adiponectin-stimulated NO release. This study also examined whether globular adiponectin really induces endothelial-dependent vasodilation using rings from rat thoracic aorta. It was observed that globular adiponectin caused dose-dependent vasorelaxation in the aorta. These results indicate that stimulated HSP90 binding to eNOS and activation of the PI3–Akt pathway contribute to globular adiponectin-induced eNOS phosphorylation and NO production, and to endothelium-dependent vasorelaxation.

Keywords: Heat shock protein 90; Akt; eNOS; Adiponectin

Adiponectin is an important adipocytokine specifically secreted by adipocytes that circulates at relatively high levels in the bloodstream [1]. Adiponectin has potent anti-inflammatory and atheroprotective effects on vascular tissue, and has an insulin-sensitizing effect on tissue involved in glucose and lipid metabolism [1–4]. Adiponectin is reduced in patients with increased insulin resistance, such as obesity, type 2 diabetes, coronary artery disease, and hypertension [1–4]. Vascular endothelial dysfunction plays a pivotal role in the pathogenesis of atherosclerosis and increases the risk of future cardiovascular events [5,6]. Adiponectin has been shown to stimulate nitric oxide (NO) production in vascular endothelial cells [7,8] and additionally, endothelial dysfunction in humans has been linked with hypoadiponectinemia [9,10]. Thus, the observed relationship between insulin resistance and vascular endothelial cell dysfunction may be related to decreased levels of adiponectin.

A proteolytic cleavage product of adiponectin containing its globular head, known as globular adiponectin, has been found to circulate in human plasma [11]. Recent studies have shown that recombinant globular adiponectin is pharmacologically active and induces free fatty acid oxidation in incubated mouse muscle and cultured muscle cells [11]. In addition, when administered to mice fed a high fat meal, globular adiponectin was observed to cause a modest decline in plasma-free fatty acid and glucose levels, while chronic administration

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was observed to cause weight loss without diminished food intake [11]. Yamauchi et al. [12] have reported significantly greater potency of globular adiponectin in reversing insulin resistance than uncleaved adiponectin. We also reported that globular adiponectin potently upregulates NO production in vascular endothelial cells [8].

Recent studies indicate that eNOS is highly regulated by post-translational modifications, such as Akt-induced phosphorylation [13,14] and interaction with several regulatory proteins such as heat shock protein 90 (HSP90) [15–17]. Binding of HSP90 ensures recruitment of activated Akt to the eNOS–HSP90 complex and phosphorylation of eNOS [15]. HSP90 inhibitors, such as radicicol and geldanamycin, can increase eNOS-dependent superoxide anion production by uncoupling eNOS [18,19]. These observations suggest that the association of HSP90 and Akt with eNOS is critical in eNOS-associated NO production.

The present study examines potential interactions between eNOS, HSP90, and Akt in vascular endothelial cells stimulated to produce NO by globular adiponectin. We also examined whether globular adiponectin is capable of inducing endothelial-dependent vasodilation. For this, rings from rat thoracic aorta were exposed to globular adiponectin and endothelial vasodilator function was evaluated.

Materials and methods

Cell culture. Bovine aortic endothelial cells (BAECs) were allowed to grow to confluence in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B, and 10% fetal bovine serum. These cells exhibited immunohistochemical and morphological characteristics of endothelial cells. All the cells in this experiment were used within 3–5 passages and were examined to ensure that they demonstrated the specific characteristics of endothelial cells.

NO_x measurement. To detect NO production within endothelium monolayers, BAECs were cultured in 24-well dishes and studied 1 day after confluence. After 1 h of incubation, nitrite and nitrate levels (NO_2^- and NO_3^-) in the medium were measured with an automated NO detector/high-performance liquid chromatography system (ENO10, Eicom).

Determination of eNOS activity. Citrulline synthesis was measured by modification of a previously described technique [20,21]. Cell monolayers were incubated at 37 °C for 30 min in Hanks' balanced salt solution (pH 7.4) containing 0.5% FBS. Subsequently, cells were incubated with globular adiponectin and/or Ca ionophore in the presence of 10 μM L-arginine and 3.3 μCi/ml L-[^{3}H]arginine. After 15 min, the reaction was stopped with cold phosphate-buffered saline (PBS) containing 5 mM L-arginine and 4 mM EDTA, after which the cells were denatured with 96% ethanol. After evaporation, the soluble cellular components were dissolved in 50 mM Hepes, 5 mM EDTA (pH 5.5), and applied to 2-ml columns of Dowex AG50WX-8 (Na^+ form). Radioactivity corresponding to [^{3}H]citrulline within the eluate was quantified by liquid scintillation counting. This was expressed as femtomoles/mg of cell protein. Basal [^{3}H]citrulline synthesis was determined from L-NAME (1 mM, 30-min preincubation)-inhibitable radioactivity in unstimulated cells, which was not always detectable.

Immunoprecipitation and immunoblotting of eNOS, HSP90, and Akt. eNOS complex was incubated with anti-eNOS antibody in the corresponding NOS reaction buffer at 4 °C for 2 h, and then with protein G–agarose beads at 4 °C overnight. The immunoprecipitates were subjected to SDS–PAGE and then blotted onto polyvinylidene difluoride membranes. The blots were incubated with the primary antibody at 4 °C overnight and then probed with secondary antibody linked to peroxidase. Immunoreactive proteins were visualized on X-ray film by an enhanced chemiluminescent method. Anti-eNOS antibody and anti-HSP90 antibody were obtained from BD Transduction Laboratories (San Diego, CA), and anti-phospho-Ser-1179 eNOS polyclonal antibody and anti-Akt antibody, anti-phospho-Ser-473 Akt antibody were obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology.

Organ chamber experiments. Organ chamber experiments were performed as previously described [22]. Animals were anesthetized with pentobarbital and exsanguinated. The thoracic aortas were carefully dissected and all perivascular tissue was removed under a microscope in a physiological salt solution (PSS) of the following composition (in mmol/L): NaCl 121, KCl 4.7, $NaHCO_3$ 24.7, $MgSO_4$ 12.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, and glucose 5.8, aerated with 95% O_2 and 5% CO_2. The rings from each thoracic aorta (5 mm in length) were mounted vertically between two hooks in organ chamber myographs (Medical Supply), which were filled with PSS and kept at 37 °C. Isometric tension was measured with force transducers (Nihon Kohden). Each preparation was stretched in a stepwise manner to an optimal length, at which point the force induced by 118 mmol/L KCl became maximal and constant. After equilibration for at least 30 min, the rings were then pre-contracted with prostaglandin F2 (3–10 μmol/L). After a stable contraction was achieved, the rings were exposed to acetylcholine (ACh, 10^{-10} to 10^{-5} mol/L) and globular adiponectin to evaluate endothelial vasodilator function. The effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis, were also evaluated to show that vasodilatation is caused by NO.

Materials. Globular adiponectin was obtained from PeproTech EC. (London, UK). This recombinant protein is derived from mouse globular domain ACRP30 cDNA and is endotoxin-free according to the limulus test (Sigma; sensitivity, 0.06 U/ml).

Statistical analysis. Data are presented as means ± SEM. Multiple comparisons were evaluated by ANOVA followed by Fisher's protected least significant difference test. A value of $P < 0.05$ was considered statistically significant.

Results

Incubation of BAE with globular adiponectin increased the concentration of bioactive NO in the supernatant of the cells (as measured by NO_2^- and NO_3^- levels). Examination of the time course showed a substantial increase in NO production for 1 h, after which only a modest elevation in NO production was observed (3–8 h). As shown in Fig. 1A, incubation of BAE with globular adiponectin (0.01–2.5 μg/ml) increased NO production in a concentration-dependent manner. To measure eNOS activity, BAE were incubated with globular adiponectin (1 μg/ml) for 15 min, after which citrulline synthesis in the cells was measured. eNOS activity, measured in terms of citrulline production, was observed to significantly increase with globular adiponectin treatment (Fig. 1B).

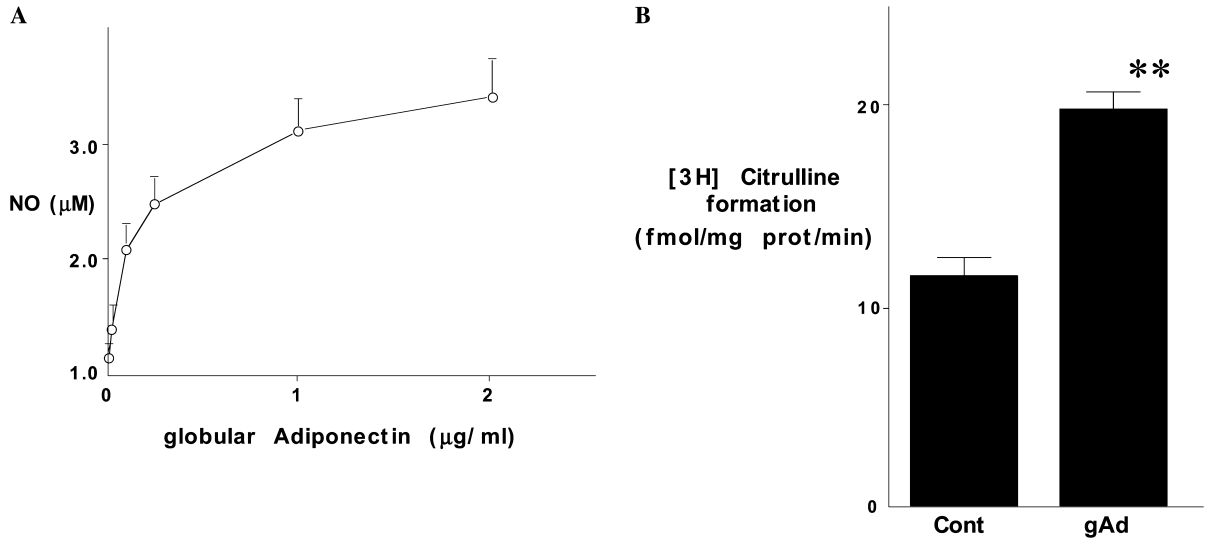

Fig. 1. (A) Effect of globular adiponectin on NO production. BAE were incubated with different concentrations of globular adiponectin for 60 min. NO_x in the medium was measured with an automated NO detector/high-performance liquid chromatography system. (B) Effects of globular adiponectin on eNOS activity. BAE were incubated with globular adiponectin for 15 min, after which eNOS activity was assayed by measuring the formation of L-[^{3}H]citrulline from L-[^{3}H]arginine. Values are means ± SE ($n = 3$). $^{**}P < 0.01$ compared with basal.

The time course of globular adiponectin-induced eNOS–HPS90–Akt complex formation is shown in Fig. 2A. HSP90 increased 5 min after globular adiponectin and remained high for 20 min, while Akt was maximal at 5–10 min after globular adiponectin administration and had returned nearly to baseline by 15 min. Fig. 2B shows globular adiponectin-induced eNOS activation, eNOS–HSP90–Akt complex formation, and the effects of gelnadamycin (GA). Globular adiponectin-induced eNOS activation concomitant with formation with HP90 and activated Akt. GA disrupted association of HSP90, activated Akt with eNOS.

Next, the effects of GA and wortmannin on globular adiponectin-induced NO production in BAE were examined. Cells were pretreated with GA (1 μg/ml) or wortmannin (500 nM) for 30 min followed by globular adiponectin for 60 min. Pretreatment with GA or wortmannin significantly suppressed globular adiponectin-induced NO release (Fig. 3).

We then examined whether globular adiponectin is capable of inducing endothelial-dependent vasodilation. For this, the rings from rat thoracic aorta were exposed to globular adiponectin to evaluate endothelial vasodilator function. First, the rings were exposed to acetylcholine (ACh) as control, where ACh induced relaxation of aortic rings in a dose-dependent manner (Fig. 4). Then globular adiponectin was examined to determine whether it causes vasorelaxation of aortic rings. It was found that globular adiponectin caused a dose-dependent vasorelaxation (Fig. 4). Thirty-eight percent vasorelaxation was elicited at 2 μg/ml globular adiponectin, which corresponds to an Ach dose of ~30 nmol/L.

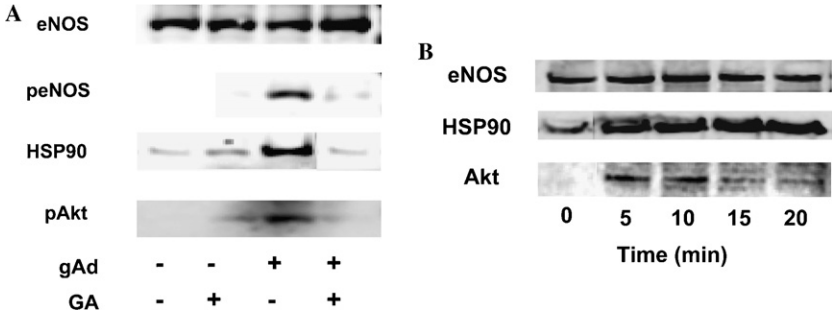

Fig. 2. (A) Time course of eNOS–HSP90–Akt complex formation. BAE were stimulated with globular adiponectin for the indicated times. eNOS was immunoprecipitated at each time point, and eNOS, HPS90, and Akt were evaluated by immunoblotting. (B) Globular adiponectin-induced eNOS activation and eNOS–HSP90–Akt complex formation in BAE. BAE were pretreated with GA and then stimulated with globular adiponectin for 10 min. eNOS was immunoprecipitated, and eNOS, peNOS, HSP90, and pAkt were evaluated by immunoblotting.

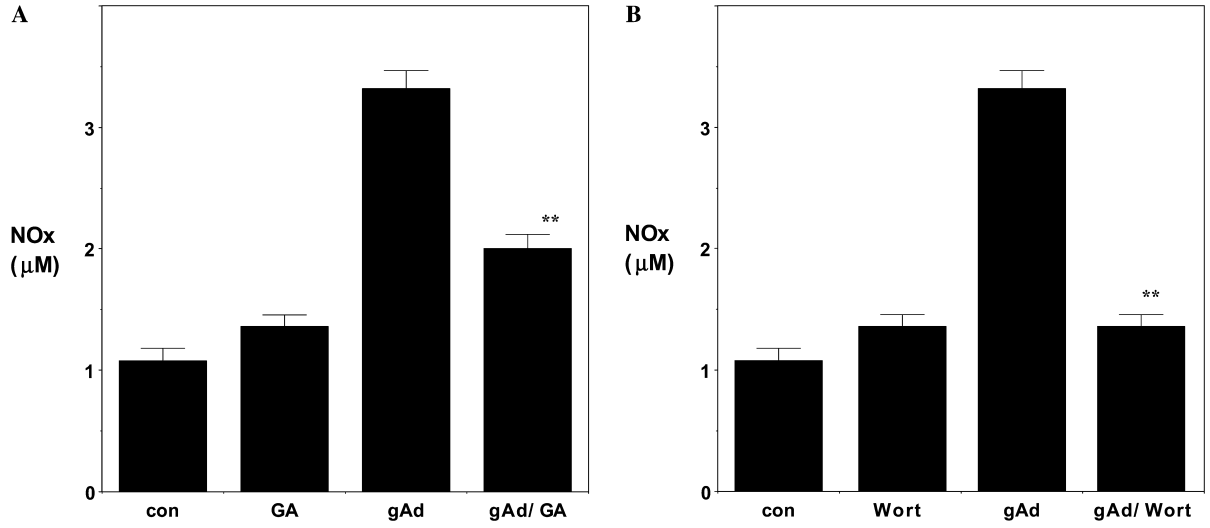

Fig. 3. Effect of PI3 kinase and HSP90 inhibitors on globular adiponectin-stimulated NO production. Cells were pretreated with geldanamycin (GA, 1 μg: A) or wortmannin (Wort, 50 nM: B) for 30 min followed by globular adiponectin for 60 min. NO_x in the medium was measured with an automated NO detector/high-performance liquid chromatography system. Data are represented as means ± SE ($n = 4$). $^{**}P < 0.01$ compared with globular adiponectin treatment.

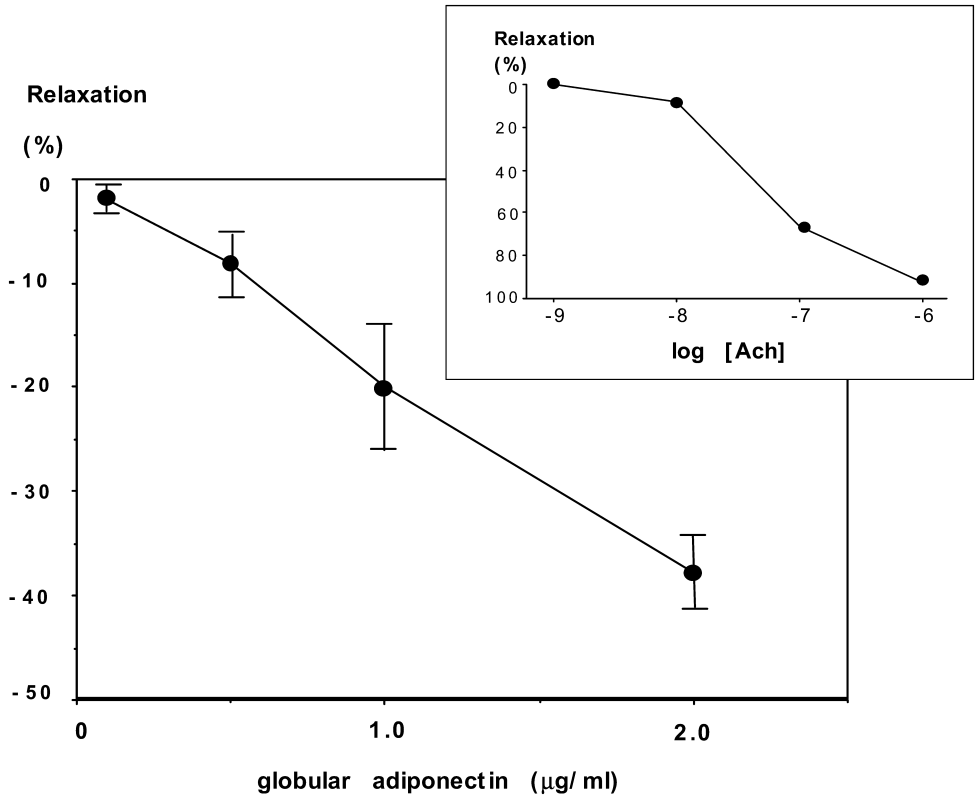

Fig. 4. Endothelium-dependent relaxation in response to acetylcholine (ACh) and globular adiponectin. The rings were exposed to ACh (10^{-10} to 10^{-5} mol/L) and globular adiponectin (0 to 2.0 μg/ml) to evaluate endothelial vasodilator function. Data are represented as means ± SE of 6–8 vascular rings.

This globular adiponectin-mediated vasodilation was abolished by administration of L-NAME (100 μmol/L). Further, vasorelaxation was not caused by globular adiponectin in aortic rings without endothelium (data not shown).

Discussion

Endothelial nitric-oxide synthase (eNOS) is the enzyme responsible for physiological production of NO in the vasculature [23]. eNOS is regulated by subcellular

localization, post-translational modification such as phosphorylation by Akt [24–26], and interactions with several regulatory proteins, such as heat shock protein 90 (HSP90) [14,27,28]. The present study demonstrates that globular adiponectin stimulates eNOS phosphorylation and that this phosphorylation is accompanied by eNOS–HSP90–Akt complex formation, resulting in a dose-dependent increase in NO release. Further, we show that globular adiponectin stimulated binding of HSP90 to eNOS and that inhibition of HSP90 significantly suppressed globular adiponectin-stimulated NO release. Globular adiponectin also caused Akt phosphorylation and blockade of this with a PI3 kinase inhibitor significantly suppressed globular adiponectin-stimulated NO release. These results indicate that activation of the PI3–Akt pathway and HSP90 binding to eNOS contribute to globular adiponectin-induced eNOS phosphorylation and NO production. It has recently been shown that HSP90 and Akt synergistically increase eNOS activity both in vitro and in intact endothelial cells at physiological Ca^{2+} concentrations. This is accompanied by the formation of a ternary complex comprised of HSP90, Akt, and calmodulin-bound eNOS [29]. GA abolishes this cooperative activation of eNOS by HSP90 and Akt, as does substitution of inactive Akt in eNOS activity assays, supporting the idea that HSP90 binding to eNOS and eNOS Ser-1179 phosphorylation by Akt are both essential for their synergistic effects on eNOS activity [29]. Our data therefore support the importance of HSP90 and Akt working together to activate eNOS when cells are stimulated by globular adiponectin.

We also demonstrated that globular adiponectin caused a dose-dependent vasorelaxation in rat aorta. This globular adiponectin-mediated vasodilation was abolished by administration of L-NAME (100 μmol/L). Vasorelaxation was not caused by globular adiponectin in aortic rings without endothelium, indicating that the effect of globular adiponectin on endothelial function was mediated in a NO-dependent mechanism. Thirty-eight percent vasorelaxation was elicited at 2 μg/ml globular adiponectin (120 nmol/L), which corresponds to approximately 30 nmol/L of Ach. Adiponectin is abundantly present in human plasma (range 3–30 μg/ml) and is present in serum as a trimer, hexamer, or high molecular weight form. Levels of globular adiponectin are normally low in serum [11,30] but could be higher at sites of inflammation. Indeed, generation of globular adiponectin by leukocyte elastase secreted by monocytic cell lines has recently been shown [31]. Adiponectin cleavage by leukocyte elastase could be one potential mechanism for the generation of globular adiponectin in plasma [31]. Circulating globular adiponectin may act on endothelial cells and induce vasorelaxation under such conditions.

Recently, it was demonstrated that adiponectin can directly stimulate the production of NO through AMP-activated protein kinase (AMPK)–PI3K–Akt–eNOS signalling axis [7,32]. It was also shown that adiponectin dose-dependently suppressed apoptosis and caspase-3 activity in human umbilical vein endothelial cells [30]. Further, transduction with dominant-negative AMPK abolished the suppressive effect of adiponectin [30]. We confirmed that mRNA of AdipoR1, which has a high affinity for globular adiponectin, is predominantly expressed in endothelial cells, while AdipoR2 mRNA levels are very low. Thus, globular adiponectin exerts its effect at least partly through AMPK activation, which leads to HSP90 binding to eNOS and activation of the PI3–Akt pathway, resulting in eNOS phosphorylation and NO production by globular adiponectin-stimulated endothelial cells bringing about endothelial-dependent vasorelaxation.

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