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

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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.

Keywords

Atherosclerosis; nitric oxide; growth factors; apoptosis; oxidative stress

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

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Disclosures
None

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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-NAME [8] or the inactive enantiomer, 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 provided in Supplement (Supplemental Table 1, 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 48h 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 16h and gene expression analysis was performed by quantative 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

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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 uM, 1h) served as the negative control for anti-N-tyrosine IHC (Supplemental Figure 1A). Normal mouse IgG was the control for anti-α-SMA and anti-8-oxo-dG antibody (Supplemental Figure 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.

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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 NaNO3 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 ± SEM. 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 L-NAME effects were statistically significant, one-way ANOVA with Bonferroni posttest was applied. Analysis for presence of potential outliers in the data-set was performed with Grubb's test.

3. Results

3.1. L-NAME reduced nitric oxide bioavailability and increased blood pressure

Apoe$^{-/-}$ mice infused with human recombinant IGF-1 had a 60±9% (D-NAME group) or 74±12% increase (L-NAME group) in total (mouse plus human) serum IGF-1 levels compared to saline-infused controls (P<0.001) (Figure 1A). Mouse (endogenous) IGF-1 levels were reduced in IGF-1-infused mice compared to saline controls (D-NAME group, ng/ml, IGF-1: 206.0±28.6 vs. Saline: 280.7±22.6, P<0.05; L-NAME group, ng/ml, IGF-1: 174.7±19.3 vs. Saline, 276.0±18.75, P<0.01), consistent with a normal negative feedback effect. IGF-1 increased body weight in both D- or L-NAME-treated mice (Figure 1B). L-NAME administration had no significant effect either on circulating IGF-1 levels or mouse body weight but caused an elevation in blood pressure (BP) compared to D-NAME controls. The L-NAME-induced BP increase reached statistical significance on the 3rd, 4th and 5th weeks and there was a strong trend for BP elevation in the L-NAME groups on the 7th, 8th and 10th weeks of the experiment (Figure 1C). This effect was consistent with prior reports that NO deficiency elevated blood pressure in mouse models [11] [12]. We measured urinary nitrate/nitrite (NOx) (Figure 1D), a systemic index of NO bioavailability [13]. L-NAME administration suppressed basal NOx levels by 30±4% (Saline/L-NAME vs. Saline/D-NAME, P<0.01). IGF-1 increased NOx in the D-NAME group (65±8% increase vs. Saline, P<0.01) after 8 weeks of infusion and this effect was completely blunted by L-NAME administration. IGF-1 also increased NOx levels in the D-NAME group (but not in L-NAME-treated animals) after 4 and 12 weeks of infusion (data not shown).

IGF-1 induced a weak "insulin-like" effect on blood glucose levels. Glucose levels at 12 weeks were slightly reduced in IGF-1-infused mice (D-NAME, mg/dL, IGF-1: 96±24 vs. Saline: 146±52, P=NS; L-NAME, IGF-1:155±37 vs. Saline: 200±61, P=NS). L-NAME induced a modest increase in blood glucose levels but this effect was not statistically significant. IGF-1 slightly reduced total cholesterol levels (Supplemental Figure 2) but this effect was not statistically significant, consistent with our previous report [9]. IGF-1 did not alter cholesterol content in serum VLDL, LDL and HDL fractions (data not shown) and

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serum lipid profiles were not affected either by IGF-1 or L-NAME as assessed by FPLC-based serum lipoprotein fractionation (Supplemental Figure 3).

3.2. L-NAME did not alter IGF-1's effects on atherosclerotic plaque burden, plaque composition and cell apoptosis

Atherosclerosis burden was measured by quantification of surface lesion area on Oil Red-stained whole aortas (*en face* analysis) and also by measuring plaque size on H&E-stained aortic valve cross-sections. IGF-1 reduced atherosclerosis in the D-NAME group (*en face*: 37% decrease, aortic valve cross-sections: 10% decrease), and this IGF-1-induced anti-atherogenic effect was not altered by L-NAME (*en face*: 39.5% decrease, aortic valve cross-sections: 29% reduction) (Fig.2) suggesting that NO does not mediate IGF-1-induced reduction of atherosclerosis. L-NAME increased atherosclerotic burden of whole aortas by 65% (Saline/L-NAME vs. Saline/D-NAME, $P<0.05$) but L-NAME did not significantly change aortic valve atherosclerotic burden. We have shown previously that SMC-specific IGF-1 overexpression increases plaque SMC and collagen levels in Apoe$^{-/-}$ mice suggesting that IGF-1 increased atherosclerotic plaque stability [14]. In the current study we determined whether an increase in circulating IGF-1 also produced these effects and whether they were NO-dependent. We found that plaque SMC (α-SMA staining) were significantly upregulated by IGF-1 in both D-NAME- (2.05±0.22-fold increase) and L-NAME-treated animals (1.82±0.21-fold increase) (Fig.3A,B) indicating that NO did not mediate the ability of IGF-1 to increase α-SMA expression. IGF-1 increased the number of α-SMA/DAPI-double positive cells in the atherosclerotic plaque and L-NAME did not change this effect (D-NAME, Saline, 47.9±11.9; IGF-1, 94.5±17.3, $P<0.05$; L-NAME, Saline, 50.0±10.9, IGF-1, 135.3±22.3, $P<0.01$). Interestingly, IGF-1-treated mice had only a trend to slightly increased plaque collagen levels in both D-NAME and L-NAME groups (Fig.3A,C) suggesting that the increase in circulating IGF-1 was not sufficient to increase collagen, in contrast to the effect of SMC-specific IGF-1 overexpression [14].

Total plaque cell apoptosis was reduced by IGF-1 in both D-NAME- and L-NAME-treated mice (37±5% decrease compared to saline, $P<0.05$ and 30±4% decrease, $P<0.05$, respectively) (Fig.3D). Interestingly, IGF-1 had no effect on SMC cell apoptosis (data not shown) indicating that the anti-apoptotic effect of IGF-1 was exerted on non-SMC plaque cells.

3.3. Nitric oxide is required for IGF-1-induced anti-oxidant effects

We have shown previously that IGF-1 reduced superoxides in the plaque-free aortic wall and suppressed systemic oxidative stress in Apoe$^{-/-}$ mice [9]. In the current study IGF-1-induced anti-oxidant effects were assessed directly in the atherosclerotic plaque by immunostaining with anti-8-oxo-d-guanosine (8-oxo-dG) and anti-N-tyrosine (NT) antibodies (Fig.4A), to measure oxidative stress-induced DNA and protein modification, respectively. Eight-oxo-dG- and NT-immunopositivity was markedly stronger in the atherosclerotic plaque region (especially, in the plaque cap) compared to the adjacent aortic valve intima suggesting increased oxidative stress in atherosclerotic plaque (data not shown). IGF-1 reduced 8-oxo-dG levels in the D-NAME group (51.8% decrease vs. saline control, $P<0.01$) and this effect was markedly blunted by L-NAME (17.9% decrease, P=NS) (Fig.4B). Similarly, IGF-1 reduced the NT signal in D-NAME-treated mice (54.2% reduction vs. saline control, $P<0.05$) and this effect was also inhibited by L-NAME (35% decrease, P=NS) (Fig.4C). These data show that IGF-1-induced reduction in oxidative stress is NO dependent.

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3.4. Profiling of atherosclerosis-related molecules

To identify potential mediators of IGF-1-induced effects we measured aortic gene expression of 86 atherosclerosis-related molecules using a PCR array (Supplemental Table 3). IGF-1 decreased expression of the pro-atherogenic molecules macrophage scavenger receptor 1 (MSR-1/SR-A) (L-NAME group) and kinase insert domain protein receptor (VEGFR2) (D-NAME group) and, surprisingly, IGF-1 upregulated expression of the chemokine RANTES (CCL5) in the L-NAME group. IGF-1 decreased TNF-α expression and downregulated TNF-α-induced protein 3 mRNA levels in the D-NAME group only. To confirm this effect of IGF-1 on TNF-α expression we perform independent real-time PCR analysis of aortic TNF-α expression (Fig. 5A). IGF-1 markedly reduced aortic TNF-α mRNA levels (consistent with our previous report [9]) and this effect was blunted by L-NAME. Furthermore, IGF-1 reduced circulating TNF-α levels (34% decrease vs. saline), and this effect was also blunted by L-NAME (Fig.5B). Lipoprotein lipase (LPL) is a key pro-atherogenic enzyme [15] secreted by macrophages in high levels [16] [17]. Our PCR arrays indicated that IGF-1 reduced LPL expression in both D-NAME and L-NAME groups (Supplemental Table). We confirmed these findings using independent real-time PCR and found that IGF-1 suppressed aortic LPL expression to a similar extent in the control group (D-NAME) (46% decrease vs. saline, $P<0.05$) and in mice treated with L-NAME (59% decrease, $p<0.05$) (Fig.5C) indicating that this effect was not dependent on nitric oxide.

We performed experiments with cultured human THP-1 macrophages to determine whether IGF-1 regulates TNF-α and LPL expression in this cell model and to determine if these effects were NO-dependent. IGF-1 downregulated TNF-α expression in macrophages ($P<0.05$) and this effect was completely blocked by the NOS inhibitor (Fig.6A). IGF-1 also reduced expression of LPL in D-NAME-treated cells; however this effect was not altered by L-NAME (Fig.6B).

4. Discussion

In our first report that IGF-1 reduced atherosclerosis in Apoe$^{-/-}$ mice fed a Western diet [9] we had identified multiple potential mechanisms, including an anti-oxidant effect of IGF-1 as evidenced by reduced aortic superoxide and urinary 8-isoprostane levels, and an increase in NO bioavailabilty, resulting perhaps in part from IGF-1 induced increase in vascular eNOS expression and activation. Recently we found that congenic 6T/Apoe$^{-/-}$ mice with a ~20% decline in circulating IGF-1 had a significant increase in atherosclerotic burden and reduced vascular eNOS expression [18]. These findings suggested that the ability of IGF-1 to increase NO bioavailability could play an important role in the atheroprotective actions of IGF-1. Indeed there is abundant data indicating that NO has atheroprotective effects. NOS1-knockout mice exhibited accelerated neointimal formation following carotid artery ligation [19], indicating a vasculoprotective effect of neuronal NOS *in vivo*. On the contrary, NOS2 deficiency resulted in reduced atherosclerosis in Apoe$^{-/-}$ mice [20] suggesting a pro-atherogenic role of this enzyme. A deficiency of NOS3/eNOS accelerated atherosclerotic lesion formation in Apoe$^{-/-}$ mice [6] although in the setting of hypercholesterolemia a depletion of BH4, an essential cofactor for the enzyme, may lead to eNOS “uncoupling”, superoxide production and pro-atherogenic effects of eNOS [21] [22]. The overall contribution of all three NO synthases and the role of systemic NO deficiency in atherosclerosis were elucidated by using triple NOS$^{-/-}$ mice which had increased blood pressure and markedly elevated atherosclerosis burden following a high cholesterol diet, consistent with a major anti-atherogenic role of nitric oxide [23]. Similar results were obtained using L-NAME to inhibit endogenous NO [24]. Interestingly our current study indicates that although L-NAME produced an expected increase in atherosclerotic plaque burden (Fig.2C) it did not abrogate the ability of IGF-1 to reduce plaque burden indicating that the atheroprotective effect of IGF-1 was NO independent.

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We reported recently that SMC-specific IGF-1 overexpression increased features of atherosclerotic plaque stability [18], in particular, plaque SMC content and collagen expression. Our current data indicates that IGF-1 infusion similarly increased plaque SMC content as demonstrated by an increase in α-SMA staining and in the number of α-SMA/DAPI double-positive cells. Our finding is consistent with a recent report that infusion of a stable IGF-1 analogue in Apoe$^{-/-}$ mice increased SMC content in the advanced plaque and reduced the rate of intra-plaque hemorrhage [25]. In addition to increasing SMC content IGF-1 reduced plaque apoptosis (Fig 3D). IGF-1 has been reported to prevent apoptosis in multiple cell culture models including SMC [26], THP-1 macrophages [27] and cardiac myocytes [28]; however to our knowledge our current findings are the first to demonstrate that IGF-1 reduces total cell apoptosis *in vivo* in the atherosclerotic plaque. Both the effects of IGF-1 to increase SMC content and to reduce plaque apoptotic rate were NO-independent suggesting that both could potentially contribute to the anti-atherosclerotic effect of IGF-1. However it is pertinent to note that SMC-specific IGF-1 overexpression, while markedly increasing the number of differentiated SMC, did not reduce total plaque burden [14], suggesting that the ability of infused IGF-1 to reduce plaque burden is unlikely to be due mainly to the increase in SMC content. Of note, contrary to the effects of IGF-1 overexpression in SMC, an increase in circulating IGF-1 did not significantly increase plaque collagen content (Fig 3).

We have shown previously that IGF-1 reduced superoxides in the plaque-free aortic wall in Apoe$^{-/-}$ mice on a Western diet [9]. We now demonstrate that IGF-1 inhibits oxidative stress specifically in the atherosclerotic plaque. The IGF-1-induced antioxidant effect was NO-dependent (at least in part) since the ability of IGF-1 to reduce oxidative stress was significantly blunted by L-NAME (Fig. 4). This finding is consistent with the ability of IGF-1 to upregulate eNOS expression and activity [29] [30] leading to increased NO levels *in vivo* and *in vitro* [31] [32] [33]. Contrary to its anti-oxidant effect, IGF-1-induced suppression of atherosclerosis was NO-independent. IGF-1 induced a similar reduction in atherosclerosis in L-NAME-treated mice compared to control indicating that IGF-1's anti-atherogenic effect does not depend on nitric oxide. IGF-1-induced suppression of cell apoptosis and downregulation of LPL were also NO-independent and as such could be involved in the ability of IGF-1 to suppress atherosclerosis. Macrophage-specific LPL is known to increase binding of oxidized lipids to macrophages thereby promoting formation of foam cells and accelerating atherosclerosis in Apoe$^{-/-}$ mice[34]. We found that IGF-1 suppresses LPL expression in cultured macrophages and also in atherosclerotic aortas and the latter effect correlated with decreased aortic lipid levels (measured by staining with Oil Red O). Patients with growth hormone deficiency had reduced serum IGF-1 levels and higher macrophage LPL expression and macrophages from these patients were more susceptible to foam cell formation [35]. These data, taken together with our findings, suggest a novel link between IGF-1, LPL downregulation and atherosclerosis suppression.

In summary, we have demonstrated that IGF-1 increases NO levels, suppresses plaque cell apoptosis and downregulates TNF-α in Apoe-null mice, however NO is not required for IGF-1-induced anti-atherogenic effects. IGF-1 reduces oxidative stress and increases SMC levels in the atherosclerotic plaque and also downregulates LPL expression *in vivo* and in cultured macrophages. IGF-1 downregulation of LPL is NO-independent and represents a novel potential mechanism mediating IGF-1's anti-atherosclerotic effects.

Supplementary Material

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List of 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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Highlights

- IGF-1 increases nitric oxide and suppresses atherosclerosis.
- IGF-1-induced anti-atherosclerotic effect does not depend on nitric oxide bioavailability.
- IGF-1 decreases cell apoptosis and reduces oxidative stress in the atherosclerotic plaque.
- IGF-1's anti-oxidant effect is partially nitric oxide-dependent.
- IGF-1 downregulates lipoprotein lipase *in vivo* and in cultured macrophages and these effects are nitric oxide-independent.

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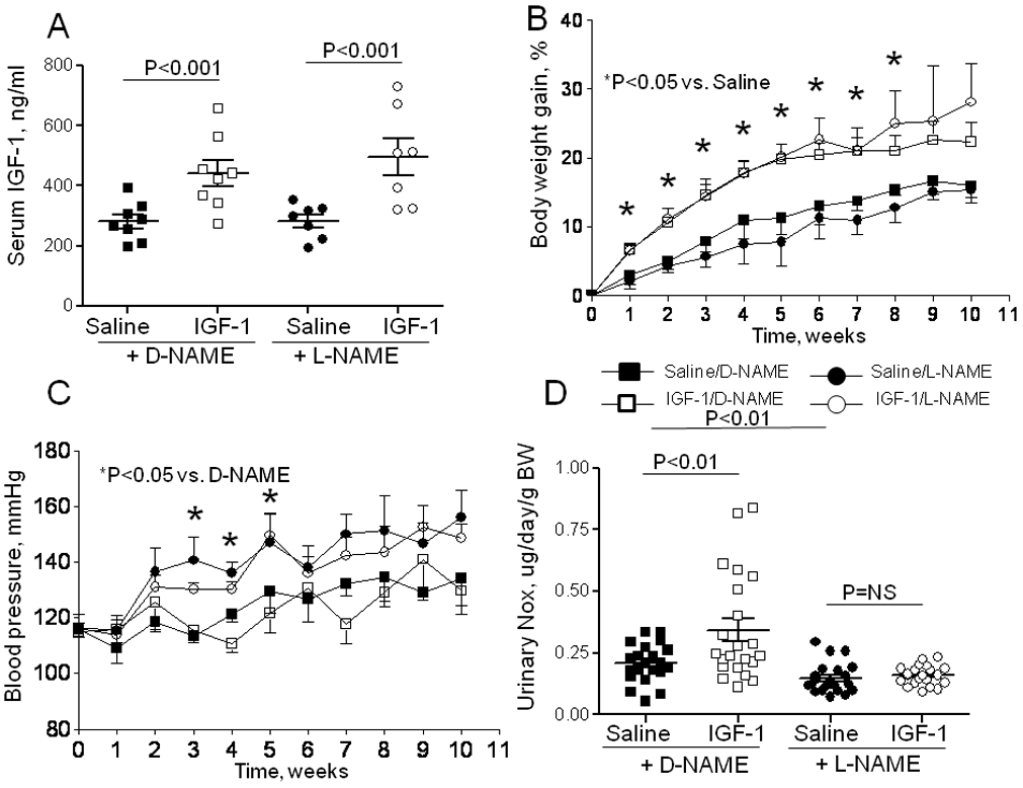

Figure 1. Serum IGF-1 levels (A), body weight (B), blood pressure (C), and urinary nitrates/ nitrites (NOx) levels (D)

Apoe-null mice were infused with saline or IGF-1 using osmotic mini-pumps and fed a Western-type diet for 12 weeks in presence of nitric oxide synthase inhibitor L-NAME or D-NAME (control). Mouse body weight were measured every week and expressed as weight gain ratio (%) per body weight at day of pumps implantation. IGF-1 levels were measured by ELISA (n=8) and urinary NOx levels by colorimetric assay followed by normalization to animal body weight. Data are shown from representative experiment (A, B, C) and NOx data (D) combined from 2 independent experiments (D).

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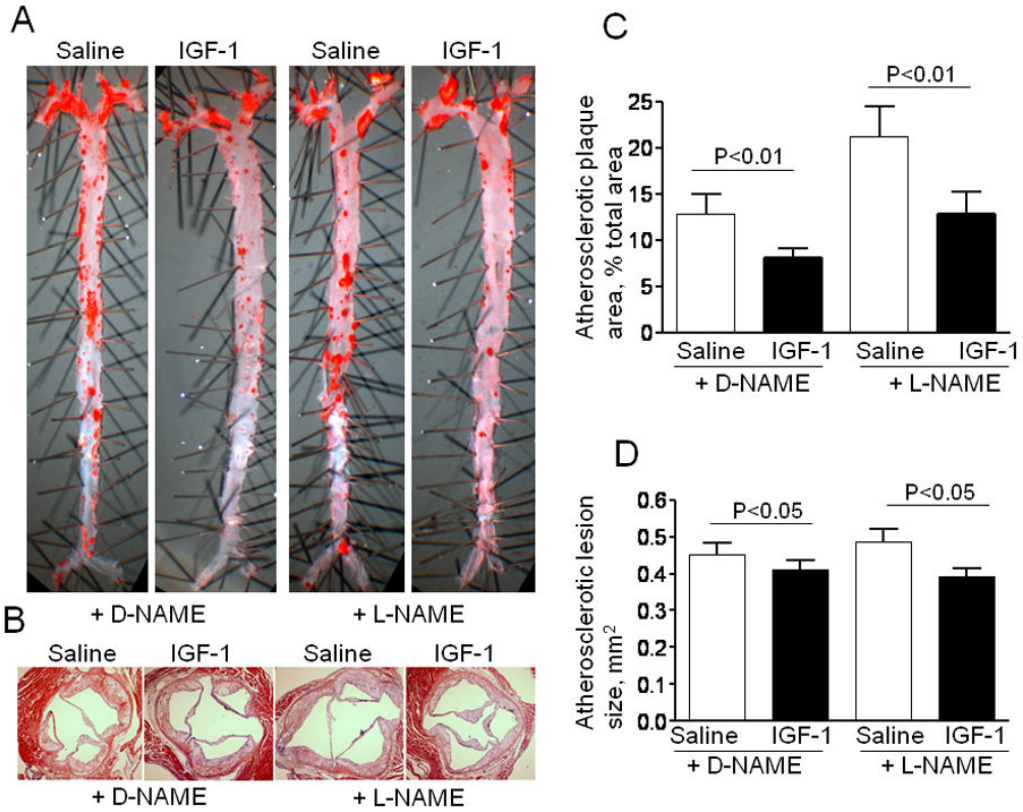

Figure 2. IGF-1 reduced atherosclerotic lesion surface area on whole aortas (A,C) and decreased lesion size on aortic valve cross-sections (B,D) in L-NAME-treated mice and, similarly, in control group

Apoe-null mice were infused with saline or IGF-1 and fed a Western-type diet in presence of L-NAME or D-NAME (control). Animals were perfused/fixed and hearts and aortas were dissected. Aortas were stain *en face* with Oil Red O (A) and hearts were paraffin-embedded and aortic valves cross-sections were stained with H&E (B). Lesion surface area (C) or cross-sectional size (D) was quantified by manual outlining in Image Pro software. Data are shown from representative experiment (n=12).

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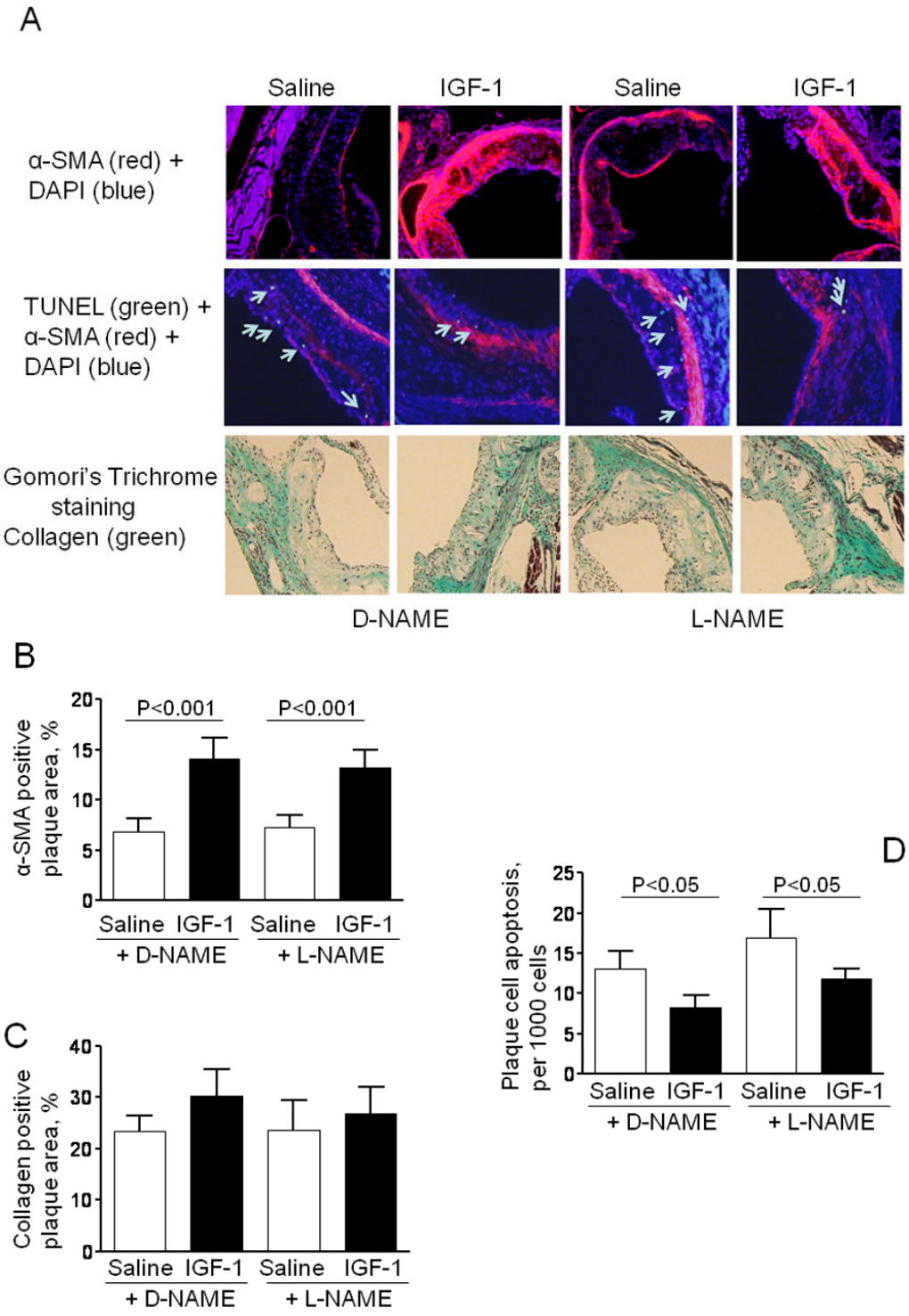

Figure 3. IGF-1 increases plaque SMC content (A,B), suppresses cell apoptosis (A,D) and elevates collagen levels (A, C) in L-NAME-treated mice and, similarly, in control group

Aortic valves cross-sections were stained with anti-α-smooth muscle actin (α-SMA) antibody to identify SMC or co-stain with anti-α-SMA antibody and TUNEL stain to identify apoptotic cells and apoptotic SMC in the plaque. Gomori's Trichrome stain was used to identify plaque collagen. α-SMA positive (B) or collagen (C) plaque area was

manually outlined in Image Pro and normalized per plaque area. Apoptotic cell number was normalized per total plaque cell number (D) or SMC number in the plaque using DAPI. Representative images are shown for each group (n=12).

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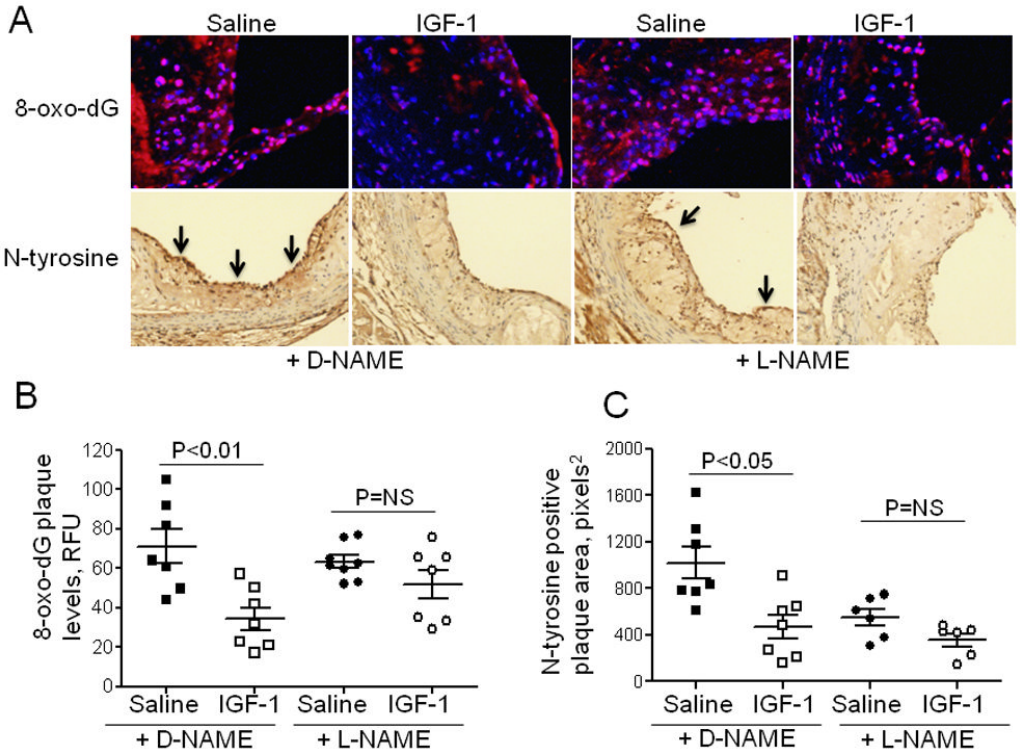

Figure 4. IGF-1 decreases oxidative stress in the atherosclerotic plaque and this effect is partially blocked by L-NAME

Aortic valves cross-sections were stained with anti-8-oxo-dG antibody (A, B) to measure oxidative stress-induced DNA damage or with anti-N-tyrosine antibody (A, C) to quantify oxidative modification of proteins. Streptavidin-Alexa594 (red) (for 8-oxo-dG) or streptavidin-HRP/DAB (brown) (for N-tyrosine) signals have been quantified with Image Pro. Black arrows on N-tyrosine-stained sections indicate strong positivity for this marker in plaque endothelial cells layer. Representative images are shown for each group (n=6–8).

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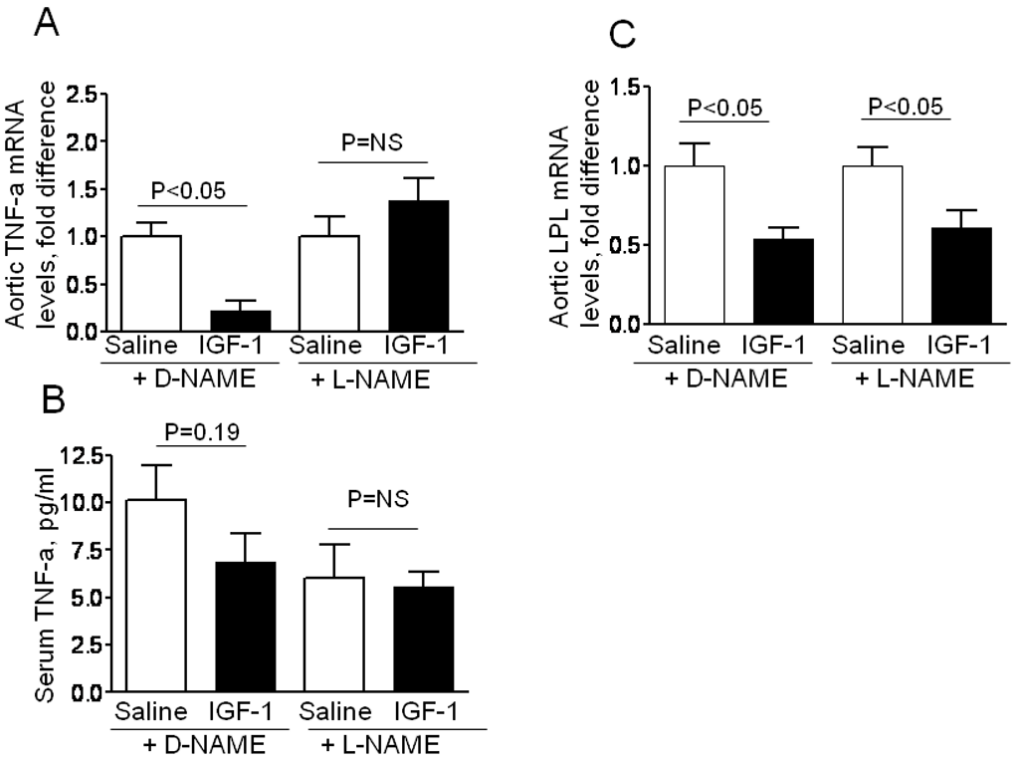

Figure 5. IGF-1-induced reduction in TNF-α but not in lipoprotein lipase (LPL) is nitric oxide-dependent

Aortic TNF-α and LPL mRNA levels (A,C) were measured using real time RT-PCR and serum TNF-α levels (B) with ELISA kit (n=5).

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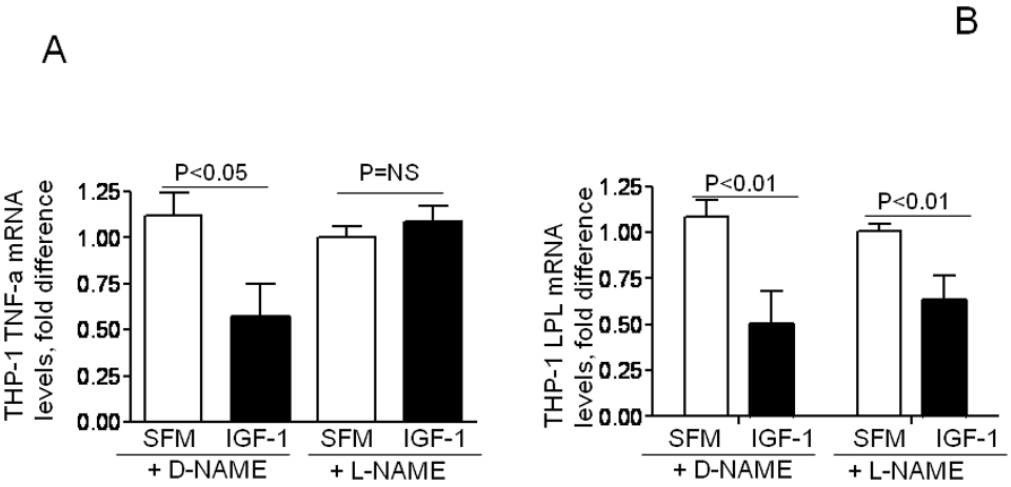

Figure 6. IGF-1-induced reduction in macrophage-specific TNF-α but not in macrophage lipoprotein lipase (LPL) is nitric oxide-dependent

Human THP-1 cells were differentiated into macrophages with phorbol 12-myristate 13-acetate and treated with serum-free media (SFM, control) or with 50 ng/ml human recombinant IGF-1 and gene expression analysis was performed by quantative real-time RT-PCR. The experiment was repeated four times (n=4 per group) and results of a representative experiment are shown on the graph.