IGF-1 Reduces Inflammatory Responses, Suppresses Oxidative Stress, and Decreases Atherosclerosis Progression in ApoE-Deficient Mice

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Objective—Whereas growth factors, via their ability to stimulate vascular smooth muscle cell (VSMC) proliferation and migration, have been thought to play a permissive role in atherosclerosis initiation and progression, the role of insulin-like growth factor-1 (IGF-1) is unknown. Here we report for the first time that IGF-1 infusion decreased atherosclerotic plaque progression in ApoE-deficient mice on a Western diet.

Methods and Results—ApoE-null mice (8 weeks) were infused with vehicle or recombinant human IGF-1 and fed a high-fat diet for 12 weeks. Analysis of aortic sinuses revealed that IGF-1 infusion decreased atherosclerotic plaque progression and macrophage infiltration into lesions. Furthermore, IGF-1 decreased vascular expression of the proinflammatory cytokines interleukin-6 and tumor necrosis factor-α, reduced aortic superoxide formation and urinary 8-isoprostane levels, and increased aortic pAkt and eNOS expression and circulating endothelial progenitor cells, consistent with an antiinflammatory, antioxidant, and prorepair effect on the vasculature.

Conclusions—Our data indicate that an increase in circulating IGF-1 reduces vascular inflammatory responses, systemic and vascular oxidant stress and decreases atherosclerotic plaque progression. These findings have major implications for the treatment of atherosclerosis. **(*Arterioscler Thromb Vasc Biol*. 2007;27:2684-2690.)**

Key words: insulin-like growth factor ■ atherosclerosis ■ apolipoprotein E ■ inflammatory response ■ oxidative stress

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,[2–5] in which oxidative stress plays a key initiating role. Thus, multiple oxidative stimuli including oxidation of low-density lipoprotein (LDL) in the subendothelial space can result in endothelial cell adhesion molecule expression, monocyte and T cell recruitment, and macrophage lipid accumulation and foam cell formation.[6] Growth factors, cytokines, chemokines, and proteases modulate many steps in the atherosclerotic process including the migration and proliferation of smooth muscle cells. Furthermore, vascular and extravascular progenitor cells contribute to atherogenesis, and these include circulating endothelial progenitor cells (EPCs) that are likely part of a vascular repair system.[7]

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 (GH) on developmental growth.[8] IGF-1 is expressed in vascular cells[9] and in monocyte/macrophages,[10,11] but its role in atherogenesis is unknown. IGF-1 is a mitogen for endothelial cells[12] and vascular smooth muscle cells,[13] and can potentiate endothelial cell TNF-α–induced c-Jun and NF-κB activation.[14] Furthermore, IGF-1 has potent survival effects on vascular cells and prevents oxidized LDL–induced apoptosis of vascular smooth muscle cells.[15] We have previously shown that oxidized LDL downregulates IGF-1 and IGF-1 receptor expression in vascular smooth muscle cells[16,17] and that expression of these molecules is reduced in areas of advanced human plaque staining positive for oxidized LDL,[18] suggesting that decreased IGF-1 activity could contribute to the atherosclerotic process. Similar findings were reported in cultured plaque-derived smooth muscle cells.[19] To investigate the potential role of IGF-1 in atherogenesis we infused recombinant human IGF-1 in ApoE-deficient mice. Our findings demonstrate that increased circulating IGF-1 reduces systemic and vascular oxidant stress and vascular cytokine expression and decreases atherosclerosis progression. These

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findings provide fundamental new insights into biological effects of IGF-1.

Methods

Material

Recombinant human IGF-1 was from Tercica. Anti–phospho-Akt, Akt, phospho-eNOS, eNOS, Sca-1, and Flk-1 antibodies were from BD Biosciences. Dihydroethidium (DHE) was from Invitrogen. L-NAME and D-NAME were from Sigma-Aldrich.

Animals

All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. ApoE-null mice (C57BL/6, 8 weeks of age) were infused with saline or IGF-1 (1.5 mg/kg/d) and fed a Western-type diet (42% of total cal from fat; 0.15% cholesterol) for 12 weeks. Mice were housed individually and maintained on a 12-hour light–dark cycle.

Preparation of Native and Oxidized LDL

Native LDL (nLDL) was separated from human plasma of healthy donors (purchased from The Blood Center, New Orleans, LA) by sodium bromide stepwise density gradient centrifugation. Oxidized LDL (OxLDL) was prepared as previously described.[17] Briefly, an aliquot of the nLDL fraction was passed through a 10-DG desalting column, and then incubated with 5 μmol/L $CuSO_4$ at 37°C for 3 hours. The value for thiobarbituric acid-reactive substances (TBARS) in OxLDL was 37.2±1.2 nmol/L malondialdehyde per mg protein. TBARS was not detectable in nLDL.

Atherosclerosis Quantification

Under anesthesia, IGF-1– or saline–infused mice were perfused with saline then 4% buffered paraformaldehyde plus 5% sucrose and the heart dissected, fixed overnight, and paraffin embedded. Serial sections (6 μm) were taken throughout the entire aortic valve area (AVA) as per Paigen et al,[20] and routinely stained with hematoxylin and eosin (H&E). To quantitate plaque size, images of 3 sections of each AVA were acquired with a DP70 digital camera connected to a microscope (Olympus), and lesion areas determined using Image-Pro plus v 6.0 software (Media Cybernetics Inc). The mean value of plaque cross-sectional areas from 3 sections was used to estimate the extent of atherosclerosis for each animal. We also quantified atherosclerosis using Oil Red O–stained frozen sections of aortic valves. Serial sections (20 μm) were cut from the site where the aorta valve cusps appear to the aorta root, and every other section was stained with Oil Red O (5 mg/mL for 12 minutes) and counterstained with hematoxylin before quantification. For a limited number of animals atherosclerosis was quantified by measuring surface area of Oil Red O–positive lesions on en face preparations of whole aortas. Under anesthesia, the mouse was perfused through the left ventricle as mentioned above then the entire aorta was dissected and placed overnight in formaldehyde. The adventitial fat was dissected, the aorta was stained with Oil Red O and opened longitudinally, pinned en face, and photographed. The total arterial surface area and total lesion area were determined using Image-Pro Plus. The extent of lesion development was defined as percentage of the total area of a given artery that was occupied by Oil Red O–positive atherosclerotic lesions.

Immunohistochemical Analysis

Serial 6-μm paraffin-embedded cross sections were taken throughout the entire AVA, and 3 sets of serial sections obtained at 60-μm intervals were used for measurement of macrophage lesion area. Every first section in each set was stained with H&E, and plaque area size was estimated as described above. The second and third sections in each set were incubated with rat anti-mouse Mac-3 monoclonal antibody (1:20, M3/84 clone, BD Pharmingen) or with isotype-matched rat IgG (Abcam) followed by incubation with biotinylated secondary antibody and avidin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories Inc). Sections were developed with DAB substrate kit, and counterstained with hematoxylin.

Aortic Superoxide Measurement

Aortic superoxide levels were measured with DHE on serial frozen sections (10 μm) obtained from the root of aorta. DHE specificity was previously validated using smooth muscle cells exposed to OxLDL.[21] Two serial sections each from IGF-1–infused and saline-infused mice were tested in parallel. One pair of sections from IGF-1 and saline-infused mice were pretreated with superoxide scavenger polyethylene glycol (PEG)-superoxide dismutase (PEG-SOD, 100 U, Sigma)[22] in buffer (50 mmol/L Tris-HCL, pH 7.4), and another pair of sections was preincubated with buffer only. All 4 sections were stained by DHE (2 μmol/L, 45 minutes, 37°C) in the dark in a humidified chamber, briefly washed, and quickly imaged with a fluorescent microscope keeping the same exposure for every section. DHE fluorescence was quantified by averaging the mean fluorescence intensity within 3 identical circles placed on a plaque-free area of aortic wall using Image-Pro Plus. The superoxide-induced DHE signal was expressed as PEG-SOD-inhibitable fluorescence after subtraction of the DHE signal obtained from scavenger-pretreated section. Three to 5 sections from each animal (n=4 for each saline-or IGF-1-infused group) were analyzed using this procedure and the average superoxide-induced DHE fluorescence was calculated.

Urine 8-Isoprostane Assay

Urine was collected for 24 hours in the presence of antioxidant butylated hydroxytoluene (BHT) (1 mmol/L), filtered with 0.2 μm filters, and frozen at −80°C. Commercially available ELISA (Cayman Chemical) was used to measure urinary 8-isoprostane (8-IP) levels, and the results were expressed relative to the urinary creatinine levels determined by colorimetric assay (Cayman Chemical). All assays were repeated 3 times.

Quantitative Real-Time RT-PCR

Total RNA extraction and real-time PCR was performed as previously described.[21] Briefly, total aortic RNA was isolated using the TriPure Isolation Reagent (Roche) followed by purification with the RNeasy mini kit (Qiagen). cDNA was synthesized using the First Strand cDNA Synthesis kit (Amersham) and used for the 40-cycle 2-step PCR with sequence-specific primer pairs in the iCycler IQ Real-Time Detection System (Bio-Rad).

Flow Cytometry

White blood cells in EDTA-treated whole blood were immunostained with R-phycoerythrin (PE)-conjugated anti-mouse Flk-1 monoclonal antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse Sca-1 monoclonal antibody and were analyzed by flow cytometer immediately. Sca-1+/Flk-1+ cells were considered to be endothelial progenitors, and were counted to evaluate circulating EPC number.

Cell Culture

Human aortic endothelial cells (HAECs) were obtained from Lonza and maintained in EGM-2 medium with supplements. Cells at passages 2 to 10 were used for experiments. Cultured human aortic smooth muscle cells (HASMCs; Cambrex Bio Science, Walkersville, Md) were grown in SmBM medium supplemented with 5% fetal calf serum, antibiotics, and supplements. The cells were used for experiments at passages 4 to 10.

Quantification of Reactive Oxygen Species Production

Generation of intracellular superoxide was measured as described by Higashi et al,[17] using DHE fluorescence. HAECs were grown to confluence on a 96-well plate and were washed once with phenol red-free EGM medium. The cells were preincubated in the same medium containing 100 ng/mL IGF-1 and 100 μmol/L D-NAME or L-NAME for 1 hour, and subsequently treated with 60 μg/mL native

LDL or OxLDL for 2 hours. Superoxide formation was determined by staining cells with 5 μmol/L DHE for 30 minutes. The fluorescent intensity was read directly from the culture plate at emission wavelength of 590 nm and excitation wavelength of 485 nm. HASMCs were preincubated for 24 hours with 0 to 100 ng/mL IGF-1 before exposure to 60 μg/mL native LDL or OxLDL or 100 nmol/L Ang II and reactive oxygen species (ROS) production was measured as described above.

Statistical Analysis

All numerical data are expressed as mean±SEM. Two-tailed unpaired Student *t* tests were performed to determine statistical significance. Differences were considered significant at $P<0.05$.

Results

IGF-1 Reduces Macrophage Infiltration, TNF-α Levels, and Decreases Atherosclerosis Progression in ApoE$^{-/-}$ Mice

IGF-1 and saline-infused ApoE-deficient mice fed a Western diet developed early stages of atherosclerotic lesions (presence of foam cells were evident) and advanced lesions (cholesterol crystals and acellular areas were present). Morphometric analysis of atherosclerotic plaque accumulation on the intimal surface of the aortic valve revealed that IGF-1-infused mice had a 27% reduction in lesion size compared with control mice (0.259±0.018 mm^2 versus 0.356±0.031 mm^2; Figure 1A). En face analysis of whole aortas revealed that IGF-1–infused mice had a similar 28% reduction in lesion size (IGF-1, 8.61±0.53% versus control 12.01±2.76%, n=7), although this effect was not statistically significant ($P=0.22$). We estimated macrophage levels within the atherosclerotic lesions of IGF-1–infused and control mice as an index of atherosclerotic burden and also as an indicator of inflammatory responses. The plaque area staining positive for macrophages in IGF-1–infused mice was decreased by 36% compared with control (0.050±0.003 mm^2 versus 0.078±0.007 mm^2; Figure 1B) with a slight reduction in macrophage area/lesion area ratio (20.2±1.7% versus 25.3±1.8%, $P=0.07$). TNF-α–immunopositive plaque area was also decreased by 58.3% in IGF-1–infused mice compared with control ($P<0.05$; Figure 1C). These data demonstrate that IGF-1 reduced macrophage infiltration and atherosclerotic plaque progression over 12 weeks, consistent with an antiinflammatory effect.

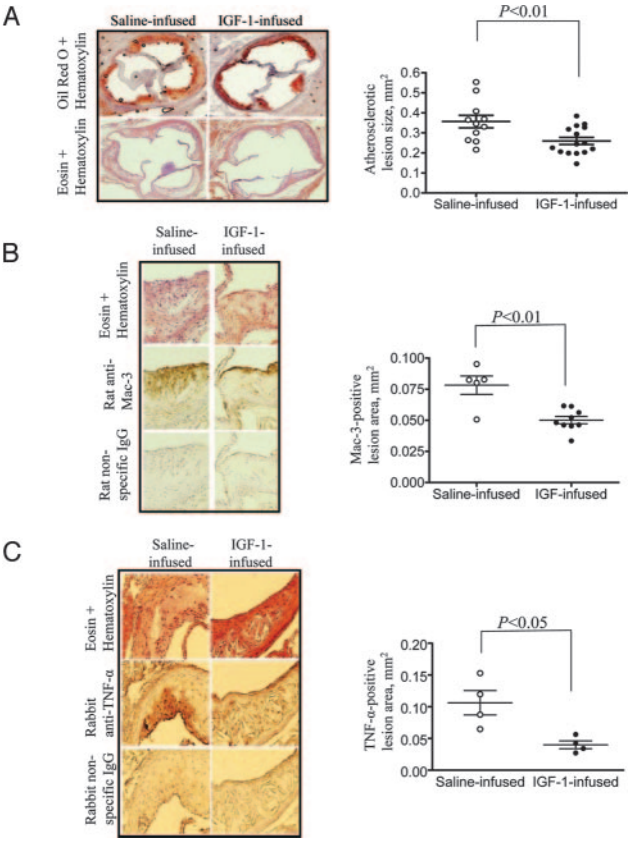

Figure 1. IGF-1 suppresses atherosclerotic plaque progression, reduces macrophage accumulation, and decreases TNF-α level in aortic valves of ApoE-deficient mice. A, Frozen or paraffin-embedded cross sections from saline- or IGF-1–infused ApoE-deficient mice were obtained throughout the aortic valve area and stained with Oil Red O and hematoxylin or H&E, respectively, then imaged and quantified with Image-Pro Plus. Magnification ×100. B and C, IGF-1 reduces macrophage accumulation (B) and decreases TNF-α level (C) within atherosclerotic plaque. Paraffin-embedded serial sections were stained with H&E, rat anti-mouse Mac-3 antibody, and with rat non-specific IgG (B) or rabbit anti-mouse TNF-α antibody and rabbit non-specific IgG, respectively (C). Magnification ×400. Aortic lesion size, Mac-3– and TNF-α–positive lesion areas are shown for each mouse from saline-infused group (empty circles) or IGF-1-infused group (solid circles). Vertical bars are mean±SEM per group.

IGF-1 Suppresses Oxidative Stress in ApoE$^{-/-}$ Mice and in Cultured Endothelial Cells

8-isoprostane levels are an index of systemic oxidative stress that correlate with the severity of atherosclerosis in humans and in animal models.[23] We found that IGF-1 infusion decreased urine 8-IP levels by 39% (Figure 2A). To measure the effect of IGF-1 on aortic superoxide levels, frozen sections from the aortic roots of IGF-1–infused and control mice were stained with DHE with/without pretreatment with the superoxide-specific scavenger PEG-SOD (Figure 2B and 2C). IGF-1 markedly suppressed superoxide levels in the aortae of ApoE-deficient mice (85% decrease compared with saline-infused mice; Figure 2C).

We explored whether IGF-1 could reduce oxidative stress in cultured HAECs or HASMCs. Consistent with previous reports[24,25] oxidized LDL induced superoxide formation in endothelial cells after 2 hours of incubation. Intriguingly, coincubation with IGF-1 suppressed oxLDL-induced superoxide generation (66% decrease with 100 ng/mL; n=4, $P<0.001$; Figure 2D). L-NAME, a nitric oxide (NO) synthase inhibitor, did not significantly blunt this antioxidant effect of IGF-1. Superoxide levels with L-NAME tended to be higher than those with the inactive isomer D-NAME, but not significantly so. OxLDL or angiotensin II (Ang II) markedly increased cellular superoxides in HASMCs, however unlike endothelial cells, pretreatment with IGF-1 had no effect on oxLDL- or Ang II–induced superoxides in this cell model (supplemental Figure IA and IB, available online at http://atvb.ahajournals.org). These data suggest that IGF-1–dependent suppression of aortic superoxide levels is primarily mediated via the endothelium.

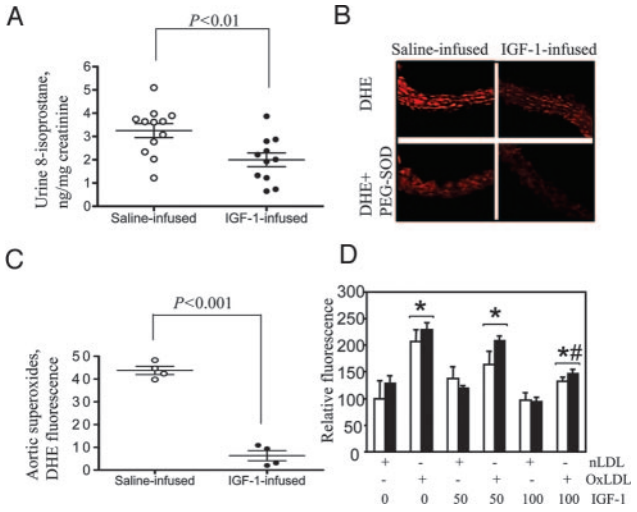

Figure 2. IGF-1 inhibits oxidative stress in vivo and in cultured endothelial cells. A, IGF-1 decreases urine 8-isoprostane levels, an index of systemic oxidative stress. Urine 8-isoprostane (8-IP) levels were normalized to creatinine levels. All assays were repeated 3 times and mean values are shown. B and C, IGF-1 decreases aortic superoxide levels. Aortic superoxides were measured by staining aortic root sections with DHE with or without pretreatment with PEG-SOD. B, Representative images; C, Superoxide-induced DHE signal is expressed as PEG-SOD-inhibitable mean fluorescence for each mouse from saline-infused group (empty circles) or IGF-1–infused group (solid circles). Vertical bars are mean±SEM per group. D, IGF-1 reduces oxLDL-induced ROS generation in human aortic endothelial cells. Human aortic endothelial cells were incubated with indicated concentration (ng/mL) of recombinant human IGF-1 and D-NAME (empty bar; 100 μmol/L) or L-NAME (solid bar; 100 μmol/L) for 1 hour and for another 2 hours with nLDL or OxLDL (60 μg/mL) in addition to IGF-1 and D-/L-NAME. Superoxide generation was determined by coincubating the cells with 5 μmol/L DHE for 30 minutes followed by fluorescent intensity determination. *P<0.001 vs nLDL-treated controls (n=4). #P<0.001 vs OxLDL and no IGF-1–treated cells (n=4).

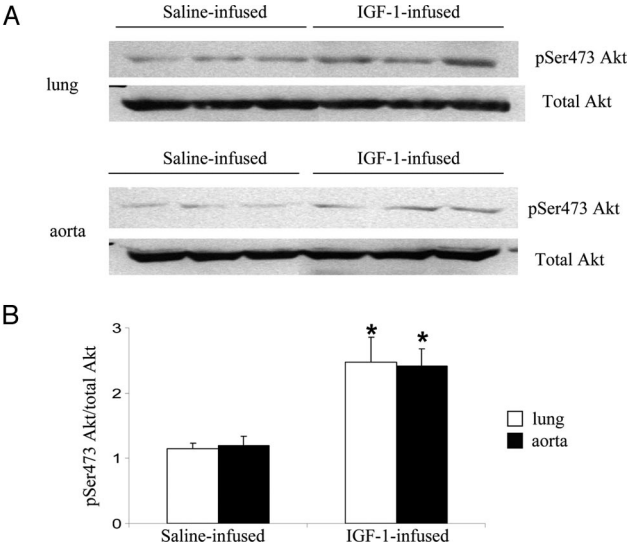

Figure 3. IGF-1 increases Akt phosphorylation in mouse lung and aorta. Lung and aortic homogenates were subjected to immunoblotting analysis with rabbit anti–phospho-Akt and mouse anti-total Akt antibody. Representative Westerns (A) and densitometry data (n=3, B) are shown. *P<0.05 vs saline-infused mice.

IGF-1 Downregulates Vascular Proinflammatory Cytokine Gene Expression and Increases Endothelial NO Synthase Expression and Circulating Endothelial Progenitor Cells

IGF-1 infusion did not change body weight or total cholesterol levels (Table 1), indicating that the atheroprotective effects of IGF-1 were not attributable to elimination of the hypercholesterolemic source of vascular injury in ApoE-deficient mice. Additionally, lipid profiles were not affected by IGF-1 as assessed by high-performance liquid chromatography (HPLC)-based serum lipoprotein fractionation (supplemental Figure II and supplemental Table). We also found no statistically significant difference in blood glucose levels between saline- and IGF-1–infused groups (266±45 mg/dL, n=7 and 233±43 mg/dL, n=5, respectively). However, mouse (endogenous) IGF-1 levels were significantly lower in IGF-1–infused mice (P<0.001, n=7), probably because of a negative feed-back effect. The total concentration of IGF-1 (mouse IGF-1 plus human IGF-1) in IGF-1–infused mice was approximately twice that of saline-infused mice (Table 1). Using Western ligand blot analysis we detected >60% increase in IGF-1 binding protein(s) (IGFBPs) sized ≈30 kDa (n=7, P<0.01) for IGF-1 mice compared with control (data not shown), consistent with an increase in IGFBP-5. Furthermore, IGF-1 infusion produced a 2.5-fold increase in lung and aortic levels of phosphorylated Akt, a key downstream molecule in the IGF-1 signaling pathway (Figure 3).

To further determine mechanisms of the antiatherogenic effect of IGF-1, we measured tissue and circulating levels of IL-6 and TNF-α, proinflammatory cytokines involved in atherosclerotic lesion development. No changes were detected in serum levels of these cytokines; however, aortic IL-6 and TNF-α mRNA levels were reduced 2.9-fold and 4.2-fold, respectively (Table 2), consistent with a reduction in macrophage and TNF-α immunopositivity (see Figure 1B and 1C).

Table 1. Serum IGF-1, Total Cholesterol, and Proinflammatory Cytokines

	Saline-Infused	IGF-1–Infused	P Value
Body weight, g	25.2±0.5	26.6±0.8	0.15
Total cholesterol, mg/dL	617±28	653±27	0.38
Human IGF-1, ng/mL	N.D.	487±78	
Murine IGF-1, ng/mL	287±8	178±15	<0.001
TNF-α, pg/mL	23.2±14.3	16.1±5.2	0.65
IL-6, pg/mL	60.1±12.7	69.2±13.5	0.63

For the body wt, Saline infused: n=13, IGF-1 infused: n=14; For the other measurements, n=7 in each group; ND indicates not detected.

Table 2. Aortic eNOS and Proinflammatory Cytokine Gene Expression

mRNA Expression	Saline-Infused	IGF-1–Infused	P Value
eNOS	1.00	4.36±0.92	<0.05
IL-6	1.00	0.34±0.02	<0.005
TNF-α	1.00	0.24±0.15	<0.05

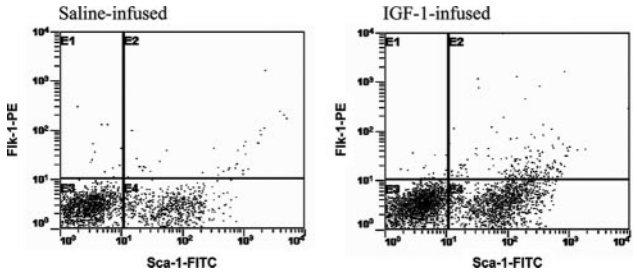

Figure 4. IGF-1 increases circulating endothelial progenitor cells. White blood cells were immunostained with anti–Sca-1 antibody conjugated with FITC and anti–Flk-1 antibody conjugated with PE, and the positively stained population was determined by flow cytometry. The left panel indicates a representative result from 7 independent analyses of the saline-infused mice, and the right from 8 independent analyses of the IGF-1-infused mice.

Circulating endothelial progenitor cells (EPCs) contribute to angiogenesis in normal vascular development and are part of a vascular repair system under pathological conditions including atherosclerosis.[26,27] We measured circulating EPC levels in IGF-1– and saline-infused mice by assessing Sca-1+/Flk-1+ cells by fluorescence-activated-cell sorter (FACS) analysis. There was a strong trend to more circulating EPCs in IGF-1–infused mice compared with control (Figure 4, 3.58±0.82% versus 1.61±0.42%, $P=0.06$, n=8 in IGF-1–infused mice and n=7 in saline-infused mice). This result suggests that IGF-1 promotes endothelial repair by increasing circulating EPCs, potentially contributing to atheroprotection.

Endothelial nitric oxide synthase (eNOS) is the major NO-producing enzyme in the vasculature and as such can exert multiple beneficial effects including an antioxidant effect and atheroprotective effect.[28] It has been reported that eNOS activity is essential for IGF-1–stimulated EPC mobilization,[29] and eNOS expression is inhibited in advanced atherosclerosis.[30] We found that IGF-1 infusion markedly upregulated eNOS gene expression in the aorta (Table 2) and IGF-1 increased eNOS Ser1177 phosphorylation in cultured endothelial cells (supplemental Figure IIIA). Because increased eNOS expression and phosphorylation could mediate an increase in NO levels, we measured plasma and urinary nitrate/nitrite (NOx) as a systemic index of NO bioavailability. The proatherogenic diet reduced urinary NOx levels by ≈2.5- to 3-fold in both saline- and IGF-1–infused mice compared with basal (pre-surgery) NOx levels (supplemental Figure IIIB). However, this reduction was significantly blunted by IGF-1 infusion (NOx levels, saline group, 14.12±1.35 μmol/d/g BW, n=8 versus IGF-1 group, 23.62±3.44 μmol/d/g BW, n=8, $P<0.05$). Consistently, plasma NOx levels were also higher in IGF-1 mice versus saline control (data not shown). These results provide initial evidence suggesting that eNOS and eNOS-derived nitric oxide may be involved in the antiatherogenic effect of IGF-1.

Discussion

Multiple growth factors may participate in the atherosclerotic process, including IGF-1, which circulates at high levels, is expressed in the arterial wall and has endocrine and autocrine/paracrine effects.[8] Growth factors such as platelet-derived growth factor and IGF-1 have traditionally been considered to contribute to the atherosclerotic process via their ability to stimulate smooth muscle cell migration and proliferation.[2] However, there is limited data to support this hypothesis as it relates to IGF-1. Of note, IGF-1 is overexpressed in the rat aorta after balloon injury.[31] However, in this model of restenosis, a stable D-peptide analogue of IGF-1 reduced intimal smooth muscle proliferation but did not significantly reduce intimal thickening.[32] Inhibition of the $\alpha V\beta 3$ integrin reduces IGF-1–mediated signaling and atherosclerotic lesion formation in hypercholesterolemic pigs.[33] However, this model includes a component of mechanical injury, and $\alpha V\beta 3$ inhibition is not specific for IGF-1 signaling. We and others have shown that IGF-1 has pleiotropic effects on vascular smooth muscle cells in vitro[15,16,18,34] including inhibition of oxidized LDL–induced apoptosis.[15] In the current study, we report that an approximate 2-fold increase in circulating levels of IGF-1 reduces atherosclerotic burden in ApoE-deficient mice, and that this reduction is associated with a constellation of potential beneficial effects including decreased expression of vascular proinflammatory cytokines, a decrease in macrophage accumulation within atherosclerotic lesions, suppression of oxidative stress, upregulation of vascular eNOS expression, and increased circulating EPCs. Of note, the reduction in atherosclerotic burden as assessed by en face analysis of whole aortas was not statistically significant, possibly because of limited sample size and significant interanimal variability of this measurement. However, we cannot exclude a preferential effect of IGF-1 on the aortic valve area.

To our knowledge, this is the first report of an antiatherogenic effect of IGF-1. Clearly, mechanisms are likely complex and represent the sum effects of IGF-1 on multiple cell types and potentially multiple signaling pathways involved in atherogenesis. Our current data suggests that one major mechanism underlying the antiatherogenic effect of IGF-1 could be an antioxidant effect targeting the endothelium and leading to decreases in aortic superoxide levels and macrophage infiltration. We demonstrated that IGF-1 protected aortic endothelial cells but not aortic smooth muscle cells from oxidized LDL-induced reactive oxygen species formation in vitro and that IGF-1 increased Ser1177 eNOS phosphorylation in endothelial cells. Because eNOS mRNA levels were increased in the aortae of ApoE-deficient mice in response to IGF-1, it is likely that increased eNOS expression or activation led to an increase in nitric oxide bioavailability and in plasma/urinary nitrate/nitrite levels. It has been reported that restoration of eNOS functional activity in animal models of atherosclerosis leads to reduced oxidative stress, inflammation, and atherosclerosis.[35,36] Furthermore, IGF-1 can increase eNOS activity through the PI 3-kinase/Akt pathway,[37] and as we demonstrated IGF-1 infusion markedly increases tissue Akt phosphorylation in ApoE-deficient mice (see Figure 3). Because eNOS appears to be essential for mobilization of EPCs,[38] our data indicating that IGF-1 increased circulating EPC levels could also potentially be explained via IGF-1–induced increase in eNOS expression or activity. Interestingly, recently Thum et al have shown that IGF-1 increased EPC levels and improved EPC function in

mice and that inhibition of eNOS abolished the stimulatory effect of IGF-1 on migration of EPCs.[29] Because EPCs play an important role in vascular repair,[26,39,40] their upregulation by IGF-1 could contribute to the antiatherosclerotic effect of IGF-1. Interestingly, in cultured endothelial cells, the ability of IGF-1 to reduce ROS was not blunted by L-NAME, suggesting that at least in vitro, the antioxidant effect of IGF-1 may be in part NO-independent. Extrapolation of this data, however, to the in vivo situation, is tenuous at best, particularly in view of the potential role of eNOS uncoupling in inflammatory states that could contribute to ROS generation.[28] Further studies using transgenic approaches will be required to determine the contribution of eNOS to the antioxidant and antiatherogenic effect of IGF-1 in vivo. In this respect, the report that IGF-1 overexpression attenuated cardiomyocyte death in diabetic mice by interfering with angiotensin II–dependent superoxide formation[41] is relevant and supports the hypothesis that IGF-1 has antioxidant effects in vivo.

Our finding that IGF infusion decreased TNF-α and IL-6 gene expression in the aorta is consistent with the report that local IGF-1 gene transfer attenuated TNF-α expression in burn wounds[42] and that IGF-1/BP-3 infusion in burned children reduced IL-6 levels.[43,44] Furthermore, the IGF-1–induced reduction in atherosclerotic burden in ApoE-deficient mice is consistent with the growing evidence from epidemiological studies that IGF-1 may be atheroprotective.[45] Thus, most studies, but not all,[46] have suggested that lower serum IGF-1 is associated with an increased risk of ischemic heart disease and congestive heart failure.[47,48]

In summary, we have demonstrated that IGF-1 infusion in ApoE-deficient mice decreases macrophage infiltration and atherosclerotic plaque burden. This beneficial effect is accompanied by a reduction in vascular IL-6 and TNF-α gene expression without changes in circulating cytokine or lipid levels. Furthermore, IGF-1 markedly reduces vascular superoxide levels and systemic oxidative stress and upregulates vascular eNOS expression and circulating EPC. These findings establish a new paradigm for biological effects of IGF-1 and have major implications for treating atherosclerosis.

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Disclosures

None.

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Arteriosclerosis, Thrombosis, and Vascular Biology

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IGF-1 Reduces Inflammatory Responses, Suppresses Oxidative Stress, and Decreases Atherosclerosis Progression in ApoE-Deficient Mice

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Supplementary Methods and Figures

Methods

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 using commercially available kits. 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.

Urinary nitrate/nitrite (NOx) levels - Twenty-four urine was collected from ApoE-deficient mice immediately before pump insertion (Basal) and after 8 weeks of infusion with saline or IGF-1. NOx levels were measured with the Griess method. Briefly, the urine was filtered, diluted with assay buffer and mixed with cofactor and nitrate reductase (NO_x 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 (BW).

Blood glucose - Blood glucose levels were determined using OneTouch^R test strips (LifeScan).

Supplementary Figures Legends

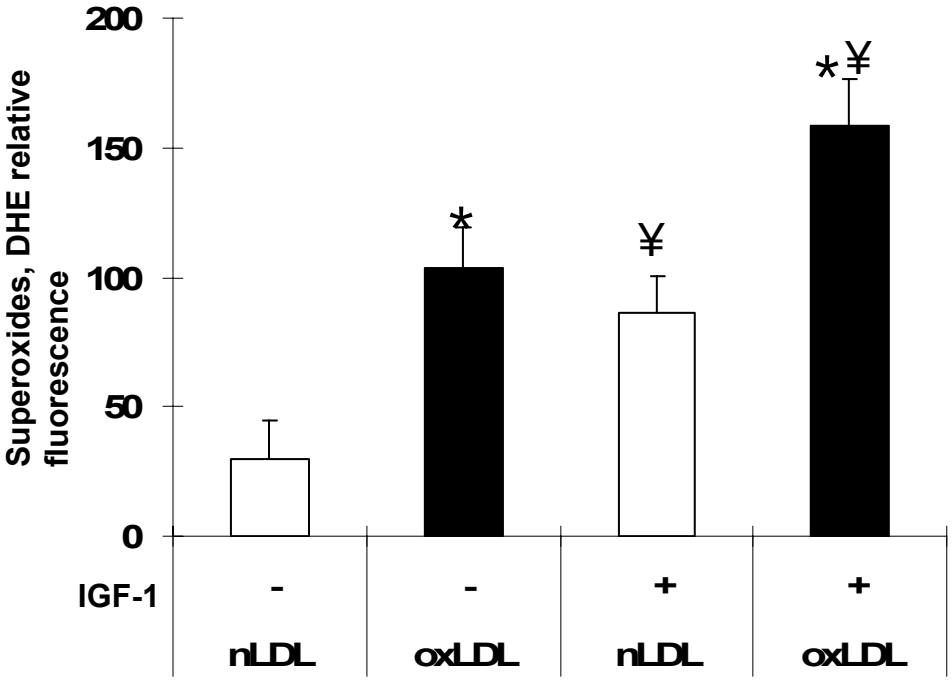
Supplemental Figure 1. IGF-1 does not alter superoxides induced by OxLDL (A) or Ang II (B) in HASMC. Confluent HASMC were pre-treated with 100 ng/ml IGF-1 for 24 h and incubated with 60 ug/ml nLDL or OxLDL for 12 h (A) or incubated with 100 ng/ml Ang II for 1 h (B). For superoxide detection cells were loaded with 5 uM superoxide-sensitive dye DHE for 30 min, washed and fluorescence signal was quantified in a microplate reader (Ex/Em=490/540 nm). *P<0.05 vs. nLDL and $^{¥}$P<0.05 vs. no IGF-1 (A). *P<0.05 vs. control. $^{\#}$IGF-1+Ang II vs. Ang II not significantly different (B).

Supplemental Figure 2. IGF-1 infusion does not alter lipoprotein profiles in ApoE-deficient mice. Serum from saline- or IGF-1-infused ApoE-deficient mice was subjected to HPLC-based lipoprotein fractionation. 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 conditions. The protein elution profiles expressed in mean ± SEM from 4 different mice in each group are almost identical, suggesting no effect of IGF-1 infusion on protein constituents in lipoprotein fractions, particularly in VLDL and IDL/LDL fractions. Triglyceride and cholesterol levels in each fraction were not significantly different between saline-infused and IGF-1-infused mice (Supplemental Table).

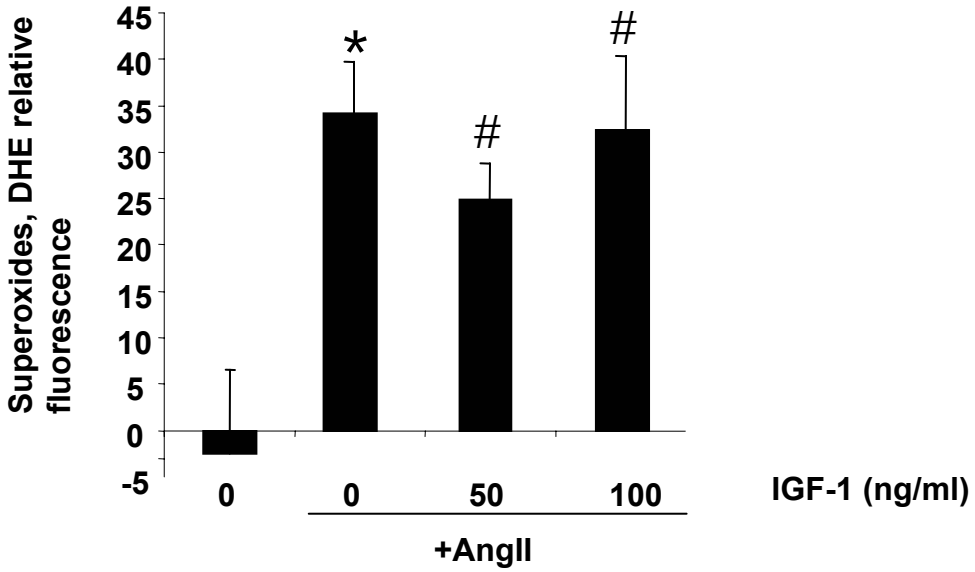
Supplemental Figure 3. IGF-1 increases eNOS phosphorylation in HAEC (A) and urinary levels of nitrates/nitrites (NOx) in ApoE-deficient mice (B). Confluent HAEC were incubated with/without 100 ng/ml IGF-1 for 2.5 h and eNOS phosphorylation was detected in cell lysates by immunoblotting with anti-pSer1177 eNOS and total eNOS antibody (BD Biosciences) (A). Twenty-four hour urine was collected from ApoE-deficient mice immediately before pump insertion (Basal) and after 8 weeks of infusion with saline or IGF-1. NOx levels were measured by colorimetric assay (Cayman Chemicals) and normalized to animal body weight (BW) (B).

Supplemental Figure 1

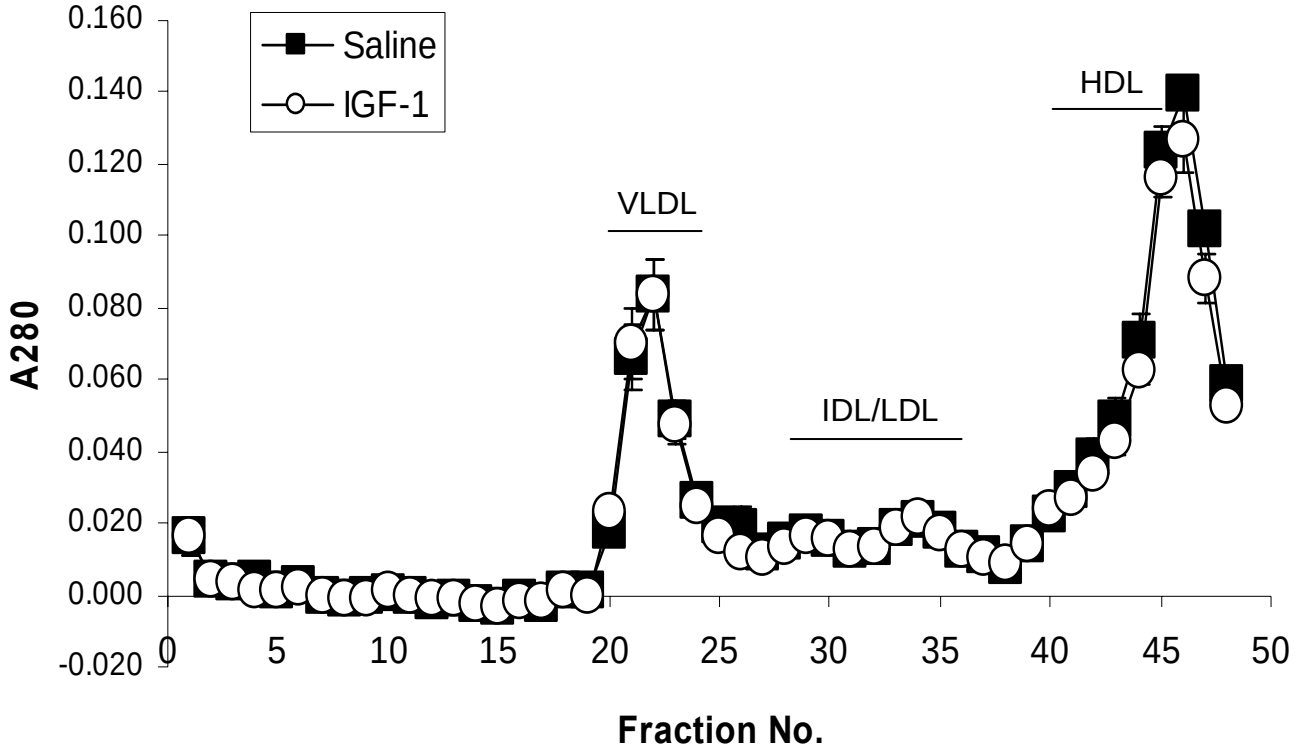
A



B



Supplemental Figure 2



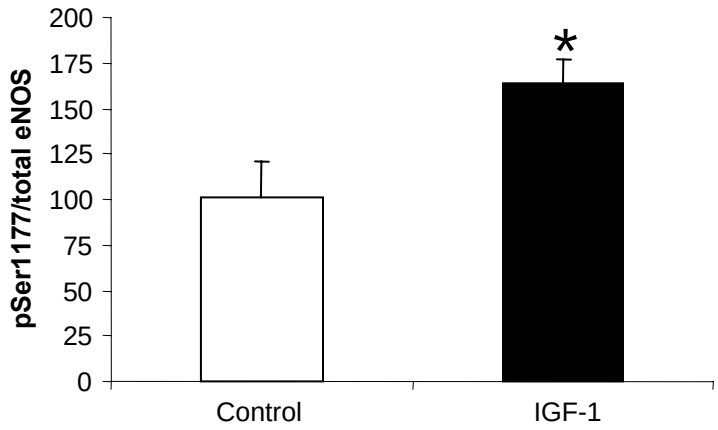
Supplemental Table. Cholesterol and triglyceride levels in lipoprotein fractions

	Saline-infused	*IGF-1-infused*
Cholesterol (mg/dL serum)		
VLDL	**263.6 ± 36.0**	**208.6 ± 45.0**
IDL/LDL	**184.7 ± 32.9**	**159.2 ± 13.9**
HDL	**8.0 ± 1.7**	**5.2 ± 0.5**
Triglyceride (mg/dL serum)		
VLDL	**23.9 ± 8.0**	**17.2 ± 2.5**
IDL/LDL	**21.5 ± 7.2**	**23.9 ± 4.8**
HDL	**Not detectable**	**Not detectable**

Values are not statistically different between saline infused and IGF-1 infused groups.
Values are expressed in mean ± sem, n=4.

Supplemental Figure 3

A



B