Adiponectin suppresses proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL

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Abstract

Adiponectin (also known as 30-kDa adipocyte complement-related protein or Acrp30) is an abundant adipocyte-derived plasma protein with anti-atherosclerotic and insulin-sensitizing properties. In order to investigate the potential mechanism(s) of the vascular protective effect of adiponectin, we used cultured bovine endothelial cells (BAECs) to study the effect of recombinant globular adiponectin (gAd) on cellular proliferation and the generation of reactive oxygen species (ROS) induced by oxidized LDL (oxLDL). By RT-PCR, we found that BAECs preferentially express AdipoR1, the high-affinity receptor for gAd. Treatment of BAECs with oxLDL (10 μg/ml) for 16 h stimulated cell proliferation by ~60%, which was inhibited by co-incubation with gAd. Cell treatment with gAd also inhibited basal and oxLDL-induced superoxide release, and suppressed the activation of p42/p44 MAP kinase by oxLDL. The effects of gAd were blocked by a specific polyclonal anti-adiponectin antibody (TJ414). OxLDL-induced BAEC proliferation and superoxide release were inhibited by the NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI), but not the eNOS inhibitor L-nitroarginine methyl ester (L-NAME). Finally, gAd ameliorated the suppression of eNOS activity by oxLDL. These data indicate that gAd inhibits oxLDL-induced cell proliferation and suppresses cellular superoxide generation, possibly through an NAD(P)H oxidase-linked mechanism.

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Adiponectin is an abundant adipocyte-derived circulating plasma protein with insulin-sensitizing metabolic effects and vascular protective properties [1]. Adiponectin is composed of two structurally distinct domains, an N-terminal collagen-like domain and a C-terminal globular domain with similarity to complement C1q. In mammalian plasma, both the full-length form of adiponectin (fAd) and the globular domain (gAd) have been observed [2]. The effects of adiponectin on glucose and lipid metabolism in liver and skeletal muscle are mediated by two receptor isoforms (AdipoR1 and AdipoR2) that have recently been cloned [3]. The globular domain of adiponectin (gAd), which we recently found to enhance glucose uptake in adipocytes [4], has a high-affinity interaction with the AdipoR1 receptor.

Adiponectin levels in human subjects with obesity, insulin resistance, and cardiovascular disease have been shown to be reduced, consistent with its putative vascular protective properties [5,6]. In mouse models, adiponectin reverses vascular neointima formation induced by balloon injury and exerts an anti-atherogenic effect in atherosclerosis-prone apoE-deficient mice [7–9]. In cultured endothelial cells, adiponectin attenuates monocyte attachment by reducing the expression of adhesion molecules on endothelial cells [10,11], and adiponectin can suppress proliferation induced by PDGF and HB-EGF in cultured vascular smooth muscle cells [12]. Consistent with these studies, low adiponectin levels have been shown to be associated with endothelial dysfunction [13,14]. Recently,
adiponectin has been shown to enhance NO production in cultured endothelial cells, an effect that is likely to contribute to the cellular mechanisms underlying its vascular protective actions [15].

The uptake and retention of low-density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis, and oxidative modification of LDL (oxLDL) appears to be a key event in this process [16]. OxLDL can potentiate the formation of foam cells, inactivate nitric oxide, induce inflammatory responses, and stimulate the vascular formation of superoxide and other reactive oxygen species (ROS) [17], processes that are widely believed to be integral to atherosclerosis [18]. Cellular ROS and oxLDL also influence the proliferation and turnover apoptosis of endothelial cells [17,19]. Oxidized LDL itself has also been shown to influence cellular ROS generation via activation of plasma membrane-bound nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase activity [20].

In the present study, we examined whether gAd could suppress the proatherogenic effects of low concentrations of oxLDL on the proliferation of BAECs and the generation of cellular superoxide formation. Our data support the hypothesis that at least some of the salutary effects of adiponectin to improve endothelial function in the presence of oxLDL are mediated by reduced cellular superoxide production, diminished endothelial proliferative responses, and enhanced generation of NO, which may help explain its role in modulating the development and progression of atherosclerosis.

Materials and methods

Materials. BAECs, human umbilical vein endothelial cells (HUVECs), and human HepG2 hepatoma cells were obtained from Cell Applications (San Diego, CA), Clonetics (Walkersville, MD), and ATCC (Manassas, VA), respectively. Escherichia coli cells (Origami B strain) were from Calbiochem (San Diego, CA). Culture media and the TRIzol reagent were from Gibco-BRL (Grand Island, NY). M-MLV reverse transcriptase was from Promega (Madison, WI). Enhanced chemiluminescence (ECL) reagents were from NEN Life Science Products (Boston, MA). Antibodies to Erk1/2 MAPK (thr-202/tyr-204) and protein control antibodies were from Cell Signaling (Beverly, MA). Horseradish peroxidase-conjugated secondary anti-mouse, anti-rabbit IgG antibodies, [3H]methyl thymidine, and LL-[3H]arginine were obtained from Amersham (Piscataway, NJ). All other chemicals and reagents, unless otherwise noted, were obtained from Sigma (St. Louis, MO).

Reverse transcription-polymerase chain reaction analysis of adiponectin receptor expression. Total RNA (1 μg) from BAEC, HUVEC, and HepG2 cells was isolated using the Trizol reagent and subjected to reverse transcription-polymerase chain reaction (RT-PCR) with oligo(dT) priming, reverse transcription with M-MLV, and PCR amplification for AdipoR1 and AdipoR2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a reference for comparison between different products and different templates. PCR primers were designed from recently published sequences [3] as follows: AdipoR1 sense, 5'-AGTGGGGACCGGTGT-3'; AdipoR1 anti-sense, 5'-ACTTCAAGAAAG-3' (product size 436 bp); AdipoR2 sense, 5'-ATAGGGATGCTGGTTGAT-3'; AdipoR2 anti-sense, 5'-TCTATG TACCACCGCAG-3' (product size 450 bp); human GAPDH sense, 5'-CGGAGTGAACGGATTGGTCTGAT-3'; and GAPDH anti-sense, 5'-AGCTTCTCCATGTTGTAAGAC-3' (product size 306 bp). RT-PCR products were electrophoresed on a 1.5% (w/v) agarose gel, stained with ethidium bromide and bands were visualized by UV light. For the human and bovine RNAs, representative amplified PCR products were gel-purified and directly subjected to DNA sequence analysis.

Recombinant adiponectin protein and antibodies. The recombinant globular domain of human adiponectin was expressed as an NHis-terminal (his)-tagged fusion protein in E. coli Origami B strain and the isolated protein was applied to an Acticlean Etox column (Sterogene Bioseparations, Carlsbad, CA) to remove endotoxin contamination as we described previously [4]. Recombinant, full-length human protein-tyrosine phosphatase 1B (PTP1B) rendered catalytically inactive by a site-directed mutation at position cys-215 [21] was produced in the same E. coli system and used as a control protein where indicated. Rabbit polyclonal antibodies (TJ 414) were raised against a recombinant full-length adiponectin, purified by protein A-Sepharose affinity chromatography (Bio-Rad, Hercules, CA), and dialed against PBS.

Preparation of oxidized LDL. Human LDL was oxidized as described [22], with slight modifications. LDL (0.3 mg protein/ml) was incubated with CuSO4 (5 μM) in PBS for 12 h at 20°C. The oxidation reaction was terminated by the addition of 1 mM EDTA and cooling at 4°C. Oxidized LDL (oxLDL) preparations were dialyzed extensively against PBS containing 0.5 mM EDTA. The degree of oxidation was quantitated by the increase in relative mobility on agarose gel, indicating an enhanced negative charge of the oxidized lipoprotein. The relative mobility of oxLDL on agarose gel electrophoresis as an index for lipoprotein oxidation was 1.7–2.3 compared with native LDL. The protein content of oxLDL and native LDL was measured by the Lowry method [23]. Oxidized LDL was stored at 4°C in the dark and used within 2 weeks. During this period, apolipoprotein B was intact and not degraded. All procedures were performed under endotoxin-free conditions.

Cell culture. HUVECs or HepG2 cells were cultured in EGM (Clonetics) or DMEM, respectively, with 10% fetal calf serum (FCS). BAECs were cultured in BGM (Cell Applications) on plates or flasks coated with 1% (w/v) gelatin. Cells were routinely studied at the fourth passage and at 80–90% confluence for cell proliferation assays or at confluence for superoxide measurements.

Cell proliferation assays. Cell proliferation was assessed by measuring [3H]methyl thymidine incorporation, the MTT assay, and direct measurement of cellular superoxide formation by BAECs was measured using the cytochrome c reduction assay as described previously [24]. The MTT assay was slightly modified from published methods [25]. Briefly, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added at a final concentration of 10 μg/ml and the cells were incubated for 3 h. The formazan precipitate formed by the live cells was dissolved in 40 mM HCl in isopropanol and the A590 was determined. BAECs treated without or with oxLDL (10 μg/ml) in the absence or presence of gAd (2.5 μg/ml) for 36 h were washed twice with PBS, detached with trypsin/EDTA, and counted using a Coulter counter [19].

Superoxide anion (O2−) formation. O2− formation by BAECs was measured using the cytochrome c reduction assay as described previously [26]. Briefly, confluent BAECs on 12-well dishes were incubated with oxLDL or its respective buffer as control in the presence or absence of the indicated amounts of gAd in phenol-red free DMEM containing 2% FCS for up to 16 h. After incubation, phenol-red free
DMEM containing 50 μM cytochrome c (final concentration) was added to each well and an additional 2h incubation was performed. Reduction of cytochrome c in 1 ml of the supernatant was detected photometrically at 550 nm. The specificity of the assay was determined by co-incubation with SOD (100 U/ml), which prevents the reduction of cytochrome c by superoxide. Nonspecific increase in absorption of cytochrome c during each incubation condition was corrected by using an SOD-blank sample. Only the SOD-inhibitable reduction of cytochrome c was used for calculation on the basis of the molar extinction coefficient of reduced ferricytochrome c (21,000 cm⁻¹ M⁻¹). O₂ formation was expressed in nanomoles of O₂ per milligram protein per hour.

**Western blotting.** Protein immunoblotting was performed essentially as previously reported [27]. Twenty-five to fifty micrograms of protein was resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes and immunoblotting was performed with the indicated monoclonal or polyclonal antibodies. Following incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were visualized by ECL, according to the instructions provided by the manufacturer. Immunoblotting signals were quantitated using an ImageStation 440 (Kodak).

**Cellular eNOS activity assay.** Confluent BAECs were incubated without or with indicated concentrations of gAd for 16 h in DMEM with 2% FCS. After incubation, BAECs were washed three times with Hepes-buffered physiologic salt solution and then eNOS activity in living BAECs was determined as described previously [28]. In some wells, 1 μM calcium ionophore (A23187) was added as a positive control during assay (15 min). The data represent the l-NAME-sensitive conversion of L-[³H]arginine to L-[³H]citrulline expressed as fmol/mg protein/min.

**Statistical analyses.** Quantitative data are presented as means ± SD or SE as indicated for 3–5 experiments. Statistical analysis was based on Student’s t test for comparison of two groups. A p value less than 0.05 was used to determine statistical significance.

**Results**

**Expression of adiponectin receptor isoforms in HUVECs and BAECs**

By RT-PCR analysis, we detected mRNA for both adiponectin receptors in BAECs, HUVECs, and human HepG2 hepatoma cells (Fig. 1). Under several PCR conditions and using GAPDH cDNA as an internal reference, we consistently found that the signal intensity of AdipoR1 was ~2.5-fold greater than that of AdipoR2 in both endothelial cell types. In contrast, AdipoR2 was ~2-fold more abundant than AdipoR1 in HepG2 cells. The sequences of the AdipoR1 and AdipoR2 PCR products from HUVECs were identical to the published human sequences and those from BAECs demonstrated 91% and 88% identity to their human homologs (GenBank Accession Nos. AY436318 and AY436319).

**Effect of oxLDL and gAd on BAEC DNA synthesis and proliferation**

Treatment of BAECs with a low concentration of oxLDL (10 μg/ml) for 16 h increased the cellular incorporation of [³H]methyl thymidine by 59.0 ± 14% (n = 9; p < 0.001). Co-incubation with gAd (2.5–5.0 μg/ml) inhibited the oxLDL-induced DNA synthesis by up to 53% in a dose-dependent manner (Fig. 2A). As a control protein expressed in the same recombinant E. coli system, inactive PTP1B had no effect on oxLDL-induced BAEC proliferation. In addition, preincubation of BAECs with our specific polyclonal anti-adiponectin antibody (TJ 414) at 10 μg/ml, but not a preimmune rabbit IgG, completely blocked the inhibitory effect of gAd on oxLDL-induced BAEC proliferation (Fig. 2B).

**Fig. 1.** Expression of adiponectin receptors, AdipoR1, and AdipoR2 in endothelial cells. RT-PCR analysis was performed with primers for AdipoR1, AdipoR2, and GAPDH, as described in Materials and methods. (A) RT-PCR products from BAECs are shown with a DNA ladder. (B) Results obtained from cDNAs from HUVECs (lanes 1 and 2) and HepG2 cells (lanes 3 and 4) without or with the RT reaction as shown. No products were observed in the negative controls performed with cellular RNA but without the RT reaction, indicating the reaction products did not arise from contaminating genomic DNA.
Role of reactive oxygen species in oxLDL-induced BAEC proliferation

To determine whether cellular reactive oxygen species (ROS) might be involved in oxLDL-induced proliferation of BAECs and its suppression by gAd, we directly measured superoxide (O$_2^-$) release in response to oxLDL treatment. After 16 h incubation with 10 or 30 µg/ml oxLDL, O$_2^-$ released from BAECs into medium was increased by 71.4% and 125%, respectively (both p < 0.01) (Fig. 3A). This was a time-dependent cellular effect, since the increased O$_2^-$ release from BAECs by low concentrations of oxLDL required more than 8 h incubation (Fig. 3B).

Cell treatment with gAd (1.25–5.0 µg/ml) in the absence of oxLDL dose-dependently decreased the basal O$_2^-$ release from BAECs by up to 37% (p < 0.01) (Fig. 3C). Co-incubation of gAd with oxLDL also significantly inhibited the oxLDL-induced O$_2^-$ release from BAECs by up to 38% in a dose-dependent manner (p < 0.01), respectively, compared to the oxLDL-treated controls (Fig. 3C). In cells incubated with 5.0 µg/ml gAd and oxLDL, the level of O$_2^-$ release was suppressed to a level similar to that observed in control cells incubated with either agent. Cell treatment with TJ414 adiponectin antibody (10 µg/ml) completely neutralized the effect of gAd to suppress basal as well as oxLDL-stimulated O$_2^-$ generation (not shown).

Effect of ROS inhibitors on BAEC proliferation

The effect of catalase and SOD on oxLDL-induced BAEC proliferation was investigated using the [3H]methyl thymidine incorporation assay. Co-incubation with catalase (200 or 400 U/ml) inhibited oxLDL-induced DNA synthesis significantly by 67–84% (Fig. 4A).

Fig. 3. Effect of oxLDL on O$_2^-$ production in BAECs. Confluent BAECs were incubated with the indicated concentrations of oxLDL for the time shown. O$_2^-$ formation by BAECs was measured as described in Materials and methods using the SOD-inhibitable cytochrome c reduction assay. O$_2^-$ formation is expressed in nanomoles of O$_2^-$ per milligram of protein per hour. (A) Dose-dependency of the effect of oxLDL on superoxide production. BAECs were stimulated with oxLDL for 16 h. (B) Time course of oxLDL-induced O$_2^-$ formation. Cells were stimulated with 10 µg/ml oxLDL for the indicated time. (C) Dose-dependency of the effect of gAd on basal and oxLDL-stimulated superoxide production. The data represent means ± SE. *p < 0.05, **p < 0.01 vs. control (at zero time or with no added oxLDL); *p < 0.05, **p < 0.01 vs. control oxLDL-treated (no added gAd).
Co-incubation with SOD (200 or 400 U/ml) had a tendency to inhibit oxLDL-induced DNA synthesis, but was not statistically significant. Cell treatment with a combination of a lower concentration of catalase (200 U/ml) and SOD (100 U/ml) completely suppressed the DNA synthesis induced by oxLDL. These data support a role for ROS, especially H2O2 derived from cellular O2, in oxLDL-induced BAEC proliferation.

Signal transduction pathways involved in oxLDL-induced BAEC proliferation and superoxide release

The potential involvement of cellular NAD(P)H oxidase and nitric oxide synthase (NOS) in oxLDL-induced BAEC proliferation was tested using the inhibitors diphenyleneiodonium (DPI) and L-nitroarginine methyl ester (L-NAME), respectively (Fig. 4B). Co-incubation with DPI (1 μM) completely blocked oxLDL-induced DNA synthesis, while L-NAME (up to 1.0 mM) had no effect. In parallel studies performed as described in Fig. 4, we also determined that the effect of oxLDL to increase the production of superoxide by BAECs by 78% (p < 0.001) was completely inhibited by DPI and that L-NAME was without effect (not shown).

Since superoxide originating from cellular NAD(P)H oxidase has been reported to induce cell proliferation in several cell types through p42/p44 MAPK activation [29], we also tested the effect of the MEK inhibitor PD98059 on oxLDL-induced BAEC proliferation. As has been shown in vascular smooth muscle cells [30], co-incubation with PD98059 (10 μM) completely suppressed BAEC DNA synthesis induced by oxLDL (Fig. 4C).

Effect of gAd on oxLDL-induced p42/p44 MAPK phosphorylation

To directly examine whether gAd could inhibit oxLDL-induced MAPK activation, Western blot analysis of phosphorylated p42/p44 MAPK was performed (Fig. 5). Treatment of BAECs for 16 h with gAd (5 μg/ml) did not alter the low basal level of p42/p44 MAPK phosphorylation. Exposure of BAECs to 50 μg/ml oxLDL for 30 min increased p42/p44 MAPK phosphorylation by 16-fold over control cells. In BAECs that had been pretreated with 2.5 or 5 μg/ml gAd for 16 h, the oxLDL-induced p42/p44 MAPK phosphorylation was suppressed to 67% of the level in cells treated with oxLDL alone (p < 0.001). Inclusion of the adiponectin antibody TJ414, but not a preimmune IgG, during the incubation with gAd abrogated its effect on MAP kinase activation. Thus, the inhibition of oxLDL-induced BAEC proliferation by gAd is coupled to a suppression of MAP kinase activation in these cells.

Effect of gAd on eNOS activity suppressed by oxLDL

Finally, to determine whether alterations in eNOS activity also contribute to the salutary effects of gAd on endothelial function, we determined the effect of oxLDL and gAd on eNOS activity in BAECs after a 16 h
incubation. Prolonged incubation with gAd alone had no effect on eNOS activity in BAECs, either without or with cell treatment with the calcium ionophore A23187 during the cell incubation with L-arginine (Fig. 6). Treatment with oxLDL suppressed eNOS activity by 45–47% in the presence or absence of the ionophore (p < 0.001), and co-incubation with gAd and oxLDL led to a significant reversal of the inhibition of eNOS activity by oxLDL, increasing the suppressed level by 46% both with and without ionophore treatment (p < 0.001).

Discussion

In the present work, we demonstrate that the high-affinity receptor (AdipoR1) for the globular form of adiponectin (gAd) is highly expressed in bovine and human endothelial cells, suggesting that this receptor mediates the multiple cellular effects of gAd that result in its salutary role in endothelial vascular dysfunction [13,14]. Our studies also provide novel evidence that gAd inhibits oxLDL-induced cell proliferation by suppressing cellular superoxide generation. Prolonged treatment with gAd also significantly reversed the inhibition of eNOS activity by oxLDL.

Increasing attention has recently been focused on the role of ROS in signal transduction, in particular with respect to the proliferative responses of vascular cell types [30,31]. Specific NAD(P)H oxidase homologs have also now been implicated in mediating the generation of ROS in vascular cells and contributing to the inflammatory milieu that is associated with LDL oxidation and atherosclerotic lesion development [32]. We found that gAd blocks the generation of ROS in endothelial cells exposed to oxLDL and suppresses oxLDL-triggered endothelial cell proliferation, and that these effects of gAd are coupled with an inhibition of oxLDL- and ROS-activated MAP kinase activation. Our data provide insight into how this circulating protein might ameliorate some of the adverse cellular effects of ROS, in particular, mitigating the influence of oxLDL. Also, these effects of adiponectin are exerted over a protracted time course that is consistent with the hypothesis that the salutary effects of gAd on vascular dysfunction are mediated tonically over a prolonged time span.

The signal transduction pathway mediating the metabolic and vascular effects of adiponectin is currently an area of intensive investigation. The rapid effects of adiponectin in a variety of cell types have been associated with activation of the widely expressed stress-activated kinase, AMP-activated protein kinase (AMPK), in skeletal muscle, liver, and adipocytes [4,33,34]. Evidence for a role for cAMP in the action of gAd to reverse TNFα-mediated signaling has also been reported [11]. Our finding that the effects of gAd on cellular ROS generation and proliferation are associated with an attenuation of MAP kinase activation suggests that gAd may impact several signal transduction pathways. In particular, suppression of MAP kinase activation may also be a key mechanism of inhibition of endothelial cell growth by gAd [31].

NO synthesis is well known to be a key regulatory function of endothelial cells. NO can quench superoxide by a direct reaction that forms peroxynitrite, and under certain conditions, the catalytic function of NO synthase may be altered so that it generates superoxide anion itself [35]. We found that prolonged exposure to gAd significantly reverses the suppression of eNOS activity by oxLDL, by a mechanism that is possibly linked to a reduction of cellular superoxide. Further work will be necessary to fully define the mechanism of gAd-induced suppression of O2− release from BAECs especially under conditions of treatment with oxLDL.

The present work provides insight into some of the mechanisms in endothelial cells that underlie the vascular protective properties of adiponectin, highlighting its effects on cellular ROS generation. As cardiovascular disease is the major cause of mortality in patients with obesity and insulin resistance [36], who also have reduced levels of circulating adiponectin, a better understanding of the pathogenesis of atherosclerosis in this setting will help in the development of more effective treatments to control these adverse outcomes. Targeting the adiponectin signaling pathway may ultimately lead to novel approaches to the management of cardiovascular risk in these individuals.

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