Insulin-Like Growth Factor-1 Receptor Expression Masks the Antiinflammatory and Glucose Uptake Capacity of Insulin in Vascular Smooth Muscle Cells

Niels Engberding, Alejandra San Martín, Abel Martin-Garrido, Mitsuhsira Koga, Lily Pounkova, Erin Lyons, Bernard Lassègue, Kathy K. Griendling

Objective—Insulin resistance of vascular smooth muscle cells (VSMCs) has been linked to accelerated atherosclerosis in diabetes; however, the effects of insulin on VSMCs remain controversial. Most VSMC insulin receptors are sequestered into insulin-insensitive hybrids with insulin-like growth factor-1 receptors (IGF1Rs). Thus we hypothesized that regulation of IGF1R expression may impact cellular insulin sensitivity.

Methods and Results—IGF1R expression was increased in aortas from diabetic mice. IGF1R overexpression in VSMCs impaired insulin-induced Akt phosphorylation. Conversely, IGF1R downregulation by siRNA allowed assembly of insulin holoreceptors, enhanced insulin-induced phosphorylation of its receptor, Akt, Erk1/2, and further augmented insulin-induced glucose uptake. IGF1R downregulation uncovered an insulin-induced reduction in activation of NF-κB and inhibition of MCP-1 upregulation in response to TNF-α.

Conclusions—Downregulation of IGF1R increases the fraction of insulin receptors organized in holoreceptors, which leads to enhanced insulin signaling and unmasks potential antiinflammatory properties of insulin in VSMCs. Therefore, IGF1R, which is susceptible to feedback regulation by its own ligand, may represent a novel target for interventions designed to treat insulin resistance in the vasculature. (Arterioscler Thromb Vasc Biol. 2009;29:408-415.)

Key Words: IGF1R ■ insulin resistance ■ diabetes ■ inflammation ■ vascular smooth muscle

Atherosclerosis is greatly enhanced in patients with type 2 diabetes mellitus (DM),¹ and these patients did not share in the overall reduction in cardiovascular disease mortality observed over the past decades.² Type 2 DM is characterized by elevated serum insulin accompanied by insulin resistance of the classical insulin targets fat, liver, and skeletal muscle, and is possibly mediated by inflammatory cytokines.³ Insulin resistance of vascular smooth muscle cells (VSMCs), a nonclassical insulin target tissue, has also been described and implicated in angiotensin II-mediated vasculopathies in diabetes.⁴

The role of insulin in the development and progression of atherosclerosis is controversial. Several large epidemiological studies reported an association of high serum insulin with increased cardiovascular events.⁵ The Quebec Cardiovascular Study found serum insulin to be an independent predictor of a first cardiovascular event in healthy participants.⁶ However, these observational data do not establish whether insulin causes cardiovascular pathology or is an innocent bystander, elevated as a consequence of whole-body insulin resistance.⁷ Moreover, a number of studies have shown that exogenous insulin is actually beneficial for vascular end points. For example, insulin treatment reduces microvascular complications in DM.⁸ Furthermore, in type 1 diabetics intensive insulin administration reduced cardiovascular end points compared to a conventional insulin protocol.⁹ In addition, patients with insulin producing tumors do not seem to have increased atherosclerosis.¹⁰ Hence, the effect of insulin on the vasculature must be clarified before enhancing vascular insulin signaling can be accepted as a desirable intervention for atherosclerosis. Current understanding deems atherosclerosis an inflammatory disease involving VSMCs, endothelial cells, and mononuclear cells. It is therefore important to characterize inflammatory responses in VSMCs in altered states of insulin sensitivity.

Although previous studies identified postreceptor targets as mechanisms of insulin resistance in VSMCs,⁴,¹¹ there is little information about the contribution of insulin-like growth factor-1 receptors (IGF1Rs) and insulin receptors (IRs) to insulin sensitivity. VSMCs express both IRs and IGF1Rs, but insulin only causes modest responses in these cells.¹² Therefore, it has been suggested that biological effects of insulin in VSMCs may be mediated mostly through cross-reaction with IGF1R.¹²,¹³ In addition, IR and IGF1R are known to randomly form hybrid receptors according to the ratio of proreceptors present in cells.¹⁴-¹⁶ Importantly, hybrid receptors behave like IGF1Rs in regard to ligand binding affinity,
with greater affinity for IGF-1 than insulin.\textsuperscript{17,18} As IGF1R may importantly contribute to insulin signaling, either by crossactivation by insulin or by formation of hybrid receptors, we sought to determine whether IGF1R expression is altered in an animal model of DM and whether manipulation of IGF1R expression affects insulin signaling in VSMCs. We found that insulin signaling is enhanced by IGF1R downregulation, and that this allows the potentially important anti-inflammatory functions of insulin to predominate. Thus, rather than propagating insulin signals, the IGF1R may contribute to insulin resistance and mask the beneficial effects of insulin.

\section*{Methods}

An expanded Materials and Methods section is available in the online data supplement at http://atvb.ahajournals.org.

\section*{Animals}

\textit{db/db} mice on a C57BLKS/J background, a model of obesity-induced Type 2 DM, were purchased from The Jackson Laboratory (Bar Harbor, Me) and bred in-house. At 12 weeks of age, aortas were harvested after CO\textsubscript{2}-euthanasia. All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee.

\section*{Serum Measurements}

Blood was collected by cardiac puncture using a 21-gauge needle and kept in an Eppendorf tube for 30 minutes before centrifugation at 3000 rpm for 20 minutes at room temperature. Serum was stored at −80°C until processing. IGF-1 and insulin levels were analyzed by radioimmunoassay and ELISA, respectively.

\section*{Immunohistochemistry}

After euthanasia, the heart and aorta were pressure perfused and harvested after CO\textsubscript{2}-euthanasia. Aortas were embedded in paraffin, and 5-μm cross sections were cut. IGF1R was immunolocalized and visualized with diaminobenzidine.

\section*{Cell Culture}

VSMCs were isolated from male Sprague-Dawley rat thoracic aortas by enzymatic digestion and grown in DMEM containing 25 mmol/L glucose.\textsuperscript{19} 80\% to 90\% confluent VSMCs between passages 6 and 15 were made quiescent in serum-free DMEM for 24 to 48 hours before stimulation with agonist at 37°C.

\section*{Protein Extraction and Immunoblotting}

Snap frozen mouse aortas were homogenized in 1\% Triton X-100 and 0.1\% SDS lysis buffer, and cultured VSMCs were lysed in 1\% Triton X-100. Solubilized proteins were quantified by the BCA assay (Pierce). Western analysis was performed as described previously.\textsuperscript{20} After incubation with secondary antibodies, proteins were detected by enhanced chemiluminescence. Band intensity was quantified by densitometry of immunoblots using NIH ImageJ for Mac, version 1.39. For immunoprecipitation, cell lysates with equal amounts of protein (500 to 1000 μg) were incubated with primary antibody at 4°C overnight, and immunocomplexes were collected with protein A agarose beads.

\section*{Small Interfering RNA Transfection Experiments}

Cells were transfected using the Nucleofector II Device (Amaxa Inc) set to program A-33 with 2.5 μg of siRNA per 2 to 4×10\textsuperscript{6} cells in nucleofector solution. Stealth siRNA against IGF1R was from Invitrogen (sense: 5′-CCUUGAGAAGUAGUUUCUGGUUU-3′; antisense: 5′-AAACGAGAACUACUUCACAGG-3′). Transfected cells were plated in 100-mm dishes in 10% calf serum DMEM 1 to 2 days before serum deprivation for experiments.

\section*{Adenovirus Transduction}

GFP-tagged IGF1R adenovirus (AdV-IGF1R) was kindly provided by Dr Jie Du (Baylor College of Medicine, Houston, Tex). VSMCs were transduced for 2 hours with AdV-IGF1R or control GFP virus\textsuperscript{4} in serum-free DMEM, and incubated for another 2 days in the same medium without virus before experiments. Transfection efficiencies were greater than 80\%, as visualized by GFP fluorescence.

\section*{Glucose Uptake Measurement}

Glucose uptake was measured as described previously\textsuperscript{4} by measuring 2-deoxy-D-[\textsuperscript{3}H]glucose during a 20 minute insulin stimulation. The amount of labeled glucose taken up by the cells was determined by scintillation counting and normalized to cell number.

\section*{NF-κB Promoter Activity}

NF-κB–mediated transcriptional induction was measured by transfection of VSMCs with a plasmid containing the luciferase reporter gene driven by an NF-κB promoter. Transfection was performed as described above using 6 μg plasmid per electroporation reaction. Luciferase activity (relative light units) was measured with the Luciferase Assay System (Promega Corporation) and normalized to protein concentration.

\section*{Real-Time Quantitative RT-PCR}

Total RNA was purified from VSMCs using the RNaseasy kit (Qiagen). 10\textsuperscript{6} copies of luciferase RNA (exogenous control) were added to 5 μg samples of VSMC RNA and reverse-transcribed with Superscript II (Invitrogen) using random primers. cDNA samples were purified and amplified in the LightCycler (Roche Diagnostics Corporation) real-time thermocycler using Platinum Taq DNA polymerase (Invitrogen). Amplification conditions for MCP-1 were: 100 nmol/L primers (upstream primer, 5′-TGTGTTGCACTAGTGTGCCGTGCCTG-3′; downstream primer, 5′-GTGCTGAAGTCCTTAGGGTTGAT-3′), 21 4 mmol/L MgCl\textsubscript{2}, 5% DMSO, annealing at 66°C; for luciferase, 75 nmol/L primers, (upstream primer, 5′-GCTGCTGTGTCGCAACCCTATTCTCTCTT-3′; downstream primer, 5′-CCGGCAAACCTTCCACTG-3′), 3 mmol/L MgCl\textsubscript{2}, and annealing at 60°C. Copy numbers of MCP-1 were corrected for copy numbers of luciferase measured in triplicate.

\section*{Statistics}

Results are expressed as mean±SE. Statistical significance was assessed by Student \textit{t} test, or by two-way ANOVA followed by contrast analysis using super ANOVA software. A value of \textit{P}<0.05 was considered statistically significant.

\section*{Results}

\subsection*{IGF1R Expression Is Increased in Diabetic Aorta}

To determine whether vascular IGF1R is altered in type 2 DM, we measured protein expression in aortas of diabetic mice. 12-week-old \textit{db/db} mice expressed significantly more IGF1R in all layers of the wall than age-matched controls (Figure 1), suggesting that increased IGF1R expression may play a role in vascular insulin resistance.

\subsection*{IGF1R Overexpression Reduces Insulin-Induced Akt Phosphorylation}

To determine the functional importance of altered IGF1R expression, we modulated IGF1R in cultured VSMCs using either adenoviral overexpression or siRNA downregulation. Overexpression of IGF1R in VSMCs to a level 2.7±0.1-fold higher than the endogenous value (supplemental Figure IA), as occurs in diabetic mice, impacts the response to insulin. Akt phosphorylation was increased 2- to 3-fold by insulin (7 nmol/L, a concentration that does not activate the IGF1R;
IGF1R Downregulation Increases Insulin-Induced IRβ Phosphorylation and Downstream Signaling

Next, we tested the converse hypothesis that IGF1R downregulation would increase insulin responsiveness. Treatment with siRNA against IGF1R (siIGF1R) reduced IGF1R protein by 70% without affecting total IR protein (supplemental Figure 1), confirming that siIGF1R is effective and specific. To determine its effect on intracellular signaling, VSMCs were stimulated with insulin after siRNA treatment. IGF1R downregulation increased insulin-induced IRβ phosphorylation and reduced the amount of IGF1R coprecipitating with IR (Figure 3A), also suggesting that, as expected, fewer hybrid receptors are present after IGF1R knockdown.

To explore further the consequences of IGF1R downregulation, we evaluated activation of downstream signaling. In accordance with the observed enhanced IR activation, IGF1R downregulation increased insulin-induced Akt phosphorylation by 250% (Figure 3B) and Erk1/2 phosphorylation by 260% (Figure 3C) compared to that in cells treated with scrambled siRNA. This increase is apparently caused by an increase in sensitivity to insulin, as confirmed by a shift in the dose response curve for Akt activation upward and to the left (Figure 3D).

IGF1R Downregulation Increases the Fraction of Insulin Holoreceptors

The observed increased IR phosphorylation does not result from IR upregulation after siIGF1R treatment (supplemental Figure 1A), but could be attributable either to higher phosphorylation of individual IRβ molecules or to an increased proportion of insulin-responsive IR holoreceptors, compared to insulin-insensitive hybrid receptors. To test these possibilities, we immunodepleted VSMC lysates to remove all receptors containing IGF1R (both IGF1R holoreceptors and IGF1R/IR hybrid receptors), leaving only IR holoreceptors in the supernatant. As shown in Figure 4, compared to scrambled siRNA, siIGF1R increased the amount of insulin holoreceptors, which are more sensitive to insulin than hybrids.18

IGF1R Downregulation Potentiates Glucose Uptake and Reduces TNF-α–Induced NF-κB Activity in Response to Insulin

To investigate the physiological significance of the observed increase in insulin signaling, we first evaluated glucose uptake in VSMCs. Consistent with its effects on IR, Akt, and Erk1/2, IGF1R downregulation increased insulin-induced uptake of 2-deoxy-glucose in VSMC (Figure 5A).

Because vascular inflammation is a hallmark of type 2 diabetes (in which IGF1R is elevated), we investigated the impact of IGF1R manipulation on an inflammatory pathway. VSMCs were treated with the prototypic inflammatory stimulus TNF-α, in the presence or absence of insulin, after downregulation of IGF1R using siRNA. TNF-α greatly enhanced NF-κB promoter activity, whereas insulin alone had no effect (Figure 5B). However, insulin markedly reduced the response to TNF-α, but only after IGF1R downregulation (Figure 5B). To confirm that insulin reduced
TNF-α proinflammatory activity, we evaluated the expression of MCP-1, a well-known NF-κB–driven inflammatory mediator, in the same experimental conditions. Consistent with the above observations, insulin reduced TNF-α-induced MCP-1 mRNA expression only when IGF1R was downregulated with siRNA (Figure 5C). Blocking Akt resulted in a reduction of MCP-1 transcription without altering the specific insulin effect (data not shown). Treatment with the Akt pathway inhibitor LY294002 (10 μmol/L) reduced TNF-α-induced MCP-1 expression to 41±4.6% compared to control (P<0.0001), indicating that Akt mediates the TNF-α response. However, there was no difference in the response to TNF-α + insulin in the presence or absence of LY294002, suggesting that insulin exerts its antiinflammatory effect via an Akt-independent pathway, most likely NF-κB.

**IGF-1 Downregulates its Receptor**

In humans, decreased circulating IGF-1 is commonly seen in DM,22 and low IGF-1 is also associated with glucose intolerance in healthy subjects.23 Because db/db mice have low circulating IGF-1, we hypothesized that IGF-1 itself may be a physiological mechanism by which IGF1R expression is regulated in VSMCs. As shown in supplemental Figure II, long-term exposure of VSMCs to IGF-1 markedly downregulated IGF1R, whereas IR expression was less affected, suggesting that physiological regulators of IGF1R expression may contribute to altering the ratio of hybrid receptors to holoreceptors.

**Discussion**

The data presented here show that IGF1R expression critically influences insulin signaling in VSMCs. IGF1R levels
are quite responsive to physiological stimuli, as they are upregulated in diabetic mice and downregulated by IGF-1. The result of this variation in IGF1R expression is an alteration in insulin responsiveness. When IGF1R are downregulated, insulin-induced IR phosphorylation, Akt and Erk1/2 phosphorylation, as well as glucose uptake, are enhanced. Strikingly, enhanced insulin signaling also results in suppression of TNF-α-induced NF-κB activation and MCP-1 expression in VSMCs. These results not only suggest that manipulating IGF1R expression may be a therapeutic target for improving insulin sensitivity, but also indicate that insulin plays a potentially important antiinflammatory role in the vasculature.

The affinities of IGF1R and IR are ~100× higher for their cognate, rather than the other, ligand.24 However, because VSMCs express ~8× more IGF1Rs than IRs, most IRs are sequestered into insulin-insensitive hybrid receptors.14,25 We observed that IGF1R downregulation increases the proportion of IR present in holoreceptors and promotes insulin signaling and antiinflammatory activity in VSMCs, suggesting that the molecular configuration of IR is functionally important in the vasculature. Our data are consistent with results from other investigators who found increased insulin signaling when IGF1R expression is reduced in breast cancer cell lines,26 adipocytes,27 and osteoblasts.28 In conjunction with the results presented here, these data suggest that IGF1R expression regulates insulin sensitivity in a variety of target tissues. However, to our knowledge, the present report is the first to show that a higher proportion of IR holoreceptors, with no change in total IR amount, may explain the improved insulin signaling. Thus, control of IGF1R expression appears to be a critical regulatory mechanism of insulin signaling (Figure 6).

Another important conclusion from our study is that insulin has effects in VSMCs that are not mediated by cross-activation of IGF1Rs, at least at the insulin concentration used in this study (7 nmol/L). In humans, normal serum insulin levels are in the low picomolar range, whereas in rodents they may reach the low nanomolar range in severe insulin resistance.29 This is particularly important because it is still unclear whether insulin exerts biological effects in VSMCs at physiological concentrations. It has been suggested that most of the effects observed in vitro with supraphysiological doses of insulin are mediated by IGF1R,12 but our data indicate that physiological concentrations of insulin in fact activate IR holoreceptors when IGF1R levels are low.
Impaired vasorelaxation is a key feature of type 2 DM and is associated with enhanced vascular oxidative stress and inflammation. Insulin stimulates vascular dilation mediated by nitric oxide. This function is impaired in vascular insulin resistance. Insulin-stimulated tyrosine phosphorylation of IRβ in the aorta of obese rats is significantly decreased compared with lean rats, although IRβ protein levels in the 2 groups are not different. Based on our data that IGF1R expression is upregulated in the vasculature of diabetic animals, we propose that hybrid receptor formation may contribute to vascular insulin resistance and pathology in vivo. This is supported by data showing that insulin-induced vasorelaxation in aortas of obese rats is significantly decreased, whereas IGF-1-induced vasorelaxation is profoundly increased, compared with that in lean rats. Similarly, IGF1R is upregulated and IGF-1–mediated aortic relaxation is increased after induction of diabetes by streptozotocin in rats.

IGF1R expression is strongly regulated by IGF-1 serum levels in vivo and in vitro. Accordingly, we find that IGF-1 pretreatment leads to autologous downregulation of IGF1R, supporting the idea that physiological regulators of IGF1R expression may impact insulin signaling. Additionally, other growth factors, such as platelet-derived growth factor, basic fibroblast growth factor, and angiotensin II stimulate IGF1R expression. Because the vascular renin–angiotensin system may be activated in DM, it could further enhance IGF1R expression in db/db mice. Indeed, angiotensin converting enzyme inhibition reduces glomerular IGF1R expression in another animal model of diabetes. Despite this relationship between increased IGF1R expression and diabetes, the impact of IGF1R upregulation on insulin signaling remains unclear. We found that in VSMCs, overexpression of IGF1R impairs, and knockdown of IGF1R increases, insulin-induced Akt phosphorylation (and in the case of siRNA, glucose uptake), suggesting that IGF1R upregulation may contribute to vascular insulin resistance.

IGF-1 effects are also enhanced in the setting of obesity and insulin resistance, which is consistent with our observation that IGF1Rs are upregulated. According to our finding that IGF-1 exerts a tight feedback control on its cognate receptor, reduced IGF-1 levels may trigger peripheral IGF1R upregulation and vascular pathology. However, we found no significant difference in serum levels of IGF-1 in our animals (supplemental Figure IIIA). It should be noted, though, that IGF-1 levels are tightly regulated by IGF-1–binding proteins, and bound IGF-1 would not be detected in our assay. Moreover, we did not measure IGF-1 content in the aorta, two points that will need to be addressed in future studies. In rat aorta after balloon injury, IGF1R mRNA is increased particularly in neointimal VSMCs. Clinical data show that high systemic levels of IGF-1 are associated with reduced risk of developing impaired glucose tolerance and type 2 DM. In addition, individuals with a genetic polymorphism in the IGF-1 promoter, predisposing to low systemic IGF-1 levels, have an increased risk for DM and myocardial infarction. Importantly, treatment of patients with DM and insulin resistance with IGF-1 led to enhanced insulin sensitivity, reduced insulin requirements, and improved glycemic control. It is conceivable that some of the effects of IGF-1 administration are related to peripheral IGF1R downregulation, leading to enhanced insulin sensitivity of peripheral tissues. This assumption warrants further studies.

The role of insulin in inflammation has recently received attention, after a clinical study in critically ill patients reported reduced morbidity and mortality with intensive insulin therapy, implying beneficial effects of insulin in severe systemic inflammation. Inhibition of inflammatory pathways in vascular cells has emerged as an antiatherosclerotic intervention. The ubiquitous inflammatory transcription factor NF-κB is activated in atherosclerotic lesions. It has been reported that insulin suppresses NF-κB in endothelial cells and mononuclear cells, two important cell types for atherogenesis. In VSMCs the data are conflicting: insulin was found to enhance NF-κB activation induced by hyperglycemia, advanced glycation end products (AGE), and angiotensin II in bovine VSMCs, but another study in rat VSMCs reported that insulin reduces AGE-induced NF-κB expression. We show that insulin reduces TNF-α–induced NF-κB activation and expression of MCP-1, a prototypic NF-κB–responsive inflammatory gene, only after IGF1R downregulation, whereas it has no effect in control cells. Baseline IGF1R expression therefore is a critical determinant of the ultimate insulin effect. Variable IGF1R expression in various experimental conditions in vitro may thus be an explanation for conflicting results regarding insulin signaling in the literature.

Reports showing that angiotensin II impairs insulin signaling in VSMCs have fueled speculation that insulin resistance...
of VSMCs may be important in the etiology of vascular diseases associated with DM.4,11 Our data suggest that enhanced insulin signaling may protect against atherosclerosis by reducing inflammation. Only a few other studies found antiatherosclerotic properties of insulin. Insulin maintains VSMC quiescence and counteracts the proatherogenic properties of PDGF, but loses this ability in insulin resistance.47 In seeming contrast, numerous in vitro studies have established that insulin stimulates growth of VSMCs, which is traditionally considered proatherogenic.48,49 However, it is questionable that one particular in vitro function is a suitable surrogate for a temporally and spatially highly complex process such as atherosclerosis. Indeed, not all growth factors promote atherosclerosis in vivo. For example, it has recently been described that IGF-1 infusion exerted antiatherosclerotic and antiinflammatory effects in ApoE-deficient mice.50 This beneficial effect was accompanied by a reduction in vascular IL-6 and TNF-α expression, vascular superoxide, systemic oxidative stress, and upregulated endothelial eNOS expression. Although insulin and IGF-1 are highly homologous and can cross-react with each other’s receptors at high ligand concentrations,51 our data clearly show that insulin exerts its antiinflammatory effects through its own receptor when IGF1R is downregulated.

Acknowledgments
We thank Dr Jie Du Baylor for the IGF1R adenovirus.

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Disclosures
None.

References


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Supplemental Material

Insulin-like growth factor-1 receptor expression masks the anti-inflammatory and glucose uptake capacity of insulin in vascular smooth muscle cells


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Methods

Materials

Anti-IRβ-subunit (SC-711), anti-IGF1R β-subunit (SC-713), anti-phospho Akt (CS-9271), anti-phospho-ERK1/2 (CS-9101), anti-Akt (CS-9272), Anti-Erk1/2 (CS-9102) were from Cell Signaling Technology, Inc. (Danvers, MA), and anti-phosphotyrosine 4G10 (05-321) antibodies were from Upstate Biotechnology (Bedford, MA). All other chemicals and reagents, including anti-β-tubulin antibody (T4026) and DMEM with 25 mmol/L Hepes and 4.5 g/L glucose, were from Sigma (St. Louis, MO).
*db/db* mice (Stock number 642) on a C57BLKS/J background, a model of obesity-induced Type 2 DM resulting from a mutation in the leptin receptor, were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in house. Mice had free access to regular chow (rodent diet 5001) from LabDiet (St. Louis, MO) and drinking water. At 12 weeks of age, aortas were harvested after CO₂-euthanasia. All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee.

**Serum measurements**

Blood was collected by cardiac puncture using a 21-gauge needle and kept in an Eppendorf tube for 30 min before centrifugation at 3,000 rpm for 20 min at room temperature. Serum was stored at –80°C until processing. IGF1 and insulin levels were analyzed in the Biomarkers Core Laboratory at Yerkes National Primate Research Center (Atlanta, GA) using a double antibody RIA kit from Diagnostic Systems Laboratories (cat.# DSL-2900) and an ELISA kit from MERCODIA (cat.# 10-1149-01), respectively.

**Immunohistochemistry**

After euthanasia, the heart and aorta were pressure perfused at 100 mmHg with 0.9% sodium chloride solution, followed by pressure fixation with a 10% formalin solution. Aortas were embedded in paraffin, and 5-µm cross sections were cut. After 10 mmol/l citrate buffer antigen retrieval, IGF-1R was immunolocalized using a polyclonal antibody (Santa Cruz) followed by anti-mouse antibody and visualized with diaminobenzidine.

**Cell culture**
VSMCs were isolated from male Sprague-Dawley (Harlan Sprague-Dawley, Indianapolis, IN) rat thoracic aortas by enzymatic digestion and grown in DMEM containing 25mM glucose. 1 80-90% confluent VSMCs between passages 6 and 15 were made quiescent in serum-free DMEM for 24 to 48 hours before stimulation with agonist at 37°C.

**Protein extraction and immunoblotting**

Snap frozen mouse aortas were minced with scissors and sonicated for 10 s on ice in 1% Triton X-100 and 0.1% SDS lysis buffer. Cultured VSMCs were lysed in the presence of 1% Triton X-100. 2 Solubilized proteins were isolated by centrifugation (15,000 g, 10 min) and quantified by the BCA assay (Pierce, Rockford, IL). Equal amounts of protein (10-20 µg) were separated using SDS-PAGE and transferred to PVDF membranes. After blocking, blots were incubated with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit (1:2500), Bio-Rad (Hercules, CA); goat anti-mouse (1:2500), Amersham (Piscataway, NJ)). After incubation with secondary antibodies proteins were detected by enhanced chemiluminescence. Band intensity was quantified by densitometry of immunoblots using NIH ImageJ for Mac, version 1.39. For immunoprecipitation, cell lysates with equal amounts of protein (500-1000µg) were incubated with rabbit anti-IRβ or anti-IGF1Rβ antibody at 4°C overnight, and immunocomplexes were collected with protein A-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA).

**Small interfering RNA transfection experiments**
Trypsinized cells were transfected in suspension by electroporation using the Nucleofector II Device (amaxa Inc, Gaithersburg, MD) set to program A-33 with 2.5 μg of siRNA per 2-4x10^6 cells in 100 μL nucleofector solution. Stealth siRNA against IGF1R was from Invitrogen (Carlsbad, CA) (sense: 5’-
CCUGUGAAAGUGAUGUUCGUU-3’, antisense: 5’-AAACGGAGAACAUCACUUUACACAGG-3’). Transfected cells were plated in 100-mm dishes in 10% calf serum DMEM, 1-2 days before serum deprivation for experiments.

**Adenovirus transduction**

GFP-tagged IGF1R adenovirus (AdV-IGF1R) was kindly provided by Dr. Jie Du (Baylor College of Medicine). VSMCs were transduced for 2 hours with AdV-IGF1R or control GFP virus in serum-free DMEM, and incubated for another 2 days in the same medium without virus before experiments. Transfection efficiencies were greater than 80%, as visualized by GFP fluorescence.

**Glucose uptake measurement**

Serum-starved VSMCs were incubated in Krebs-Ringer-Hepes buffer (in mmol/L: 15 Hepes, pH 7.4, 105 NaCl, 5 KCl, 1.4 CaCl₂, 1 KH₂PO₄, 1.4 MgSO₄, and 10 NaHCO₃) for 2 hours at 37°C in a 5% CO₂ incubator. Next, cells were incubated with insulin (7 nmol/L) and 0.2 mmol/L 2-deoxy-D-glucose containing 1 μCi/mL 2-deoxy-D-[³H]glucose for 20 minutes. Transport was stopped by removal of the buffer, followed by 3 washes with ice-cold PBS. Cells were disrupted with 0.4 mol/L NaOH, neutralized with
HCl, and the amount of labeled glucose taken up by the cells was determined by scintillation counting and normalized to cell number.

**NF-κB promoter activity**

NF-κB–mediated transcriptional induction was measured by transfection of VSMCs with a plasmid containing the firefly luciferase reporter gene driven by an NF-κB promoter (pNF-κB-Luc, # 631904), from Clontech Laboratories (Mountain View, CA).

Transfection was performed as described above using 6 μg plasmid per electroporation reaction. Luciferase activity (relative light units) was measured with the Luciferase Assay System (Promega Corporation, Madison, WI) and normalized to protein concentration.

**Real-Time quantitative RT-PCR**

Total RNA was purified from VSMCs using the RNeasy kit (Qiagen, Valencia, CA), as recommended by the manufacturer. 10⁸ copies of luciferase RNA (exogenous control) were added to 5 μg samples of VSMC RNA and reverse-transcribed with Superscript II (Invitrogen) using random primers. cDNA samples were purified using Micro Bio-Spin 30 columns (Bio-Rad) and amplified in the LightCycler (Roche Diagnostics Corporation, Indianapolis, IN) real-time thermocycler using Platinum Taq DNA polymerase (Invitrogen) with SYBR green dye. Amplification conditions for MCP-1 were: 100 nmol/L primers (upstream primer, 5'-TGTTGGTCACAGTTGCTGCCTG-3'; downstream primer, 5'-GTGCTGAAGTCTTAGGTTGAT-3')⁴, 4 mmol/L MgCl₂, 5% DMSO, annealing at 66°C; for luciferase, 75 nmol/L primers, (upstream primer, 5'-
GCTGCTGGTGCCAACCCTATTCTCCTT-3'; downstream primer, 5'-
CGCGCAACTTTTTTCGC

GTTACTTGA-3'), 3 mmol/L MgCl$_2$, and annealing at
60°C. Standard curves were generated using 10-fold serial dilutions of a plasmid
containing cloned cDNA (either MCP-1 or luciferase) and measured in each run. Copy
numbers were calculated from a linear regression of cycle threshold versus Log(copy
number). Finally, copy numbers of MCP-1 were corrected for copy numbers of luciferase
measured in triplicate.

Statistics

Results are expressed as mean ± SE. Statistical significance was assessed by Student’s t-
test. A value of P<0.05 was considered statistically significant.
Supplemental figure legends

**Supplemental Figure 1. IGF1R downregulation by siIGF1R is specific.**

**A.** VSMCs were transfected with siIGF1R or scrambled control siRNA (left panel), or transduced with control virus (AdV-GFP) or adenovirus containing IGF1R (AdV-IGF1R) (right panel). Bar graphs represent mean ± SE of values from 3 independent experiments.

**B.** Left: VSMCs were stimulated with IGF1 (20 ng/mL) or insulin (7 nM) for 5 min, and IGF1Rβ was immunoprecipitated and blotted for phosphotyrosine or IGF1Rβ. Right: VSMCs were transduced with AdV-GFP or AdV-IGF1R, and IGF1Rβ was immunoprecipitated. Blots were probed for phosphotyrosine or IGF1Rβ.

**Supplemental Figure 2. Prolonged IGF-1 stimulation downregulates its receptor.**

VSMCs were maintained in serum free medium (SF) or treated with IGF-1 (40 ng/mL) for 72 hours, stimulated with insulin (7nM) for 5 minutes, and lysed for Western analysis. Analysis of IGF1Rβ, IRβ and β-tubulin (loading control) is shown. Representative Western blots (top panel); bar graphs (bottom panel) represent mean ± SE (n=3); Open bars, no insulin stimulation; Black bars, stimulated with insulin.

**Supplemental Figure 3. Insulin and IGF-1 levels in serum of C57 and db/db mice.**

Blood was collected by cardiac puncture, serum prepared, and insulin and IGF-1 levels were measured as described in Supplemental Methods.
References


Supplemental Figure 1

A

siIGF1R : - +
IGF1Rβ → [image]
IRβ → [image]
β-tubulin → [image]

Densitometry units

P=0.002

IGF1R β
CDK4

IGF-1R β relative expression (densitometry units)
P<0.001
Supplemental Figure 1

B

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<th>Insulin</th>
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<td>[Image]</td>
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Supplemental Figure 2

[Image showing Western blots for IGF1Rβ, IRβ, and β-tubulin under different conditions: SF (serum-free) and IGF1 treatment with or without insulin (+ or -). The bar graph below the blots shows quantification of IGF1Rβ/β-tubulin ratio with error bars. The graph indicates a significant difference (P<0.05) between the insulin-treated and untreated samples in both SF and IGF1 conditions.]
Supplemental Figure 3

A

IGF-1

ng/mL

ctr  dbdb

B

Insulin

nmol/L

ctr  dbdb

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