FEBS Letters 585 (2011) 3065–3072

FEBS Letters

journal homepage: www.FEBSLetters.org

Differential requirement for nitric oxide in IGF-1-induced anti-apoptotic, anti-oxidant and anti-atherosclerotic effects

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ARTICLE INFO

Article history:
Received 2 July 2011
Revised 12 August 2011
Accepted 18 August 2011
Available online 26 August 2011

Edited by Barry Halliwell

Keywords:
Atherosclerosis
Nitric oxide
Growth factors
Apoptosis
Oxidative stress

ABSTRACT

We have shown previously that insulin like-growth factor I (IGF-1) suppressed atherosclerosis in Apoe$^{-/-}$ mice and activated endothelial nitric oxide (NO) synthase. To determine whether IGF-1-induced atheroprotection depends on NO, IGF-1- or saline-infused mice were treated with L-NAME, the pan-NO synthase inhibitor or with D-NAME (control). IGF-1 reduced atherosclerosis in both the D-NAME and L-NAME groups suggesting that IGF-1's anti-atherogenic effect was NO-independent. IGF-1 increased plaque smooth muscle cells, suppressed cell apoptosis and downregulated lipoprotein lipase and these effects were also NO-independent. On the contrary, IGF-1 decreased oxidative stress and suppressed TNF-α levels and these effects were blocked by L-NAME. Thus IGF-1's anti-oxidant effect is dependent on its ability to increase NO but is distinct from its anti-atherosclerotic effect which is NO-independent.

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1. Introduction

Atherosclerosis is the principal underlying cause of most cardiovascular disease-related deaths, the leading cause of mortality in the USA [1]. Long considered to result from progressive vascular lipid accumulation, atherosclerosis is now recognized as a chronic inflammatory disease [2]. Insulin-like growth factor-1 (IGF-1) is an endocrine and autocrine/paracrine growth factor that is the primary mediator of the effect of growth hormone on developmental growth [3]. We have demonstrated that IGF-1 suppresses atherosclerosis in Apoe$^{-/-}$ mice fed with high fat (Western-type) diet for 12 weeks. This effect was associated with a reduction in vascular and systemic oxidative stress, an increase in circulating nitric oxide (NO) bioavailability and vascular endothelial nitric oxide synthase (eNOS/NOS3) expression, the main NO-producing enzyme in the vascular wall [4]. NO exerts an array of potentially atheroprotective effects on the vasculature, including suppression of cell apoptosis, inflammation and oxidative stress [5]. A null mutation for eNOS increases atherosclerosis in Apoe$^{-/-}$ mice [6] and chronic administration of L-arginine (a NOS substrate for NO synthesis) reduces the extent of atherosclerosis [7]. To determine whether nitric oxide mediates IGF-1-induced antioxidant and/or anti-atherogenic effects, Apoe$^{-/-}$ mice were infused with IGF-1 or saline (control) in the presence of the pan-NOS inhibitor L-arginine methyl ester hydrochloride (L-NAME) [8] or the inactive enantiomer, Nω-Nitro-D-arginine methyl ester hydrochloride (D-NAME) and mice were fed with a Western diet for 12 weeks. Our findings indicate that IGF-1's anti-oxidant effect was partially blunted by L-NAME; however IGF-1-induced reduction in atherosclerosis and in plaque apoptosis were NO-independent.

2. Materials and methods

2.1. Animals

All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Apoe$^{-/-}$ mice (C57BL/6, 8 weeks of age, Jackson Lab) were infused with saline or IGF-1 (1.5 mg/kg/day) using osmotic mini-pumps (ALZET, Cupertino, CA). IGF-1 dose and drug administration protocol were selected based on our previous report [9]. Mice were fed a high-cholesterol pro-atherogenic diet (Western-type diet, 42% of calories from fat) from Harlan-Teklad (TD 88137) for 12 weeks. Diet formula and nutrient information are

Abbreviations: NOS3, endothelial nitric oxide synthase; IGF-1, insulin like-growth factor I; NO, nitric oxide; SMC, smooth muscle cells; LPL, lipoprotein lipase; L-NAME, L-arginine methyl ester hydrochloride; D-NAME, Nω-Nitro-D-arginine methyl ester hydrochloride; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor α; α-SMA, α-smooth muscle actin, NOx, nitrate/nitrite

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0014-5793/$36.00 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2011.08.029

provided in Supplementary data (Supplementary Tables 1 and 2). Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 1 mg/ml, Sigma–Aldrich, St. Louis, MO) or Nω-Nitro-D-arginine methyl ester hydrochloride (D-NAME, 1 mg/ml, Sigma–Aldrich) was added to the drinking water and water was replaced every other day. Mice were housed individually and maintained on a 12-h light-dark cycle.

2.2. Cell culture

Human THP-1 mononuclear cells were purchased from ATCC and were cultured in RPMI1640 media (ATCC) as per manufacturer's recommendations. Phorbol 12-myristate 13-acetate (PMA, 100 ng/ml, Sigma–Aldrich) was added to THP-1 monocytes for 48 h to promote cell differentiation into macrophages. PMA-treated cells were considered to be macrophages based on typical macrophage-like phenotype and immunopositivity for CD36 and CD16. THP-1 macrophages were treated with 50 ng/ml human recombinant IGF-1 (Tersica) for 16 h and gene expression analysis was performed by quantitative real-time RT–PCR with β-actin, tumor necrosis factor α (TNF-α) and lipoprotein lipase (LPL) sequence-specific primer pairs (Qiagen/SABiosciences).

2.3. Atherosclerosis burden quantification

Atherosclerosis burden was quantified by measuring the surface area of Oil Red O–positive lesions on *en face* preparations of whole aortas as previously described [9]. Additionally, serial sections (6 μm) were taken throughout the entire aortic valve area as per Paigen et al. [10] and stained with H&E for quantitation of plaque cross-sectional area as previously described [9].

2.4. Immunohistochemistry (IHC)

Serial aortic valve paraffin-embedded cross sections were stained for α-smooth muscle actin (α-SMA), 8-oxo-D-guanosine (8-oxo-dG) and N-tyrosine. Antibodies were from Chemicon/Millipore (mouse anti-α-SMA antibody and rabbit anti-N-tyrosine antibody) and Abcam (mouse anti-8-oxo-dG antibody). 4′,6-diamidino-2-phenylindole (DAPI) was from Invitrogen. Sections were also stained with Gomori's Trichrome stain to visualize collagen (Richard-Allan Scientific, Kalamazoo, MI). Antibody specificity was verified by staining of serial sections with "normal" IgG (obtained from an unimmunized animal of the same species as primary antibody) and/or by staining with blocked primary antibody. Section stained with "normal" rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or with anti-N-tyrosine antibody blocked by incubation with 3-nitro-L-tyrosine (Sigma–Aldrich, 200 μM, 1 h) served as the negative control for anti-N-tyrosine IHC (Supplementary Fig. 1A). Normal mouse IgG was the control for anti-α-SMA and anti-8-oxo-dG antibody (Supplementary Fig. 1B).

2.5. Quantification of cell apoptosis

Cell apoptosis was quantified in paraffin-embedded aortic valve cross sections with the Apoptosis TUNEL detection kit from Roche as per manufacturer's instructions. To identify apoptotic smooth muscle cells (SMC) in the atherosclerotic plaque, TUNEL-stained sections were co-stained with α-SMA antibody (1:1000) followed by incubation with biotinylated secondary antibody and streptavidin–Alexa 594 complex (Invitrogen). Sections were mounted with DAPI-contained mounting media (Vector Laboratories Inc.). Total cell apoptosis was defined as TUNEL-positive cell number per 1000 plaque cells and SMC apoptosis was measured as the number of α-SMA/TUNEL-double positive cells per 1000 α-SMA-positive cells.

2.6. Gene expression analysis

Gene expression profiling of mouse atherosclerotic aortas was performed in a 96-well plate with an Atherosclerosis RT^2 Profiler PCR Array system (Qiagen/SA Biosciences, Frederick, MD). Real-time PCR was performed using a 40 cycle two-step PCR protocol in the iCycler IQ real-time detection system (Bio-Rad, Hercules, CA). For RT array statistical analysis the cycle threshold value (Ct) of each gene-of-interest was normalized to five housekeeping genes which are included in this commercially available kit. The *P* values were calculated based on a Student's *t*-test of the replicate $2^{-\Delta Ct}$ values for each gene in the group of IGF-1 infused mice vs. saline-infused mice (control) treated either with L-NAME or D-NAME using the Superarray Analysis software provided by the manufacturer. In addition to RT array-based gene expression analysis, we measured TNF-α and LPL mRNA levels in mouse atherosclerotic aortas and in human THP-1 macrophages using specific mouse RT primer pairs for mouse TNF-α (cat# PPM03113F), LPL (cat# PPM04353E) and for human TNF-α (cat# PPH00341E) and LPL (cat# PPH00023B) from Qiagen using standard real time PCR protocol as described previously [9].

2.7. Biochemical assays

IGF-1 and TNF-α ELISA kits were obtained from Diagnostic Systems Laboratories (Webster, TX). Total cholesterol levels were measured using a commercially available kit (Cholesterol/Cholesteryl Ester Quantitation Kit, BioVision, Mountain View, CA). Blood glucose levels were determined using OneTouchR test strips (LifeScan).

2.8. Lipoprotein analysis

Serum lipoproteins were fractionated using an FPLC system (Pharmacia) with a Superose 6 column. Fifty 0.5-ml fractions were collected at a rate of 0.5 ml/min and analyzed for cholesterol and triglyceride concentrations. Individual lipoprotein fractions (VLDL, IDL/LDL, and HDL) were identified by performing Western blot analysis on ApoB and ApoA-I in each fraction and by testing purified lipoproteins (EMD Biosciences) for their elution profiles in the same running condition.

2.9. Urinary nitrate/nitrite (NOx) levels

Twenty-four urine was collected from mice after 4, 8 and 12 weeks of infusion with saline or IGF-1 and treatment with L-NAME or D-NAME. NOx levels were measured with the Griess method. Briefly, the urine was filtered, diluted with assay buffer and mixed with cofactor and nitrate reductase (NOx colorimetric assay kit, Cayman Chemical Co.). After conversion of nitrate to nitrite, total nitrite was measured by reaction with Griess reagent (sulfanilamide and naphthalene-ethylene diamine dihydrochloride). Amounts of nitrite in the urine were estimated by a standard curve obtained from enzymatic conversion of $NaNO_3$ to nitrite and normalized to animal body weight.

2.10. Statistical analysis

Four groups of mice have been used for this study ($n = 12$ per group) and the entire experiment was repeated two times. Data in graphs is shown as mean ± S.E.M. The experiment with cultured macrophages ($n = 4$ per group) was repeated four times. The IGF-1 effect, L-NAME treatment effect and potential interaction between these effects were analyzed by repeated measures analysis of variance (two-way ANOVA) using GraphPad Prism 4.0 software (GraphPad Software, Inc.) and *P* values of less than 0.05 were considered statistically significant. If interactions between the IGF-1 and

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