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Insulin-like growth factor-1 regulates glutathione peroxidase expression and activity in vascular endothelial cells: Implications for atheroprotective actions of insulin-like growth factor-1

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ABSTRACT

Oxidative stress promotes endothelial cell senescence and endothelial dysfunction, important early steps in atherogenesis. To investigate potential antioxidant effects of IGF-1 we treated human aortic endothelial cells (hAECs) with 0–100 ng/mL IGF-1 prior to exposure to native or oxidized low-density lipoprotein (oxLDL). IGF-1 dose- and time- dependently reduced basal- and oxLDL-induced ROS generation. IGF-1 did not alter superoxide dismutase or catalase activity but markedly increased activity of glutathione peroxidase (GPX), a crucial antioxidant enzyme, via a phosphoinositide-3 kinase dependent pathway. IGF-1 did not increase GPX1 mRNA levels but increased GPX1 protein levels by 2.6-fold at 24 h, and altered selenocysteine-incorporation complex formation on *GPX1* mRNA. Furthermore, IGF-1 blocked hydrogen peroxide induced premature cell senescence in hAECs. In conclusion, IGF-1 upregulates GPX1 expression in hAECs via a translational mechanism, which may play an important role in the ability of IGF-1 to reduce endothelial cell oxidative stress and premature senescence. Our findings have major implications for understanding vasculoprotective effects of IGF-1.

1. Introduction

Cardiovascular disease is the leading cause of death in the developed world, accounting for more than one third of all deaths in the United States [1]. The underlying etiology responsible for most cardiovascular disease is atherosclerosis, which has a complicated pathogenesis in which increased inflammatory responses and oxidative stress play a major role [2,3]. An initial early event in atherogenesis is the development of endothelial senescence and endothelial dysfunction [4,5], both of which have been linked to increased oxidative stress. We have previously shown that a systemic elevation of insulin-like growth factor-1 (IGF-1) by continuous infusion suppressed macrophage infiltration and oxidative stress in the vascular wall, thereby attenuating atherosclerosis in apolipoprotein E deficient ($Apoe^{-/-}$) mice [6]. However, smooth muscle specific overexpression of IGF-1 did not alter atherosclerotic burden or oxidative stress levels both in the normal vessel wall and in atherosclerotic lesions [7], suggesting that the endothelium was the major site of IGF-1's antioxidant and anti-atherogenic action. Here we sought to determine potential antioxidant effects of IGF-1 in cultured human aortic endothelial cells. We found that IGF-1 enhances endothelial antioxidant activity, primarily via upregulation of glutathione peroxidase-1 (GPX1) expression and activity. We further characterized mechanisms for IGF-1 upregulation of GPX1, and demonstrated that IGF-1 prevents oxidant stress induced endothelial cell senescence. These findings provide novel insights into mechanisms whereby IGF-1 reduces oxidant-stress induced vascular complications.

2. Materials and methods

2.1. Materials

Reagents and antibodies were obtained as follows; LY294002, SB202190, and PD98059 from EMD Millipore Chemicals (Billerica, MA); rabbit anti-SBP2 antibody used for Western blot is a generous gift from Dr. Khanna [8]. Mouse monoclonal anti-SBP2 antibody used for immunoprecipitation and mouse monoclonal anti-human GPX1 antibody are from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-human GPX4 antibody from Cayman Chemical (Ann Arbor, MI); 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) and dihydroethidium from Invitrogen (Grand Island,

Abbreviations: hAECs, human aortic endothelial cells; oxLDL, oxidized low-density lipoprotein; nLDL, native low-density lipoprotein; GPX, glutathione peroxidase; IGF-1, insulin-like growth factor 1; Apoe, apolipoprotein e; ROS, reactive oxygen species; L-NAME, NG-nitro-L-arginine methyl ester; D-NAME, NG-nitro-D-arginine methyl ester; SECIS, selenocysteine insertion sequence; SBP2, SECIS-binding protein 2; PI3k, phosphatidylinositide 3-kinase; GH, growth hormone; NRF2, nuclear factor erythroid 2-like 2; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; CHX, cycloheximide; eIF4E, eukaryotic initiation factor 4E; 4EBP1, eukaryotic initiation factor 4E binding protein 1

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NY); mouse monoclonal anti-β-actin antibody from Sigma-Aldrich (St. Louis, MO); anti-acetylated lysine, anti-phospho-Tyrosine, and anti-phospho-Ser/Thr (Akt, ATM, and ATR substrates) antibodies from Cell Signaling Technology (Danvers, MA).

2.2. Cell culture

Human aortic endothelial cells (hAECs) were purchased from Lonza (Basel, Switzerland) and maintained in Endothelial Growth Medium 2 with 2% fetal bovine serum and supplements provided by the manufacturer (Lonza). Actively dividing cells (passage 4 to 8) were used for experiments. All the experiments were conducted using fully confluent culture in serum-free/phenol red-free Endothelial Cell Basal Medium (Lonza; Basel, Switzerland) supplemented with 0.5% bovine serum albumin (fraction V; Sigma-Aldrich, St. Louis, MO).

2.3. Enzyme activity assay

Glutathione peroxidase activity, superoxide dismutase activity, and catalase activity were determined in hAECs using commercially available kits as follows; Glutathione Peroxidase Assay kit and Catalase Assay kit from Cayman Chemical (Ann Arbor, MI); and Superoxide Dismutase Assay kit from R&D Systems (Minneapolis, MN). Cellular glutathione levels were determined using Glutathione Assay kit obtained from Cayman Chemical (Ann Arbor, MI). All the assays were performed accordingly to the instructions provided by the manufacturers.

2.4. Western blot analysis

Western blot analysis was performed as described previously [9]. In brief, cells were washed with PBS and lysed in RIPA buffer, containing 150 mM NaCl, 20 mM Tris–Cl, pH 7.2, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 0.1 M okadaic acid, 0.1 μM aprotinin, 10 μg/mL leupeptin, and 10 mM NaF. Lysates were subjected to 10% SDS-PAGE and western blotting analysis. Immunopositive bands were visualized by enhanced chemiluminescence (ECL, Amersham). Blots were stripped and reprobed with monoclonal anti-β-actin antibody as a control for equal loading.

2.5. Lipoprotein preparation

Human plasma derived native LDL (nLDL) was purchased from Kalen Biomedical (Montgomery Village, MD). OxLDL was prepared as previously described. Briefly, an aliquot of the nLDL fraction was passed through a 10DG desalting column (Bio-Rad) to remove EDTA, then the nLDL fraction (0.2 mg/mL, diluted in PBS) was incubated with 5 μM $CuSO_4$ at 37 °C for 3 h. The reaction was stopped by adding EDTA (final concentration 0.25 mM). The oxLDL prepared under these conditions showed an increase in relative mobility on agarose gel electrophoresis, and the value for thiobarbituric acid-reactive substances (TBARS) in oxLDL was 37.2 ± 1.2 nmol malondialdehyde per milligram protein. TBARS were not detectable in nLDL.

2.6. Realtime PCR analysis of gene expression

After exposure to testing agents for 6–24 h, cells were lysed in Tripure reagent (Roche). Total RNA was extracted and precipitated by isopropanol, and was further purified using RNeasy kit (Qiagen). The total RNA was subjected to a reverse-transcriptase reaction using RT^2 First Strand Kit (Qiagen), followed by the realtime PCR analysis in RT^2 SYBR Green Master Mix (Qiagen) using iCycler iQ Realtime PCR Detection System (Bio-Rad). Specific primer sets for following genes were purchased from Qiagen; *GPX1* (catalog number, PPH00154F), *GPX2* (PPH01710B), *GPX3* (PPH05746F), *GPX4* (PPH05586B), *GPX5* (PPH00454A), *GPX6* (PPH06081A), *GPX7* (PPH09224F), and β-actin (used as an internal control, PPH00073G).

2.7. Immunoprecipitation of SBP2-mRNA complex and mRNA quantification

Messenger RNA–protein complexes immunoprecipitation has been performed as described [10,11]. Briefly, mRNA–protein complexes were extracted from the cells using polysome lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES pH 7.0, 0.5% IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO), 1 mM dithiothreitol, 100 units/mL RNase OUT (Invitrogen, Grand Island, NY), and 0.2% Ribonucleoside Vanadyl Complex, protease inhibitor cocktail (Halt Protease Inhibitor Cocktails, Thermo Scientific, Rockford, IL)). Protein contents in the extract were determined using RC DC Protein Assay kit (Bio-Rad, Hercules, CA), and the equal amount of protein for each sample was subjected to an immunoprecipitation. Immunoprecipitation reaction was achieved by mixing the extract with anti-SBP2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.05% IGEPAL CA-630, 15 mM EDTA, 1 mM dithiothreitol, 100 units/mL RNase OUT (Invitrogen, Grand Island, NY), and 0.2% Ribonucleoside Vanadyl Complex containing protease inhibitor cocktail for 18 h<at 4 °C. The antibody-SBP2-mRNA complexes were collected by Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) and extensively washed using a buffer solution with the same composition of the immunoprecipitation mix. The resulted immunoprecipitates were extracted for RNAs by using Tripure reagent (Roche) and further purified using RNeasy kit (Qiagen). *GPX1* and *GPX4* mRNA levels were determined as described above by quantitative realtime-PCR. Mouse non-immune IgG was used replacing the anti-SBP2 antibody in the immunoprecipitation procedure to confirm specific precipitation of SBP2–mRNA complexes, and produced no detection of *GPX1* or *GPX4* mRNA (data not shown).

2.8. Senescence associated β-galactosidase expression

Semi-confluent hAEC culture was exposed to IGF-1 for 24 h, followed by exposure to 100 μM hydrogen peroxide for 1 h. Subsequently, cells were trypsinized and counted to re-plate in an equal cell number on a new culture dish. Cells were then maintained for a week in the regular Endothelial Growth Medium 2 containing 2% fetal bovine serum and supplements, and then stained for senescence associated β-galactosidase expression using Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA). Positively stained cells were captured in images using a DP70 digital camera connected to a microscope (Olympus) and counted using ImageJ software. Data are expressed as a % of positively stained cells in a total cell count.

2.9. Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed using one-way ANOVA or Student's *t*-test, with $P<0.05$ considered significant. All experiments were performed a minimum of three times.

3. Results

3.1. IGF-1 reduces reactive oxygen species levels in cultured human aortic endothelial cells

To assess potential IGF-1 effects on reactive oxygen species (ROS) levels in endothelial cells, cultured human aortic endothelial cells (hAECs) were exposed to native or oxidized LDL and intracellular ROS levels were probed using 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA; Fig. 1A and C). Consistent with previous reports [12,13] oxidized LDL enhanced ROS generation (Fig. 1A and