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# Smad5 regulates Akt2 expression and insulin-induced glucose uptake in L6 myotubes

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ABSTRACT

Insulin-induced glucose uptake by skeletal muscle results from Akt2 activation and is severely impaired during insulin resistance. Recently, we and others have demonstrated that BMP9 improves glucose homeostasis in diabetic and non-diabetic rodents. However, the mechanism by which BMP9 modulates insulin action remains unknown. Here we demonstrate that Smad5, a transcription factor activated by BMP9, and Akt2, are upregulated in differentiated L6 myotubes. Smad5, rather than Smad1/8, is downregulated "in vivo" and "in vitro" by dexamethasone. Smad5 knockdown decreased Akt2 expression and serine phosphorylation and insulin-induced glucose uptake, and increased the expression of the lipid phosphatase Ship2. Additionally, binding of Smad5 to Akt2 gene is decreased in dexamethasone-treated rats and increased in L6 myotubes compared to myoblasts. The present study indicates that Smad5 regulates glucose uptake in skeletal muscle by controlling Akt2 expression and phosphorylation. These finding reveals Smad5 as a potential target for the therapeutic of type 2 diabetes.



## 1. Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF-β) superfamily that play a determinant role in cell differentiation and growth (Miller et al., 2000; von Bubnoff and Cho, 2001). Recently, BMP9 has been shown by us and others to play a central role in glucose homeostasis. We have demonstrated that BMP9 expression and processing in the liver is controlled by glucose and insulin, and that its neutralization using antibodies elicits glucose intolerance and insulin resistance (Caperuto et al., 2008). Previously, treatment with recombinant BMP9 has shown to improve glucose intolerance in diabetic mice and to increase insulin response in L6 myotubes (Chen et al., 2003). The description of these features has highlighted the hypothesis of BMP9 as putative candidate for the hepatic insulin sensitizing substance (HISS). HISS action is believed to account for ~55% of the insulin-induced glucose disposal mainly by favoring insulin action in skeletal muscle (Lautt, 1999).

Apart from these recent and concordant data regarding the insulin sensitizing properties of BMP9, no information has emerged in order to clarify the molecular mechanisms by which these effects occur. In muscle cells, insulin stimulates glucose uptake by a well characterized intracellular mechanism that culminates with the translocation of glucose transporter (GLUT4) enriched vesicles to cell surface. The serial events begin with activation of transmembrane insulin receptor (IR) and tyrosine phosphorylation of IR substrates (IRS). Once phosphorylated, the IRS proteins bind to the regulatory subunit of the phosphatidylinositol-3-kinase (PI3-K), releasing its catalytic subunit. Activation of PI3-K targets downstream activation of Akt, a limiting step for insulin-stimulated GLUT4 translocation to membrane and glucose uptake (Taniguchi et al., 2006).

The canonical BMP intracellular signaling, in contrast to that of insulin, involves two distinct transmembrane serine/threonine kinase receptors, classified as type I and type II receptors (von Bubnoff and Cho, 2001). BMP first bind to a type II receptor, which then recruits and activates a type I receptor, forming a ligand/type II/type I ternary complex. The formation of this complex is a pre-requisite for downstream phosphorylation of mothers against decapentaplegic homolog proteins type 1, 5 and 8 (Smad1, Smad5 and Smad8), known as BMP receptor-related Smad (BR-Smad) (von Bubnoff and Cho, 2001). Phosphorylated Smads are released by the receptor and sequentially dimerize, associate to Smad4 (also known as Co-Smad), translocate to the nucleus and, together with other transcription factors such as p300 and CBP, regulate gene expression (Zwijsen et al., 2003).

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In the present study we demonstrated that Smad5 regulates insulin signaling. Concomitantly with improved insulin response throughout formation of L6 myotubes it was observed the upregulation of Smad5, but not others BR-Smads. Exposure to dexamethasone, a classical inducer of insulin resistance, resulted in decreased Smad5 expression in muscle cells. Using chromatin immunoprecipitation and small interference RNA techniques we showed that Smad5 directly binds to Akt2 promoter and positively regulates Akt2 and, by extension, glucose uptake in L6 myotubes.

## 2. Materials and methods

### 2.1. Animals and L6 cell culture and treatments

Two-month-old male Wistar rats were treated with dexamethasone for 5 consecutive days (1 mg/kg body weight each day, ip) and sacrificed by decapitation. Soleus muscles were removed as previously described (Anhê et al., 2007). L6 skeletal muscle cell line was maintained in DMEM containing 10% FBS, 25 mM glucose, penicillin (100 IU) and streptomycin (100 μg/mL) in a humidified atmosphere with 5% $CO_2$ at 37 °C. L6 myoblasts (80% confluent) were allowed to differentiate in DMEM containing 2% FBS for 4–6 days. Cells were harvested at three distinct times: myoblasts (MB), 2-day-differentiated myotubes (MT2) and 4-day-differentiated myotubes (MT4 or MT). Dexamethasone treatment (200 nM) was performed in myotubes for 48 h in DMEM containing 2% FBS and glucose 5.6 mM. Insulin challenge of myotubes was performed after a 45 min pre-incubation period with Krebs–Henseleit, pH 7.4, at 37 °C, and saturated with a 95% $O_2$:5% $CO_2$ mixture. The buffer was then replaced by fresh Krebs with or without insulin (100 nM) and cells were incubated at 37 °C under a 5% $CO_2$ atmosphere. After 10 min, myotubes were rapidly harvested in ice-cold PBS and pulled down with a 1000 × *g* centrifugation at 4 °C for 10 min. Cell pellets were processed for further experiments.

### 2.2. L6 myoblast transfection with small interference RNA (siRNA)

Small interference RNAs (siRNA) were synthesized using the Block-iT RNAi TOPO Transcription Kit and Block-iT Dicer RNAi Kit (Invitrogen, Carlsbad, CA), strictly following the manufacturer's instructions. Template cDNA used for the synthesis of siRNA was obtained by amplification of a 577 bp fragment from the Smad5 codifying region using the following primers: sense 5′-GCCGTGAAGCGATTGTTGG-3′ and antisense 5′-GGGAGTTGGGATATGTGCTGC-3′; *lacZ* siRNA was synthesized with cDNA and primers provided in the kit. L6 myoblasts were washed twice with serum free medium (Opti-MEM, Invitrogen, Carlsbad, CA) and then incubated in 1 mL of the same medium containing the siRNA targeted to *lacZ* or Smad5 (approximate final concentration of 100 nM) previously mixed with 2 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA), or Lipofectamine only (CTL). After 6 h, the medium containing siRNAs and Lipofectamine was added of 1 mL DMEM containing 4% FBS and 50 mM glucose and the cells were incubated for additional 12 h. Thereafter the cells were allowed to differentiate for 4–6 days and used for further experiments.

### 2.3. RNA isolation and RT-PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, USA). Conventional RT-PCR analysis was performed as previously described (Bordin et al., 2004). The results were expressed by a ratio between target genes and the housekeeping RPL37a. The primer sequences used, as well as melting points and lengths were as follows: Activin-like Receptor 2 (ALK2) sense 5′-GGATCGCAGAACTTCCACCTG-3′ and antisense 5′-GAACCACGACTTCTCATCACGG-3′, 57 °C, 393 bp; ALK3 sense 5′-AAGTTGACATACCCTTGAACACC-3′ and antisense 5′-CCAGCGGTTAGAGACGATTG-3′, 55 °C, 284 bp; ALK6 sense 5′-TTGACATTCCACCCAACACCC-3′ and antisense 5′-CCTGGAGGCAGGATTATGCG-3′, 57 °C, 353 bp; Activin Receptor 2 (ACVR2) sense 5′-AACAATCTGACAGCTTGCATTG-3′ and antisense 5′-CCATCTGCAGCAGTACAACGAG-3′, 55 °C, 224 bp; Smad1 sense 5′-AGTGACAGCAGCATCTTCGTGC-3′ and antisense 5′-CGGGTGTATCTCAATCCAGCAG-3′, 57 °C, 276 bp; Smad5 sense 5′-GGAGGAGTTGGAGAAAGCCTTG-3′ and antisense 5′-GGGAGTTGGGATATGTGCTGC-3′, 57 °C, 470 bp; Smad8 sense 5′-AGAAGGCACATTGGAAAGGGTG-3′ and antisense 5′-CATCCTGGCGATGATACTCGG-3′, 59 °C, 319 bp; AKT2 sense 5′-TTCTACAACCAGGACCACGAGC-3′ and antisense 5′-TGATGCTGAGGAAGAACCGATG-3′, 55 °C, 311 bp; RPLA37a sense 5′-CAAGAAGGTCGGGATCGTCG-3′ and antisense 5′-ACCAGGCAAGTCTCAGGAGGTG-3′, 57 °C, 290 bp. AKT2 expression was analyzed by real-time PCR using the 2(-Delta Delta C(T)) method.

### 2.4. Protein extraction and western blotting

Soleus muscles and myotubes were homogenized in SDS lysis buffer (1% SDS, 100 mM Tris pH 7.4, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA and 10 mM sodium orthovanadate). After homogenization, samples were processed as previously described (Anhê et al., 2007) and resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dried milk diluted in 10 mM Tris–HCl, 100 mM NaCl, 0.05% Tween-20 (TBST) and probed with antibodies against Smad1 (Cell Signaling, 1:1000), Smad5 (Cell Signaling, 1:1000), Smad8 (Santa Cruz Biotechnology, 1:1000), Ship2 (Santa Cruz Biotechnology, 1:1000), Pten (Santa Cruz Biotechnology, 1:1000), pPten (Ser 380) (Cell Signaling, 1:1000), phospho-Akt1/2/3 (Ser 473) (Santa Cruz Biotechnology, 1:100), Akt1 (Upstate Biotechnology, 1:5000), Akt2 (Upstate Biotechnology, 1:5000), IRS1 (Upstate Biotechnology, 1:1000) and IR (Santa Cruz Biotechnology, 1:1000) diluted in TBST with 3% BSA. Chemiluminescence was performed after 1h incubation with a secondary antibody conjugated to HRP (GE Healthcare) diluted in TBST containing 1% non-fat dried milk. Band intensities were visualized in X-ray sensitive films and quantified by Scion Image software (Scioncorp, USA).

### 2.5. Total Akt activity

Akt activity was measured using the Akt Kinase Assay Kit (Cat. No. 9840, Cell Signaling, USA). Briefly, cells were homogenized in 1× cell lysis buffer and incubated with anti-Akt1/2/3 antibody cross-linked to agarose beads. Agarose beads were then incubated with 1× kinase buffer, ATP and GSK3 fusion protein. Reaction was terminated by the addition of SDS sample buffer. The supernatants were resolved in SDS-PAGE and transferred to nitrocellulose membranes. Phospho-GSK3 was detected using monoclonal anti-phospho-GSK3 antibody, secondary HRP-conjugated antibody and LumiGLO substrate provided in the kit.

### 2.6. Akt2 immunoprecipitation

Myotubes were sonicated in immunoprecipitation buffer (1% Triton X-100, 100 mM Tris pH 7.4, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate and 2 mM PMSF) and processed for immunoprecipitation using 2 μg of Akt2 antibody (Upstate Biotechnology) and 500 μg of total protein. After incubation, samples were treated with Protein A-Sepharose (GE Healthcare) and 5× Laemmli buffer containing DTT as previously described (Anhê et al., 2007). Supernatants were processed for western blot to detect Akt2 serine phosphorylation using anti-phospho-Akt1/2/3 Ser (Santa Cruz Biotechnology, 1:100) as primary antibody.

### 2.7. 2-Deoxy-D-[2,6-$^3$H]glucose uptake

Myotubes seeded in six-well plates were pre-incubated for 6 h in DMEM without serum. Next, cells were incubated for 2 h in DMEM without glucose and serum. DMEM was then removed and cells were washed and pre-incubated with Krebs–Henseleit at 37 °C for 10 min with or without insulin (100 nM). The buffer was replaced by a fresh one containing 5.6 mM glucose and 0.2 mCi/mL 2-DG, and cells were incubated for additional 20 min at 37 °C. After that, cells were washed in ice-cold PBS and the radioactivity associated with the cells was determined by cell lysis with 1 M KOH at 70 °C for 20 min, followed by liquid scintillation of aliquots of each sample (100 μL). The uptake measurement was performed in triplicate. Protein concentration of each sample was determined by Bradford method. Results were normalized by milligrams of total protein.

### 2.8. Chromatin immunoprecipitation assay (ChIP assay)

Skeletal muscle and L6 myoblasts and myotubes were processed using buffers and reagents from EZ ChIP Chromatin Immunoprecipitation Kit (Upstate, Lake Placid, NY, USA) according to manufacturer instructions. Briefly, cells were fixed in DMEM containing 1% formaldehyde for 10 min at room temperature and transferred to lysis buffer. Muscles were minced in PBS containing 1% formaldehyde and treated as L6 cells. DNA was sheared to fragments of ~200–1000 bp by applying 4 bursts of sonication for cells or 6 bursts of sonication for muscle fragments (20% of power, 10 s each). Samples were diluted in dilution buffer and pre-cleared for 1 h at 4 °C with protein G-agarose (50% slurry) saturated with salmon sperm DNA. An aliquot of 10 μL were collected as "input". The remaining supernatants were submitted to immunoprecipitation with protein G-agarose saturated with salmon sperm DNA and 5 μg of anti-Smad5 antibody (Santa Cruz Biotechnology, sc-26418). In parallel, one sample was incubated with protein G-agarose only in order to generate the negative control (no-AB). Agarose pellets were then washed with buffers provided in the kit and treated with elution buffer. Supernatants were submitted to cross-linking reversal and RNase A treatment. DNA was purified using phenol–chloroform and resuspended in 20 μL of $ddH_2O$. DNA samples were amplified for detection of Akt2 and Id1 genes, the latter used as control gene. A 146 bp fragment corresponding to nucleotides −281 to −126 of the rat Akt2 promoter was amplified by conventional (40 cycles) and real-time PCR. The sequences of the primers were: Akt2 sense 5′-GCCGCTTAGGAAGCCTTCAG-3′ and antisense 5′-TCCAGTTCCGAGCTTGAGTGC-3′, 56 °C; and Id1 sense 5′-TGAAGGTCGCCAGTAGCAGTG-3′ and antisense 5′-AGCCGTTCATGTCGTAGAGCAG-3′, 60 °C, 192 bp. The products were visualized as described for RT-PCR. Amplification values were normalized by input values and DNA content.