



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

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## 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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### Abstract

**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 (IGF1R). 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- $\kappa$ B and inhibition of MCP-1 upregulation in response to TNF- $\alpha$ .

**Conclusions**—Downregulation of IGF1R increases the fraction of insulin receptors organized in holoreceptors, which leads to enhanced insulin signaling and unmasks potential anti-inflammatory 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.

### Keywords

IGF1R; insulin resistance; diabetes; inflammation; vascular smooth muscle

### Introduction

Atherosclerosis is greatly enhanced in patients with type 2 diabetes mellitus (DM),<sup>1</sup> and these patients did not share in the overall reduction in cardiovascular disease mortality observed over the past decades.<sup>2</sup> 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.<sup>3</sup> Insulin resistance of vascular smooth muscle cells (VSMCs), a non-classical insulin target tissue, has also been described and implicated in angiotensin II-mediated vasculopathies in diabetes.<sup>4</sup>

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The role of insulin in the development and progression of atherosclerosis is controversial. Several large epidemiologic studies reported an association of high serum insulin with increased cardiovascular events.<sup>5</sup> The Quebec Cardiovascular Study found serum insulin to be an independent predictor of a first cardiovascular event in healthy participants.<sup>6</sup> 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.<sup>7</sup> Moreover, a number of studies have shown that exogenous insulin is actually beneficial for vascular endpoints. For example, insulin treatment reduces microvascular complications in DM.<sup>8</sup> Furthermore, in type 1 diabetics intensive insulin administration reduced cardiovascular endpoints compared to a conventional insulin protocol.<sup>9</sup> In addition, patients with insulin producing tumors do not seem to have increased atherosclerosis.<sup>10</sup> 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 and mononuclear cells. It is therefore important to characterize inflammatory responses in VSMCs in altered states of insulin sensitivity.

Although previous studies identified post-receptor targets as mechanisms of insulin resistance in VSMCs,<sup>4, 11</sup> 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.<sup>12</sup> Therefore, it has been suggested that biological effects of insulin in VSMCs may be mediated mostly through crossreaction with IGF1R.<sup>12, 13</sup> In addition, IR and IGF1R are known to randomly form hybrid receptors according to the ratio of proreceptors present in cells.<sup>14-16</sup> Importantly, hybrid receptors behave like IGF1Rs in regard to ligand binding affinity, with greater affinity for IGF-1 than insulin.<sup>17, 18</sup> As IGF1Rs 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.

## Methods

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

## Animals

*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<sub>2</sub>-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 by radioimmunoassay and ELISA, respectively.

## Immunohistochemistry

After euthanasia, the heart and aorta were pressure perfused and fixed with a 10% formalin solution. Aortas were embedded in paraffin, and 5- $\mu$ m cross sections were cut. IGF-1R was immunolocalized and visualized with diaminobenzidine.

## Cell culture

VSMCs were isolated from male Sprague-Dawley rat thoracic aortas by enzymatic digestion and grown in DMEM containing 25 mM glucose.<sup>19</sup> 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 homogenized in 1% Triton X-100 and 0.1% SDS lysis buffer, and cultured VSMCs were lysed in 1% Triton X-100. Solubilized proteins quantified by the BCA assay (Pierce, Rockford, IL). Western analysis was performed as described previously.<sup>20</sup> 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 (500-1000  $\mu$ g) were incubated with primary antibody at 4°C overnight, and immunocomplexes were collected with protein A-agarose beads.

## Small interfering RNA transfection experiments

Cells were transfected using the Nucleofector II Device (Amaxa Inc, Gaithersburg, MD) set to program A-33 with 2.5  $\mu$ g of siRNA per 2-4 $\times$ 10<sup>6</sup> cells in nucleofector solution. Stealth siRNA against IGF1R was from Invitrogen (Carlsbad, CA) (sense: 5'-CCUGUGAAAGUGAUGUUCUCCGUUU-3'; antisense: 5'-AAACGGAGAACAUCACUUUCACAGG-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<sup>4</sup> 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

Glucose uptake was measured as described previously<sup>4</sup> by measuring 2-deoxy-D-[<sup>3</sup>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.

## NF- $\kappa$ B promoter activity

NF- $\kappa$ B-mediated transcriptional induction was measured by transfection of VSMCs with a plasmid containing the luciferase reporter gene driven by an NF- $\kappa$ B promoter. Transfection was performed as described above using 6  $\mu$ g 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).  $10^8$  copies of luciferase RNA (exogenous control) were added to 5  $\mu$ g 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, Indianapolis, IN) real-time thermocycler using Platinum Taq DNA polymerase (Invitrogen). Amplification conditions for MCP-1 were: 100 nmol/L primers (upstream primer, 5'-TGTTGTTACAGTTGCTGCCTG-3'; downstream primer, 5'-GTGCTGAAGTCCTTAGGGTTGAT-3')<sup>21</sup>, 4 mmol/L MgCl<sub>2</sub>, 5% DMSO, annealing at 66°C; for luciferase, 75 nmol/L primers, (upstream primer, 5'-GCTGCTGGTGCCAACCCTATTCTCCTT-3'; downstream primer, 5'-CGCGCAACTTTTTGCGGGTTGTTACTTGA-3'), 3 mmol/L MgCl<sub>2</sub>, and annealing at 60°C. Copy numbers of MCP-1 were corrected for copy numbers of luciferase measured in triplicate.

## Statistics

Results are expressed as mean  $\pm$  SE. Statistical significance was assessed by Student's t-test. A value of  $P < 0.05$  was considered statistically significant.

## Results

### 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 *db/db* mice expressed significantly more IGF1R in all layers of the wall than age-matched controls (Fig. 1), suggesting that increased IGF1R expression may play a role in vascular insulin resistance.

### 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  $2.7 \pm 0.1$ -fold higher than the endogenous value (Suppl. Fig 1A), as occurs in diabetic mice, impacts the response to insulin. Akt phosphorylation was increased 2-3 fold by insulin (7 nM, a concentration that does not activate the IGF1R, Suppl. Fig 1B) in VSMCs transduced with control virus (Fig. 2). In contrast, transduction with IGF1R-overexpressing adenovirus increased baseline Akt phosphorylation, but prevented further stimulation by insulin.

### IGF1R downregulation increases insulin-induced IR $\beta$ 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 (Suppl. Fig. 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 IR $\beta$  phosphorylation and reduced the amount of IGF1R coprecipitating with IR (Fig. 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% (Fig. 3B) and Erk1/2

phosphorylation by 260% (Fig. 3C) compared to that in cells treated with scrambled siRNA. This increase is apparently due to an increase in sensitivity to insulin, as confirmed by a shift in the dose response curve for Akt activation upward and to the left (Fig. 3D).

### IGF1R downregulation increases the fraction of insulin holoreceptors

The observed increased IR phosphorylation does not result from IR upregulation after siIGF1R treatment (Suppl. Fig. 1A), but could be due either to higher phosphorylation of individual IR $\beta$  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 $\beta$  (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.<sup>18</sup>

### IGF1R downregulation potentiates glucose uptake and reduces TNF- $\alpha$ -induced NF- $\kappa$ B 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 (Fig. 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- $\alpha$ , in the presence or absence of insulin, after downregulation of IGF1R using siRNA. TNF- $\alpha$  greatly enhanced NF- $\kappa$ B promoter activity, while insulin alone had no effect (Fig. 5B). However, insulin markedly reduced the response to TNF- $\alpha$ , but only after IGF1R downregulation (Fig. 5B). To confirm that insulin reduced TNF- $\alpha$  proinflammatory activity, we evaluated the expression of MCP-1, a well-known NF- $\kappa$ B-driven inflammatory mediator, in the same experimental conditions. Consistent with the above observations, insulin reduced TNF- $\alpha$ -induced MCP-1 mRNA expression only when IGF1R was downregulated with siRNA (Fig. 5C). Blocking Akt resulted in a reduction of MCP-1 transcription without altering the specific insulin effect. Treatment with the Akt pathway inhibitor LY294002 (10  $\mu$ M) reduced TNF- $\alpha$ -induced MCP-1 expression to 41 $\pm$ 4.6% compared to control,  $p < 0.0001$ , indicating that Akt mediates the TNF- $\alpha$  response. However, there was no difference in the response to TNF- $\alpha$  + insulin in the presence or absence of LY294002, suggesting that insulin exerts its anti-inflammatory effect via an Akt-independent pathway, most likely NF- $\kappa$ B.

### IGF-1 downregulates its receptor

In humans, decreased circulating IGF-1 is commonly seen in DM,<sup>22</sup> while low IGF-1 is also associated with glucose intolerance in healthy subjects.<sup>23</sup> 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 Suppl. Fig. 2, long-term exposure of VSMCs to IGF-1 markedly downregulated IGF1R, while IR expression was less affected, suggesting that physiologic 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- $\alpha$ -induced NF- $\kappa$ B 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 anti-inflammatory role in the vasculature.

The affinities of IGF1R and IR are about a hundred times higher for their cognate, rather than the other, ligand.<sup>24</sup> However, since VSMCs express about 8 times more IGF1Rs than IRs, most IRs are sequestered into insulin-insensitive hybrid receptors.<sup>14, 25</sup> We observed that IGF1R downregulation increases the proportion of IR present in holoreceptors, and promotes insulin signaling and anti-inflammatory 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,<sup>26</sup> adipocytes,<sup>27</sup> and osteoblasts.<sup>28</sup> 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 (Fig. 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 nM). In humans, normal serum insulin levels are in the low picomolar range, while in rodents they may reach the low nanomolar range in severe insulin resistance.<sup>29</sup> 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,<sup>12</sup> 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<sup>29</sup>. Insulin stimulates vascular dilatation mediated by nitric oxide<sup>30</sup>. This function is impaired in vascular insulin resistance. Insulin-stimulated tyrosine phosphorylation of IR $\beta$  in the aorta of obese rats is significantly decreased compared with lean rats, although IR $\beta$  protein levels in the 2 groups are not different.<sup>31</sup> 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.<sup>32</sup> Similarly, IGF1R is upregulated and IGF-1-mediated aortic relaxation is increased after induction of diabetes by streptozotocin in rats.<sup>33</sup>

IGF1R expression is strongly regulated by IGF-1 serum levels *in vivo*<sup>34</sup> and *in vitro*.<sup>35</sup> Accordingly, we find that IGF-1 pretreatment leads to autologous downregulation of IGF1R, supporting the idea that physiologic 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.<sup>36</sup> Since 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.<sup>37</sup> 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 Fig. 3A). It should be noted, though, that IGF-1 levels are tightly regulated by IGF-1-binding proteins,<sup>36</sup> 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.<sup>38</sup> Clinical data show that high systemic levels of IGF-1 are associated with reduced risk of developing impaired glucose tolerance and type 2 DM.<sup>23</sup> 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.<sup>39</sup> Importantly, treatment of patients with DM and insulin resistance with IGF-1 led to enhanced insulin sensitivity, reduced insulin requirements, and improved glycemic control.<sup>40</sup> 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.<sup>41</sup> Inhibition of inflammatory pathways in vascular cells has emerged as an anti-atherosclerotic intervention. The ubiquitous inflammatory transcription factor NF- $\kappa$ B is activated in atherosclerotic lesions.<sup>42</sup> It has been reported that insulin suppresses NF- $\kappa$ B in endothelial cells and mononuclear cells, two important cell types for atherogenesis.<sup>43, 44</sup> In VSMCs the data are conflicting: insulin was found to enhance NF- $\kappa$ B activation induced by hyperglycemia, advanced glycation end products (AGE), and angiotensin II in bovine VSMCs,<sup>45</sup> but another study in rat VSMCs reported that insulin reduces AGE-induced NF- $\kappa$ B expression.<sup>46</sup> We show that insulin reduces TNF- $\alpha$ -induced NF- $\kappa$ B activation and expression of MCP-1, a prototypic NF- $\kappa$ B-responsive inflammatory gene, only after IGF1R downregulation, while 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 VSMC have fueled speculation that insulin resistance of VSMCs may be important in the etiology of vascular diseases associated with DM.<sup>4, 11</sup> 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.<sup>47</sup> In seeming contrast, numerous *in vitro* studies have established that insulin stimulates growth of VSMCs, which is traditionally considered proatherogenic.<sup>48, 49</sup> 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 anti-inflammatory effects in ApoE-deficient mice.<sup>50</sup> This beneficial effect was accompanied by a reduction in vascular IL-6 and TNF- $\alpha$  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,<sup>51</sup>

our data clearly show that insulin exerts its anti-inflammatory effects through its own receptor when IGF1R is downregulated.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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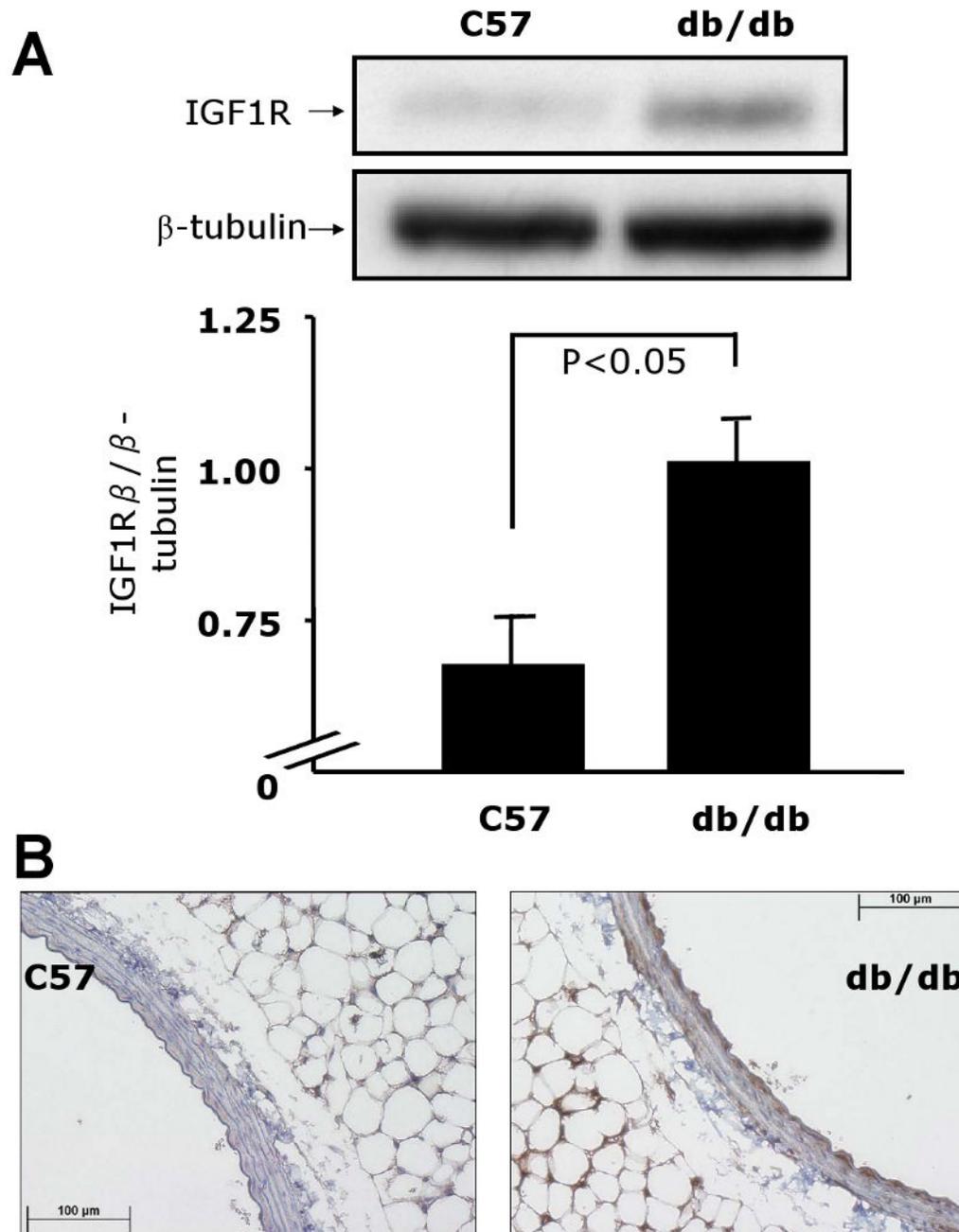
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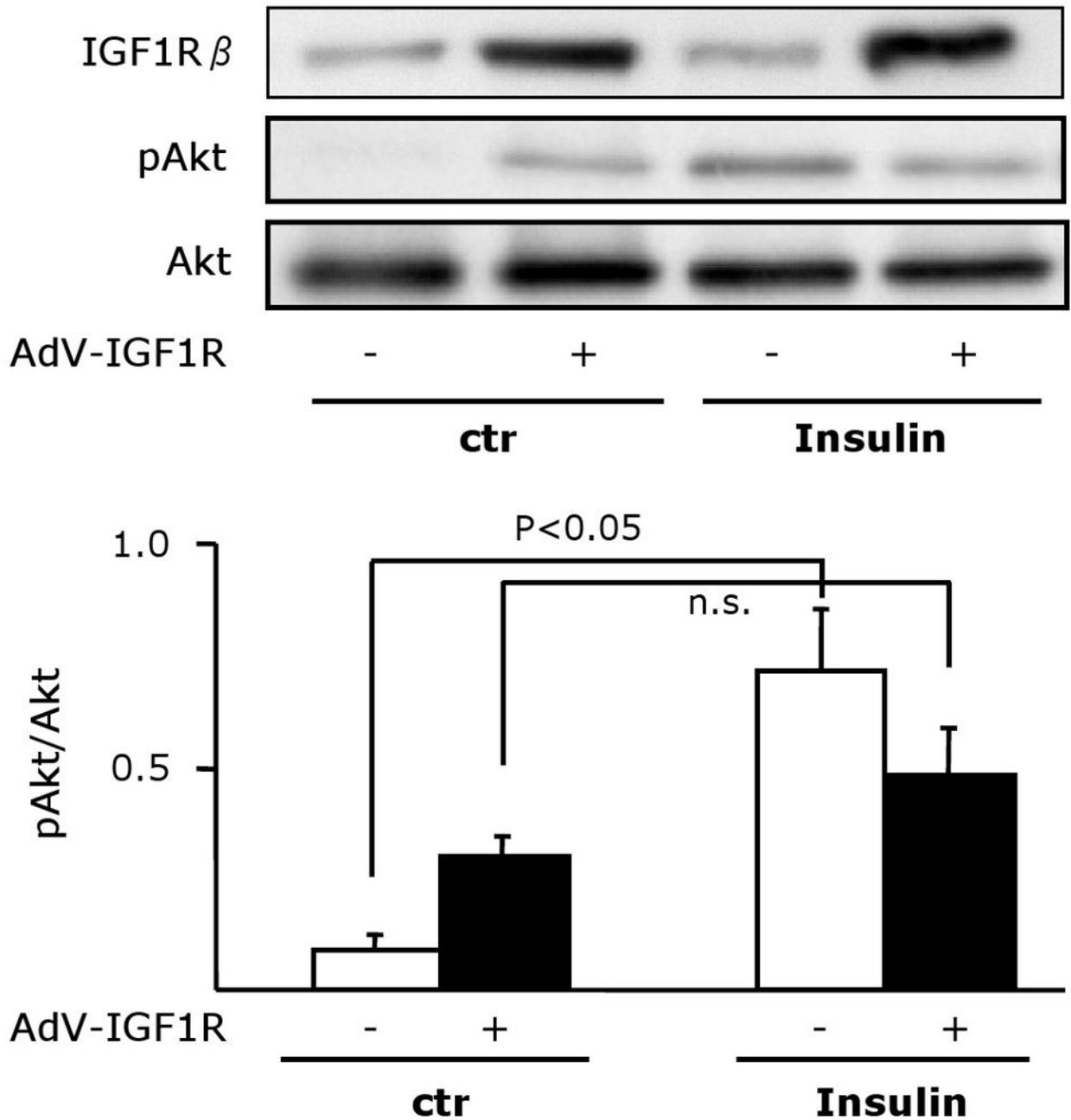
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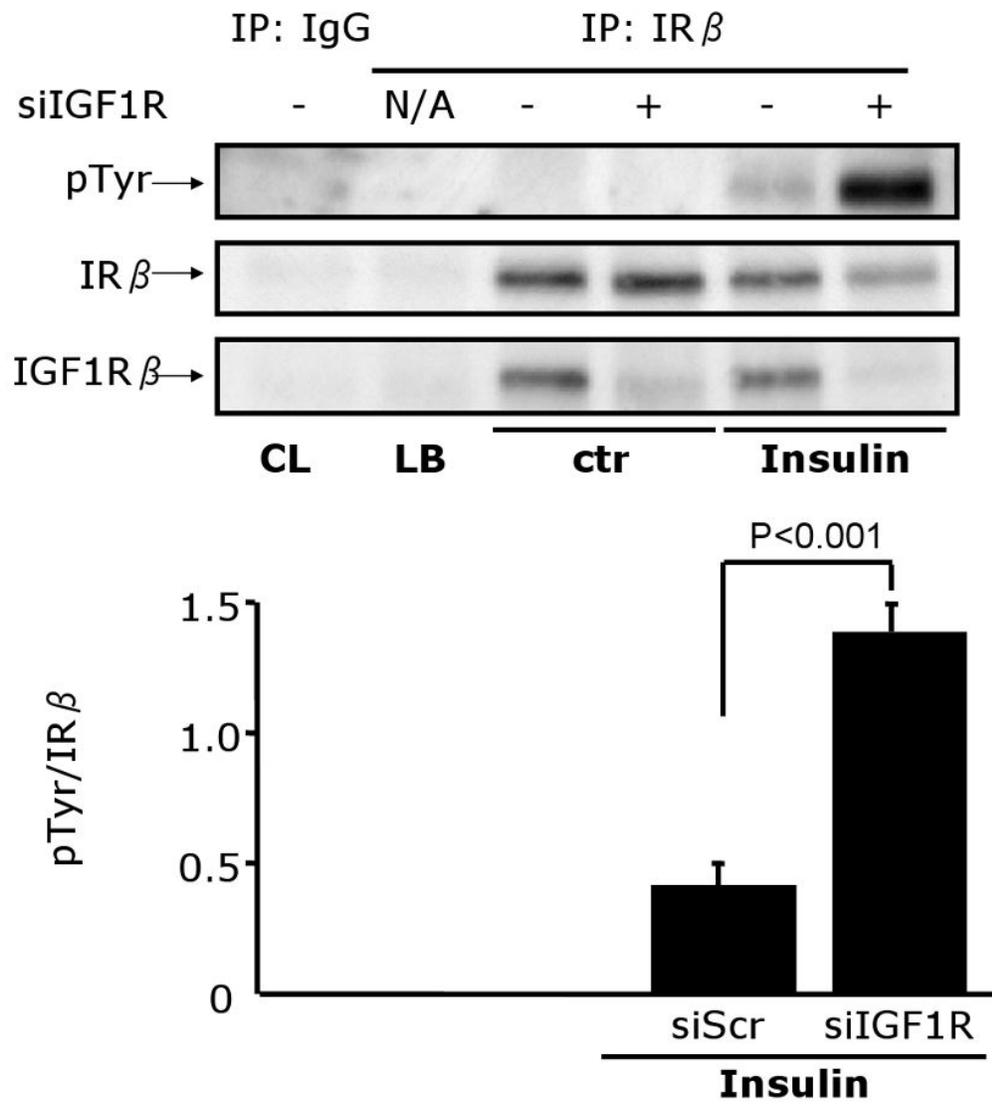
**Figure 1. Vascular IGF1R is upregulated in diabetes**

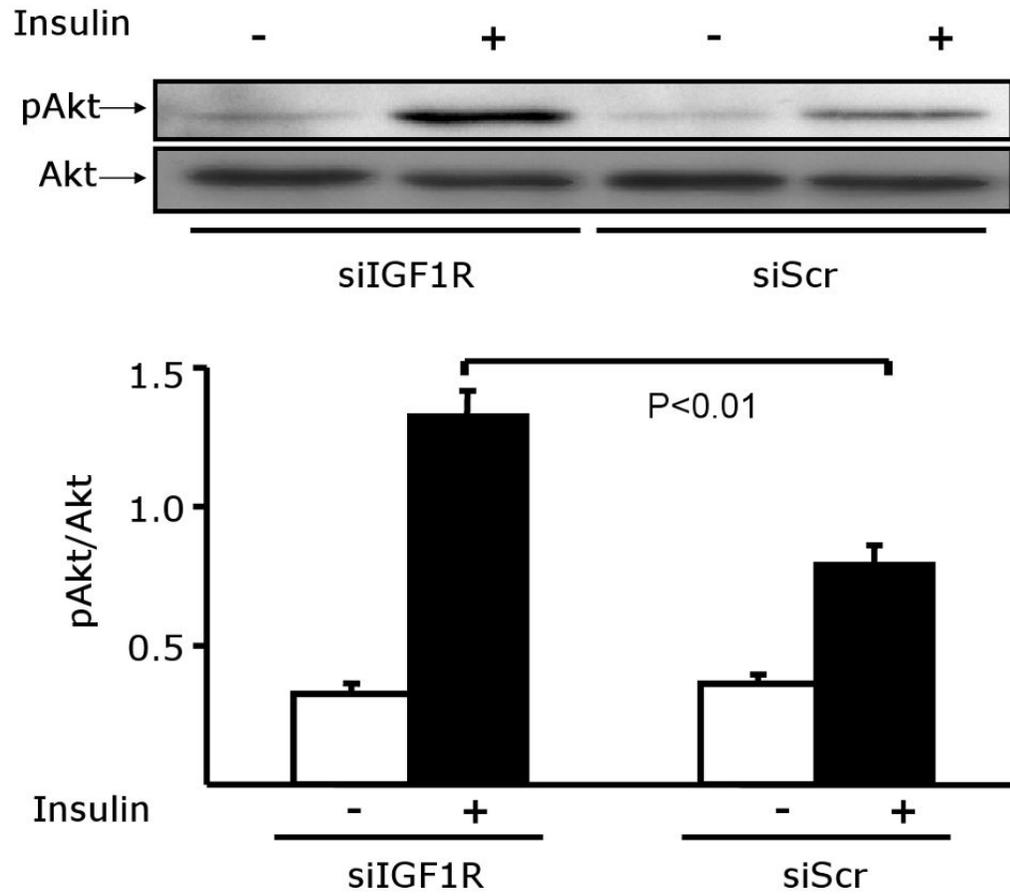
**A**, IGF1R $\beta$  expression was analyzed in aortas from control (C57) and diabetic (*db/db*) mice by Western blotting.  $\beta$ -tubulin served as loading control. Representative blots (top); bar graph (bottom) represents mean  $\pm$  SE (n=3). **B**, immunohistochemistry of IGF1R (brown precipitate) in paraffin embedded cross-sections of mouse aorta.

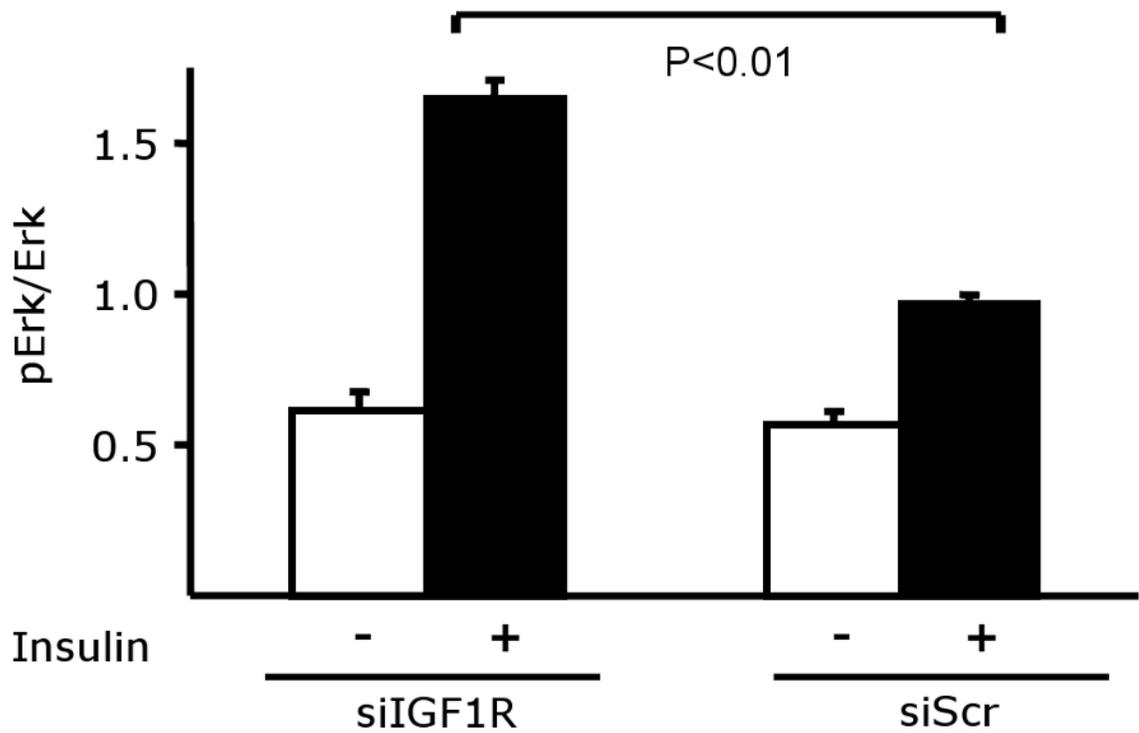
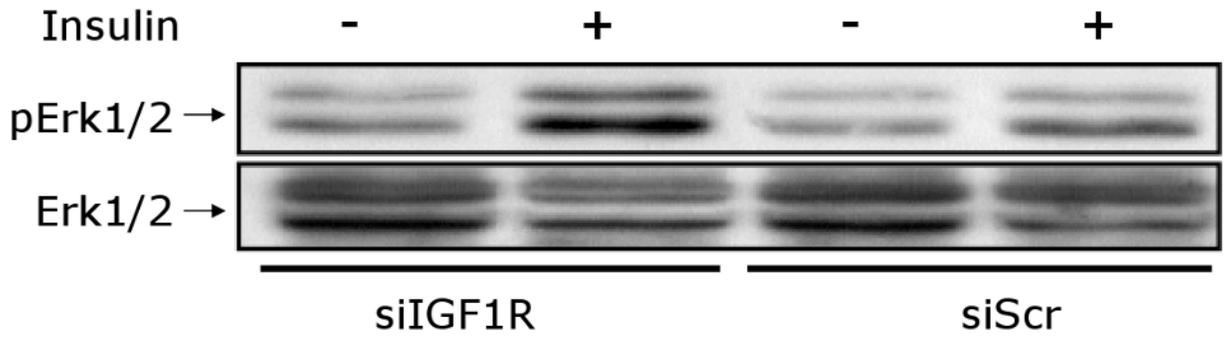


**Figure 2. IGF1R overexpression reduces insulin-induced Akt phosphorylation**

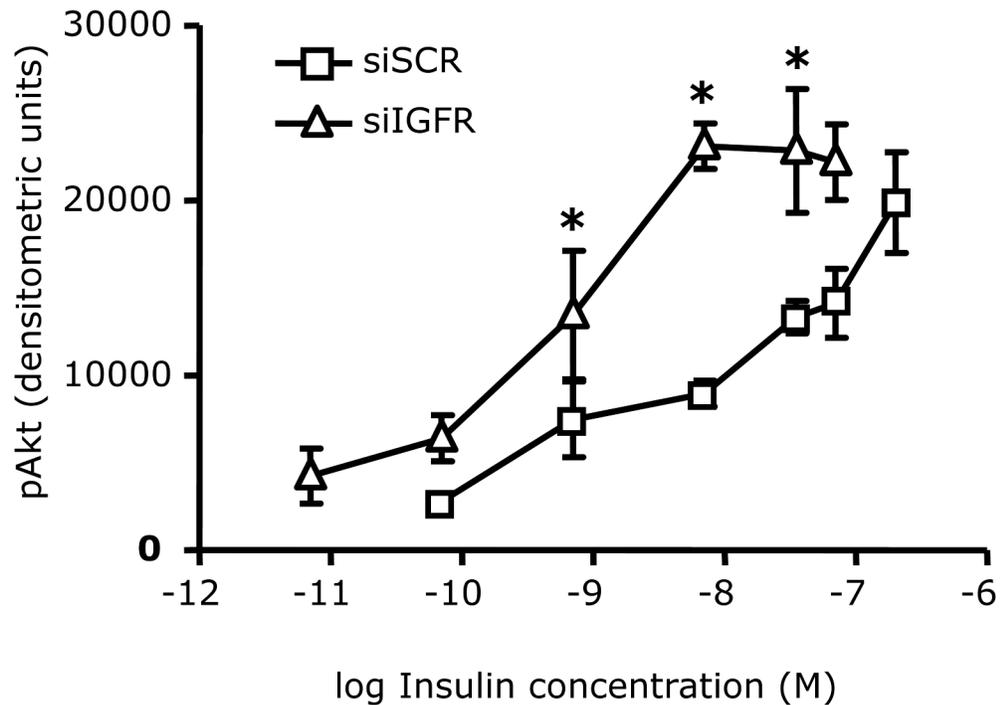
VSMCs were transduced with AdV-IGF1R or control virus and stimulated with 7 nM insulin for 5 minutes. Representative Western blot (top); bar graph (bottom) represents mean  $\pm$  SE (n=3); open bars, control virus; black bars, AdV-IGF1R.

**A**

**B**

**C**

D



**Figure 3. IGF1R downregulation increases phosphorylation of IR $\beta$ -subunit and downstream targets by insulin**

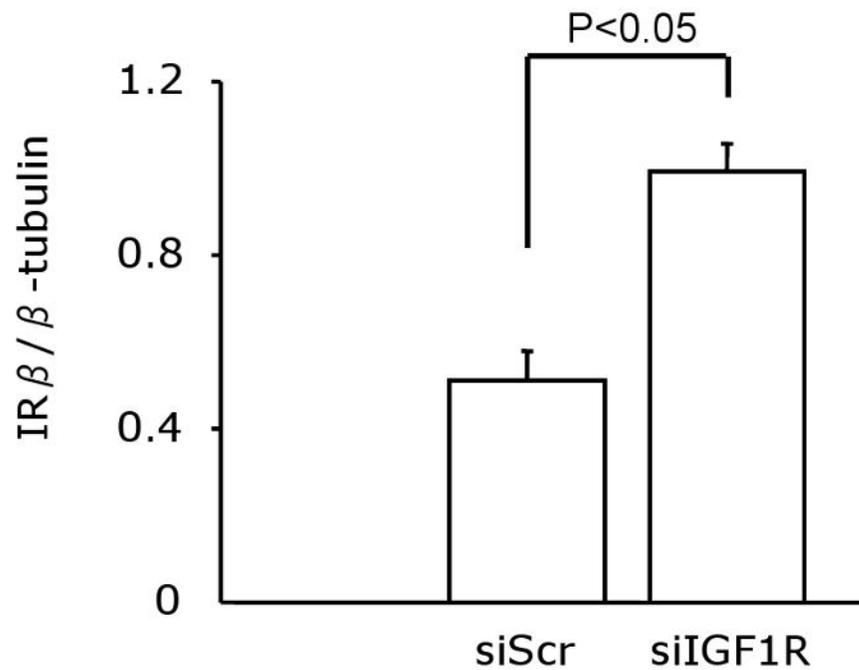
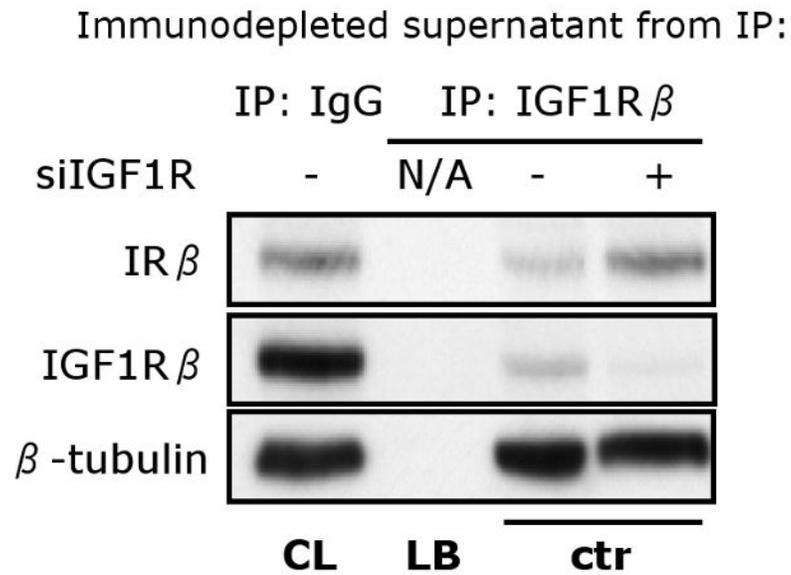
VSMCs were stimulated with 7 nM insulin for 5 minutes and harvested for protein. Representative Western blots (top panels). Bar graphs (lower panels) or line graph (D) represent mean  $\pm$  SE (n=3).

**A**, Immunoprecipitation with anti-IR $\beta$  antibody, followed by Western analysis for phosphotyrosine, IR $\beta$  and IGF1R $\beta$ .

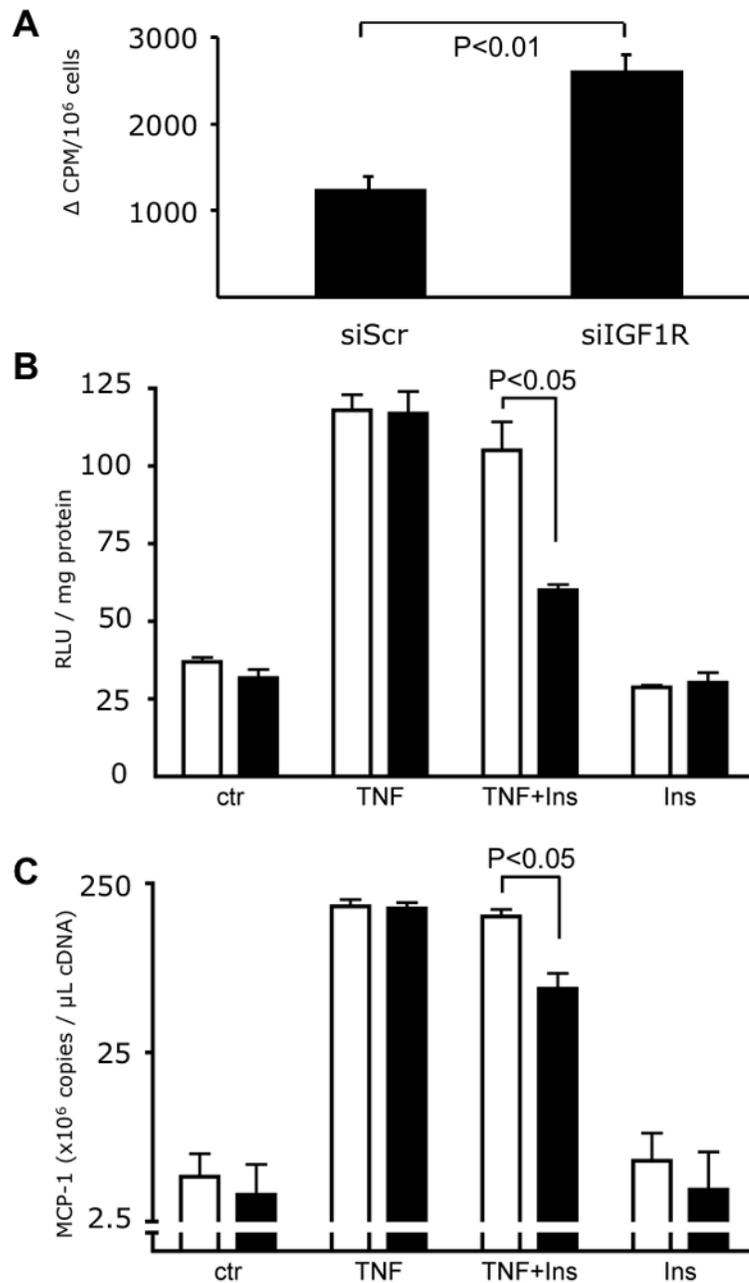
**B**, Western analysis of phospho-Akt and total Akt.

**C**, Western analysis of phospho-Erk1/2 and total Erk1/2.

**D**, Dose dependency of insulin-induced Akt activation, as measured by Western blot. IP, immunoprecipitation; ctr, control; CL, cell lysate; LB, lysis buffer; siScr, scrambled siRNA; siIGF1R, siRNA against IGF1R



**Figure 4. IGF1R downregulation increases the fraction of insulin holoreceptors**  
 VSMCs were lysed, and immunoprecipitated with anti-IGF1R $\beta$  antibody. The immunodepleted supernatant was used for Western analysis of IR $\beta$ , IGF1R $\beta$  and  $\beta$ -tubulin (loading control). Representative Western blots (top); bar graph (bottom) represents mean  $\pm$  SE (n=3). Abbreviations as in Fig. 3.



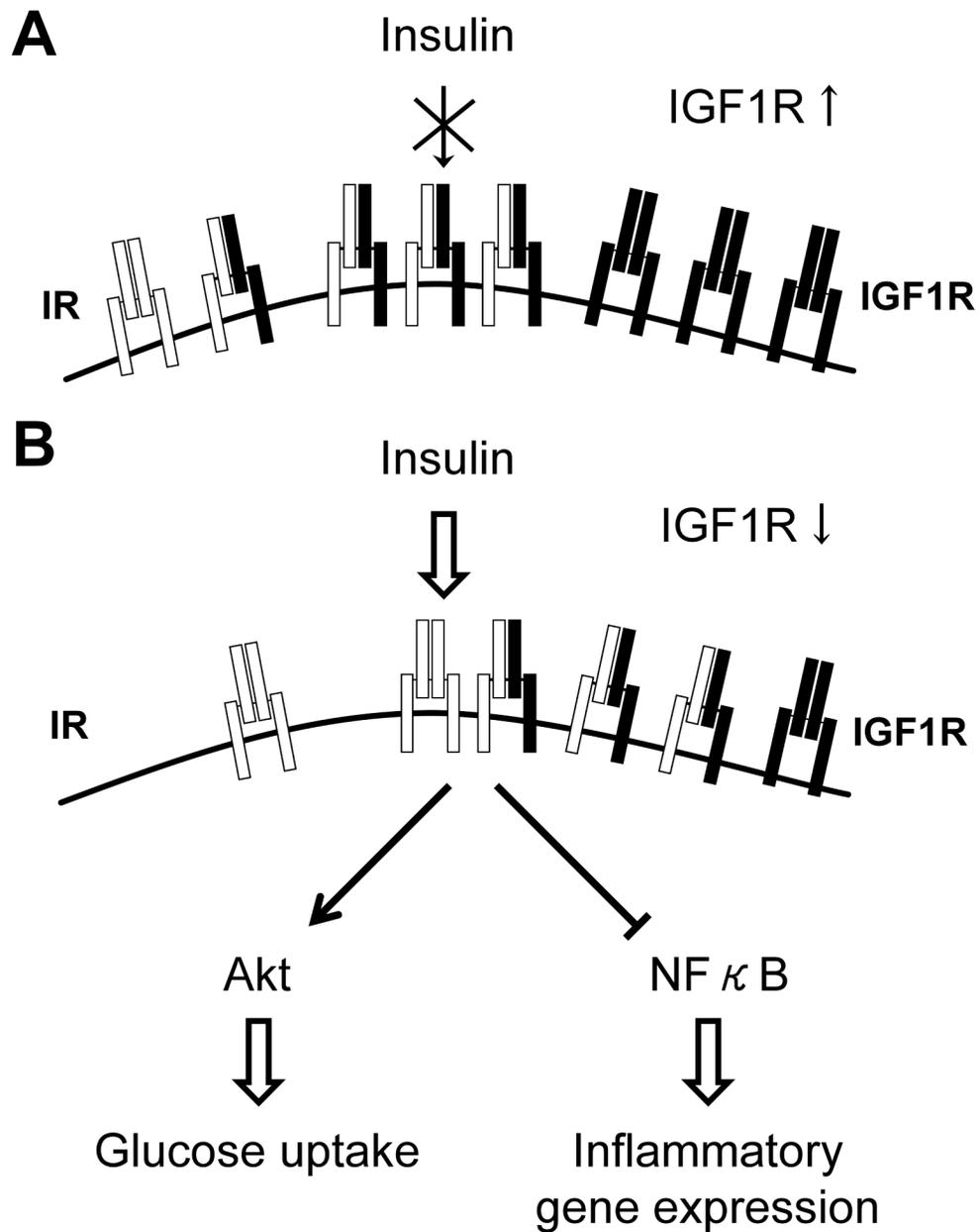
**Figure 5. IGF1R downregulation enhances biological effects of insulin**

VSMCs were transfected with siIGF1R or scrambled control (siScr).

**A**, Increases in 2-deoxy-D-[<sup>3</sup>H]glucose uptake following stimulation with 7 nM insulin for 20 min. Mean  $\pm$  SE (n=3) of counts per minute (CPM) normalized to cell number.

**B**, NF- $\kappa$ B activation was measured with a luciferase reporter assay 4 hours after TNF- $\alpha$  (10 ng/mL) and insulin (7 nM) treatments and normalized to protein. RLU indicates relative light units, mean  $\pm$  SE (n=3-5).

**C**, MCP-1 mRNA was measured using real-time RT-PCR. Data represent cDNA copies, mean  $\pm$  SE (n=3). Open bars, scrambled RNA; Black bars, siIGF1R.



**Figure 6. Ratio of hybrid receptors to holoreceptors determines cellular insulin sensitivity**  
 A. In the basal state, most insulin receptors are sequestered into hybrid receptors, and the response to insulin is very low. After IGF1R downregulation (B) more insulin-sensitive holoreceptors can form with more effective downstream signaling (e.g. Akt activation with subsequent glucose uptake or inhibition of NF- $\kappa$ B activation and inflammatory gene expression).