Adiponectin improves endothelial function in hyperlipidemic rats by reducing oxidative/nitrative stress and differential regulation of eNOS/iNOS activity

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Li R, Wang W-Q, Zhang H, Yang X, Fan Q, Christopher TA, Lopez BL, Tao L, Goldstein BJ, Gao F, Ma XL. Adiponectin improves endothelial function in hyperlipidemic rats by reducing oxidative/nitrative stress and differential regulation of eNOS/iNOS activity. Am J Physiol Endocrinol Metab 293: E1703–E1708, 2007. First published September 25, 2007; doi:10.1152/ajpendo.00462.2007.—Plasma adiponectin level is significantly reduced in patients with metabolic syndrome, and vascular dysfunction is an important pathological event in these patients. However, whether adiponectin may protect endothelial cells and attenuate endothelial dysfunction caused by metabolic disorders remains largely unknown. Adult rats were fed with a regular or a high-fat diet for 14 wk. The aorta was isolated, and vascular segments were incubated with vehicle or the globular domain of adiponectin (gAd; 2 μg/ml) for 4 h. The effect of gAd on endothelial function, nitric oxide (NO) and superoxide production, nitrotyrosine formation, gp91phox expression, and endothelial nitric oxide synthase (eNOS)/inducible NOS (iNOS) activity/expression was determined. Severe endothelial dysfunction (maximal vasorelaxation in response to ACh: 70.3 ± 3.3 vs. 95.2 ± 2.5% in control, P < 0.01) was observed in hyperlipidemic aortic segments, and treatment with gAd significantly improved endothelial function (P < 0.01). Paradoxically, total NO production was significantly increased in hyperlipidemic vessels, and treatment with gAd slightly reduced, rather than increased, total NO production in these vessels. Treatment with gAd reduced (−78%, P < 0.01) superoxide production and peroxynitrite formation in hyperlipidemic vascular segments. Moreover, a moderate attenuation (−30%, P < 0.05) in gp91phox and iNOS overexpression in hyperlipidemic vessels was observed after gAd incubation. Treatment with gAd had an effect on eNOS expression but significantly increased eNOS phosphorylation (P < 0.01). Most noticeably, treatment with gAd significantly enhanced eNOS (+83%) but reduced iNOS (−70%, P < 0.01) activity in hyperlipidemic vessels. Collectively, these results demonstrated that adiponectin protects the endothelium against hyperlipidemic injury by multiple mechanisms, including promoting eNOS activity, inhibiting iNOS activity, preserving bioactive NO, and attenuating oxidative/nitrative stress.

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barrier facility with a 12:12-h light-dark cycle. Fourteen weeks later, animals were anesthetized by intraperitoneal administration of 20% urethane. Cava1 blood was withdrawn, the plasma was immediately separated, and lipid profile, glucose, and insulin levels were determined as described below. The aortic segment from the heart to the iliac bifurcation was excised and placed in ice-cold Krebs buffer consisting of (in mM): NaCl 118, KCl 4.8, CaCl₂·2H₂O 2.5, MgCl₂·6H₂O 2.5, NaH₂PO₄·2H₂O 1.2, NaHCO₃ 8.5, and glucose·H₂O 11. The aorta was cleaned of adhering tissues, cut into rings ~2 mm in length, and incubated with vehicle or gAd (Biovision, Mountain View, CA) (1–20 μg/ml in pilot study; 2 μg/ml was selected as the optimal dose and used in the rest of the experiments) in a cell culture incubator. After 4 h of incubation, endothelial function and biochemical assays were performed as described below.

Lipid, glucose, and insulin plasma determinations. Plasma cholesterol and triglyceride levels were determined by a biochemistry analyzer (Cobas Integra 400 Plus, Roche). Fasting blood glucose and insulin levels were measured with the use of a blood glucose meter (SureStep, LifeScan) and an RIA test kit (Peninsula Laboratories), respectively.

Determination of endothelial function. Endothelial function was determined by comparing the vasorelaxation response to acetylcholine (ACh), an endothelium-dependent vasodilator, with that of S-nitroso-N-acetylpenicillamine (SNAP), an endothelium-independent vasodilator, as described previously (24). Briefly, aortic rings were mounted onto hooks, suspended in organ chambers filled with Krebs buffer and aerated with 95% O₂ and 5% CO₂ at 37°C, and connected to force transducers (WPI, Sarasota, FL) to record changes via a Maclab data acquisition system. After equilibration for 60 min at a preload of 1 g, the rings were precontracted with norepinephrine (NE; 0.1 mM). Once a stable contraction was achieved, the rings were exposed to cumulative concentrations of ACh (10⁻⁹ to 10⁻⁵ M). After the cumulative response stabilized, the rings were washed and allowed to equilibrate to baseline. The procedure was then repeated with an endothelium-independent vasodilator (SNAP, 10⁻⁹ to 10⁻⁵ M) to determine smooth muscle function and sensitivity to NO. Endothelial dysfunction was defined as a reduced vasorelaxation in response to ACh with a normal response to SNAP.

Total NO production measurement. Total NO production (NOx) by aortic segments was determined by measuring the concentration of nitrite, a stable metabolite of NO in vitro, with a modified Griess reaction method (8). Briefly, 5 min after the highest concentration of ACh (10⁻⁵ M) was added, 100 μl of buffer solution was taken from the vascular chamber and mixed with an equal volume of modified Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid). After 10 min of incubation at room temperature, the resultant chromophore was spectrophotometrically determined at 540 nm using a spectrophotometer (SpectraMax 190, Molecular Device). The nitrite concentrations in the samples were calculated from freshly prepared nitrite standard curves made from sodium nitrite with the same Krebs buffer.

Determination of NOS activity. Vascular tissue was minced and homogenized in lysis buffer (Tris 20 mM, NaCl 50 mM, NaF 50 mM, Na₃P₂O₄·10H₂O 5 mM, C₁₂H₂₈O₁₁ 25 mM, DTT 1 mM, NaVO₄ 2 mM, and 1% protease inhibitor cocktail, pH 7.4) with a Heidolph DIA900 tissue homogenizer (Heidolph Instruments, Schwabach, Germany). The homogenate was centrifuged (12,000 g, 30 min at 4°C), and total NOS activity and inducible NOS (iNOS) activity were determined using an NOS activity assay kit (tNOS, Nanjing Jiancheng Bioengineering Institute), following the manufacturer’s instructions. In brief, 100 μl of supernatant was added to the reaction buffer containing L-arginine, NADPH, calcium (not present in iNOS assay buffer), calmodulin, tetrahydrobiopterin, nitroblue tetrazolium (NBT), and phenazine methosulfate (PMS). Fifteen minutes after incubation at 37°C, reaction was stopped by adding a termination buffer. Formazan, the reaction product of NBT/ PMS with NADPH in the presence of NO, was quantified spectrophotometrically at 530 nm. One NOS enzymatic unit was defined as 1 nmol NO⁻¹·min⁻¹·mg protein⁻¹. Endothelial NOS (eNOS) activity was obtained by subtracting iNOS activity from the total NOS activity, and results were normalized against the mean value of control and expressed as fold changes.

Determination of eNOS, iNOS, and gp91phox expression by Western blot. The aortic segments were pulverized in liquid nitrogen and resolubilized in lysis buffer. Equal amounts of protein (80 μg protein/lane) were electrophoresed on a 14% SDS-polyacrylamide gel and electrophoretically transferred to a poly (vinylidene difluoride) membrane (Millipore, Billerica, MA). After blocking with 5% skim milk in Tris-buffered saline at room temperature for 1 h, we incubated the membrane with an antibody against eNOS, phosphorylated eNOS (Cell Signaling Technology, Danvers, MA), iNOS, or gp91phox (BD Bioscience Laboratories, San Jose, CA) overnight at 4°C. The membrane was then washed with PBS and incubated with horseradish peroxidase-conjugated IgG antibody (Cell Signaling) for 1 h at 37°C. The blots were developed with an enhanced chemiluminescence detection kit (PierceChem Technology, Rockford, IL). The immunoblotting was visualized with ChemiDoc XRS (Bio-Rad Laboratory, Hercules, CA), and the blot densities were analyzed with LabImage software.

Determination of tissue antioxidant capacity. Aortic vessels were rinsed, homogenized in 0.9% NaCl solution (1:10, wt/vol), and centrifuged at 3,000 g for 5 min. The pellet was discarded. Total antioxidant capacity was determined with a spectrophotometric assay kit (Nanjing Jiancheng Bioengineering Institute), following the manufacturer’s instruction. In brief, 30 μl of supernatant were added to the reaction buffer containing xanthine, xanthine oxidase, and hydroxylamine. After 40 min of incubation at 37°C, accumulation of nitrite was quantified by the Griess reaction. Tissue antioxidant capacity is inversely related to the concentration of nitrate. Results were normalized against the mean value of control and expressed as fold changes.

Quantification of vascular superoxide production. Superoxide production from aortic segments was measured by flow injection chemiluminescence as described previously (26). Superoxide production was expressed as chemiluminescence intensity (CI) per milligram of vessel weight (CI/mg tissue).

Quantitation of tissue nitrotyrosine content. Nitrotyrosine content in the aortic tissue, a footprint of in vivo ONOO⁻ formation and an index of nitrative stress, was determined using a nitrotyrosine ELISA kit (Cell Sciences, Canton, MA) as described in our previous study (23).

Statistical analysis. Values are presented as means ± SE. Data were analyzed with one-way ANOVA (GraphPad Software, San Diego, CA). A probability value of <0.05 was considered to be statistically significant.

RESULTS

Plasma lipid profile. There was no significant difference in any parameters determined before high-fat diet feeding. Compared with animals fed a normal diet, animals fed a high-fat diet exhibited a significant increase in body weight and marked elevation in plasma cholesterol, triglyceride, fasting blood glucose, and insulin concentrations (Table 1).

Treatment of hyperlipidemic aortic segments in vitro with gAd enhanced ACh-induced vasorelaxation in an NO-dependent fashion. Consistent with previously reported results (14), concentration-dependent vasorelaxation in response to ACh was impaired in vascular segments isolated from animals fed a high-fat diet (Fig. 1A). However, concentration-dependent vasorelaxation in response to SNAP, an endothelium-independent vasodilator, remained unchanged in these vessels (Fig. 1B). These results indicate that high-fat diet-induced hyperlipidemia caused significant endothelial dysfunction.
Most interestingly, in vitro treatment with gAd for 4 h improved endothelial function, as evidenced by a significant improvement of the dose-response curve to ACh (Fig. 1A). To further determine whether gAd increased ACh-induced vasorelaxation by enhancing NO production, a portion of gAd-treated aortic segments was treated with Nω-nitro-L-arginine methyl ester (L-NAME; 0.5 mM). As illustrated in Fig. 1A, addition of L-NAME completely blocked vasorelaxation in response to ACh in those vessels pretreated with gAd.

**Table 1. Metabolic profiles**

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Values are means ± SE; n = 11–15 animals/group. HL, hyperlipidemia; TG, triglyceride; FBG, fasting blood glucose. *P < 0.05 and †P < 0.01 vs. control.

Treatment with gAd significantly improved endothelial function in hyperlipidemic aortic segments in response to ACh (Fig. 1A). Having demonstrated that gAd improved endothelial function, as evidenced by a significant improvement of the dose-response curve to ACh (Fig. 1A), additional experiments were performed. As summarized in Fig. 3A, hyperlipidemia-induced reduction in total antioxidant capacity was significantly preserved after gAd treatment. In addition, a 2.7-fold increase in superoxide production was observed in aortic segments isolated from hyperlipidemic animals, and treatment with gAd almost abolished (78% reduction compared with vehicle-treated vessels) the superoxide overproduction observed in hyperlipidemic vessels (Fig. 3B). Moreover, protein expression of gp91phox, a major component of NADPH oxidase, was significantly increased in hyperlipidemic vessels. Pretreatment with gAd caused a moderate (~30%), yet statistically significant, reduction in gp91phox expression (Fig. 3C).

Treatment with gAd significantly reduced superoxide overproduction in hyperlipidemic vessels. The above-mentioned results demonstrating that endothelial dysfunction occurred despite a significant increase in NO production in hyperlipidemic vessels suggest that increased NO destruction is responsible for hyperlipidemic endothelial dysfunction. In addition, our novel observation that gAd improved endothelial function without increasing NO production indicates that gAd may improve endothelial function by preserving bioactive NO. To obtain direct evidence to support this hypothesis, several additional experiments were performed. As summarized in Fig. 3A, hyperlipidemia-induced reduction in total antioxidant capacity was significantly preserved after gAd treatment. In addition, a 2.7-fold increase in superoxide production was observed in aortic segments isolated from hyperlipidemic animals, and treatment with gAd almost abolished (78% reduction compared with vehicle-treated vessels) the superoxide overproduction observed in hyperlipidemic vessels (Fig. 3B). Moreover, protein expression of gp91phox, a major component of NADPH oxidase, was significantly increased in hyperlipidemic vessels. Pretreatment with gAd caused a moderate (~30%), yet statistically significant, reduction in gp91phox expression (Fig. 3C).

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Fig. 1. Concentration-dependent vasorelaxation of control (Con) and hyperlipidemic (HL) aortic segments in response to ACh (A) and 5-nitroso-N-acetylpenicillamine (SNAP; B) and effect of in vitro globular domain of adiponectin (gAd) treatment (HL + gAd) on hyperlipidemia-induced endothelial dysfunction. Values are means ± SE; n = 8–10 vascular segments/group from 5–7 rats. **P < 0.01 vs. control. #P < 0.05 and ##P < 0.01 vs. hyperlipidemic vessels treated with vehicle.

Fig. 2. Total nitric oxide production (NOx) by aortic segments isolated from control animals (Con) or hyperlipidemic animals (HL) treated with vehicle or gAd (HL + gAd). NOx concentration in medium containing vascular segments was determined by Griess reaction. dw, Dry weight. Values are means ± SE; n = 8–10 vascular segments/group from 5–7 rats. **P < 0.01 vs. control. "$P < 0.01 vs. HL + gAd.
Importantly, pretreatment with gAd significantly reduced nitrotyrosine content (Fig. 4).

Treatment with gAd differentially regulated eNOS and iNOS activity in hyperlipidemic vessels. The data presented above indicate that the reduction of superoxide production and prevention of NO destruction contribute to the vasculoprotective effect of gAd in hyperlipidemia. However, although gAd has been shown to stimulate NO production in cultured endothelial cells by phosphorylating eNOS (6, 25), treatment with gAd did not increase but slightly reduced total NO production in hyperlipidemic aortic vessels (Fig. 2). These results suggest that the overall effect of gAd on total NO production may involve a complex regulation of gAd on different forms of NOS under hyperlipidemic conditions. To directly investigate this novel possibility, the effect of gAd on eNOS and iNOS activity was determined. As summarized in Fig. 5, a significant reduction in eNOS activity and a marked increase in iNOS activity were observed in hyperlipidemic vessels. Pretreatment with gAd almost completely normalized eNOS activity and significantly reduced iNOS activity. These results demonstrated that gAd had opposite effects on eNOS and iNOS activity, and this differential regulatory role may explain the paradoxical finding that gAd significantly increased ACh-induced vasorelaxation but slightly reduced total NO production.

Treatment with gAd enhanced eNOS phosphorylation and inhibited iNOS expression in hyperlipidemic vessels. In a final attempt to determine, at a molecular level, how eNOS and iNOS were differentially regulated by gAd, eNOS and iNOS protein expression and eNOS phosphorylation were determined. As illustrated in Fig. 6A, neither hyperlipidemia nor treatment with gAd had a significant effect on eNOS protein expression. However, the level of phosphorylated eNOS was significantly reduced in hyperlipidemic vessels, and this pathological change was almost completely normalized by gAd treatment (Fig. 6B). Moreover, hyperlipidemia caused a >10-

![Fig. 4. Effect of gAd on hyperlipidemia-induced overproduction of nitrotyrosine in vascular segments. Values are means ± SE; n = 6–8 vascular segments/group from 5–7 rats. **P < 0.01 vs. control. #P < 0.05 and ##P < 0.01 vs. hyperlipidemic segments treated with vehicle.](http://ajpendo.physiology.org/)

![Fig. 5. Differential regulation of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) activity by gAd in hyperlipidemic vessels. Values are means ± SE; n = 6–8 vascular segments/group from 5–7 rats. **P < 0.01 vs. control. ##P < 0.01 vs. hyperlipidemic segments treated with vehicle.](http://ajpendo.physiology.org/)

![Fig. 3. Effect of gAd on hyperlipidemia-induced reduction in antioxidant capacity (A), superoxide overproduction (B), and gp91phox expression (C). Values are means ± SE; n = 6–8 vascular segments/group from 5–7 rats. *P < 0.05 and **P < 0.01 vs. control. #P < 0.05 and ##P < 0.01 vs. hyperlipidemic segments treated with vehicle.](http://ajpendo.physiology.org/)
fold increase in iNOS expression. Acute treatment with gAd moderately attenuated hyperlipidemia-induced iNOS expression (Fig. 6C).

DISCUSSION

Several important observations were made in the present study. First, we have observed for the first time that acute treatment with gAd significantly attenuated hyperlipidemia-induced endothelial dysfunction. This result raises the possibility that therapeutic application of gAd may be a useful treatment of metabolic disorders with vascular complication. Second, we have provided direct evidence that inhibiting superoxide production, preserving NO from destruction, and blocking the formation of toxic ONOO⁻ are the major mechanisms by which adiponectin exerts its vasculoprotective effect. Finally, we have demonstrated that adiponectin exerts an opposite effect on eNOS and iNOS activity. This novel result indicates that adiponectin possesses a unique property, i.e., differential regulation of eNOS and iNOS activity, that is not shared by any other cytokines identified to date.

The pathogenic relationships among obesity, the metabolic syndrome, and its cardiovascular complications are well established (7). However, mechanisms by which obesity causes vascular dysfunction are not well understood. Increasing attention has been paid to the direct vascular effects of plasma proteins that originate from adipose tissue, especially adiponectin (10). Decreased plasma adiponectin levels are observed in patients with diabetes, metabolic syndrome, and coronary artery disease (18). Moreover, many studies in animal models and human subjects have demonstrated an association between circulating adiponectin levels and endothelial function (13, 17, 21, 22). All these clinical and experimental studies strongly suggest that adiponectin is a critical vascular protective molecule whose reduction may contribute to vascular injury in metabolic disorder-related diseases. The present study took a different approach and provided the first evidence that acute treatment with gAd significantly attenuates endothelial dysfunction associated with hyperlipidemia. This result not only provides additional evidence that reduced adiponectin in metabolic disorder contributes to the development of endothelial dysfunction, but it also suggests that supplementation of gAd in patients with metabolic disorder may normalize endothelial function and prevent or reduce atherosclerosis.

We have obtained several lines of evidence indicating that gAd improves endothelial function by its novel antioxidative/antinitrative property. First, hyperlipidemia-induced reduction of total antioxidant capacity was significantly reversed by treatment with gAd. Since total antioxidant capacity can be increased by either increasing expression of antioxidant molecules or reducing production of oxidant molecules, we then determined the effect of gAd treatment on superoxide production and demonstrated that gAd significantly reduced superoxide production in hyperlipidemic vessels. It is well documented that superoxide reacts with NO at a near diffusion-limited rate, which is three times faster than the reaction between superoxide and superoxide dismutase (12). This reaction not only causes the inactivation of NO, a cytoprotective and vasodilatory molecule, but also results in the formation of ONOO⁻, a highly reactive and cytotoxic molecule (1). Thus the superoxide/NO reaction is a “toxic switch” that plays a critical pathogenic role in the development of endothelial dysfunction. We have provided direct evidence that increased ONOO⁻ formation in hyperlipidemic vessels was significantly attenuated after gAd treatment. Finally, considerable evidence now exists that NADPH oxidase is the most important source for superoxide production in vascular tissues (20). We have demonstrated that treatment with gAd moderately reduced gp91phox (a critical component of NADPH oxidase) expression in hyperlipidemic vessels. However, it should be noted that the potent antioxidant effect of gAd (~78% reduction in superoxide production) cannot be completely attributed to its inhibition of NADPH oxidase expression (~30%), and other signaling pathways that may contribute to the antioxidant effect of gAd should be investigated.

The most significant finding of the present study is that gAd differentially regulates eNOS and iNOS activity in hyperlipidemic vessels. Previous studies in cultured endothelial cells or
normal vascular tissues have demonstrated that gAd and full-length adiponectin (fAd) activate eNOS through an AMPK-Akt signaling pathway and increase NO production (6, 25). The present study demonstrates that the level of phosphorylated eNOS was significantly reduced in hyperlipidemic vessels and that treatment with gAd reversed eNOS phosphorylation and significantly increased eNOS activity. This result is consistent with the present understanding that gAd stimulates NO production by eNOS phosphorylation. However, total NO production was slightly reduced, rather than increased, after gAd treatment of hyperlipidemic vessels, suggesting that other forms of NOS are involved. Given that hyperlipidemia causes iNOS expression and increased NO production, we investigated the effect of gAd on iNOS expression and iNOS activity. Treatment with gAd resulted in a moderate reduction in iNOS expression. Surprisingly, this treatment markedly inhibited (~70%) iNOS activity in hyperlipidemic vessels. This novel result indicates that gAd inhibits iNOS activity by multiple mechanisms. Further study to explore these mechanisms is not only scientifically significant, because it may reveal new signaling pathways that regulate iNOS activity, but also clinically important, since it could provide a foundation for the application of gAd in the treatment of metabolic disorders.

Endothelial dysfunction due to reduced eNOS activity and nitrative/oxidative stress due to increased iNOS and NADPH oxidase expression and subsequent production of cytotoxic peroxynitrite are early hallmarks of vascular injury in patients with metabolic syndrome. The present study demonstrated for the first time that adiponectin is a unique cytokine that improves endothelial function by enhancing eNOS activity and attenuates oxidative/nitrative stress by blocking iNOS and NADPH oxidase expression and ONOO⁻ production. Loss of this dual-protective effect of adiponectin because of reduced adiponectin production and/or development of adiponectin resistance in patients with metabolic syndrome may play a critical pathogenic role in atherosclerosis and vascular injury.

REFERENCES


