Supplementary Data

Insulin signaling in the heart is directly and early impaired by growth hormone

Marina C Muñoz1, Verónica G Piazza1, Valeria Burghi1,a, Jorge F Giani1,b, Carolina S Martinez1,c, Nadia S Cicconi1, Nadia V Muia1, Yimin Fang2, Sergio Lavandero3,4, Ana I Sotelo1, Andrzej Bartke5, Patricia A Pennisi5, Fernando P Dominici1, Johanna G Miquet1.

1 Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Instituto de Química y Fisicoquímica Biológicas (IQUIFIB), Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Buenos Aires, Argentina
2 Department of Internal Medicine, Geriatrics Research, Southern Illinois University School of Medicine, Springfield, IL, USA
3 Advanced Center of Chronic Diseases (ACCDiS), Facultad de Ciencias Químicas y Farmacéuticas y Facultad de Medicina, Universidad de Chile, Santiago, Chile.
4 Department of Internal Medicine, Cardiology Division, University of Texas Southwestern Medical Center, Dallas, TX, USA.
5 Centro de Investigaciones Endocrinológicas 'Dr. César Bergadá' (CEDIE) CONICET - FEI - División de Endocrinología, Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina

a V Burghi is now in Department of Pharmacology and Moores Cancer Center, University of California, San Diego, La Jolla, California, USA.
b JF Giani is now in Departments of Biomedical Sciences and Pathology, Cedars-Sinai Medical Center, Los Angeles, CA, USA.
c CS Martinez is now in Departamento de Ciencia y Tecnología, Laboratorio de Bio-Nanotecnología, Universidad Nacional de Quilmes, Buenos Aires, Argentina.

Correspondence should be addressed to Johanna G Miquet: jmiquet@qb.ffyb.uba.ar
Detailed Materials and methods

Experimental models

**Transgenic mice overexpressing GH (GH-Tg)**

Transgenic mice containing the bovine GH (bGH) gene fused to control sequences of the rat phosphoenolpyruvate carboxykinase (*Pepck*) were used. PEPCK-bGH mice have chronically elevated bGH and IGF1 circulating level, significantly increased body weight and organomegaly. Mouse generation, breeding system, feeding conditions and animal housing were previously described (McGrane *et al*. 1988; Miquet *et al*. 2013; Bacigalupo *et al*. 2019). Briefly, hemizygous transgenic mice were produced by mating transgenic males with normal C57BL/6 × C3H F1 hybrid females purchased from the Jackson Laboratory. Matings produced approximately equal proportion of transgenic and normal (non-transgenic) progeny. Normal siblings of transgenic mice (littermates that exhibited normal size) were used as controls. Mice were housed 3–5 per cage in a room with controlled light (12 h light/day) and temperature (22 ± 2°C). The animals had free access to a balanced diet (Rodent Laboratory Chow 5001 with 23.4% protein, 5% fat, and 5.8% crude fiber; LabDiet, PMI Feeds, St. Louis, MO, USA) and water. Female 3-4-month-old mice were used.

**Liver IGF1 deficient mice (LID)**

The generation of liver IGF1 deficient (LID) mice using a Cre/loxP recombination system has been previously reported (Yakar *et al*. 1999). These animals have a 75% reduction in circulating IGF1 levels, with a concomitant increase in GH circulating levels, but exhibit normal growth and development. Mice that do not express the Cre transgene were used as controls. LID and control mice backcrossed into the C57BL/6 background were kindly donated by Dr. S Yakar.
Animal husbandry and PCR genotyping were previously described (Fernández et al. 2012). Briefly, mice were housed 3–5 per cage in standard conditions of 12h light/day and controlled temperature (22 ± 2°C), with free access to a nutritionally balanced diet (Alimento Balanceado Cooperación with 23% protein, 5% fat and 6% crude fiber; ACA Nutrición Animal, San Nicolás de los Arroyos, Buenos Aires, Argentina) and water. For this work, female mice (2-4 months old) were used. Considering the well-known sexual dimorphism in many of GH actions (Liu et al. 2000; Tang et al. 2005; Liu et al. 2016), some determinations were also corroborated in male mice of the same age.

**Swiss-Webster mice treated with GH**

Female Swiss-Webster mice (2-3 months old) were used. Animal housing and feeding conditions were as previously described (Bacigalupo et al. 2019). Briefly, mice were housed 3–5 per cage in a room with controlled light (12h light: 12h darkness cycle) and temperature (22±2°C). Mice had free access to a nutritionally balanced diet (Alimento Balanceado Cooperación with 23% protein, 5% fat and 6% crude fiber; ACA Nutrición Animal, San Nicolás de los Arroyos, Buenos Aires, Argentina) and water. Mice were treated with highly purified porcine GH (Zamira Life Sciences, Knoxfield, Victoria, Australia) for four days. The hormone (2mg/kg/day) was administered by two daily subcutaneous injections (8 am and 5 pm), control animals received saline solution (vehicle). The first injection was administered in the afternoon of the first day of treatment, and on the fifth day, the last injection was administered in the morning after which food was removed to allow a 6-h fasting prior to insulin stimulation and tissue collection.

**Primary culture of rat cardiomyocytes treated with GH**

The isolation of neonatal rat cardiomyocytes was previously detailed (Santos et al. 2014). Briefly, hearts from 1- to 3- day old Sprague Dawley rats were obtained, ventricles were excised and washed in cold phosphate saline buffer, and pieces were digested in a balanced
salt solution containing collagenase type IV (1 mg/mL, Worthington, Lakewood, NJ, USA). Cells were collected by centrifugation and resuspended in DMEM-F12 containing 10% FBS, 1% v/v ampicillin and streptomycin, 10 μg/mL holo-transferrin, 10 μg/mL insulin and 100 μM bromodeoxyuridine. Cells were plated into a Petri dish and incubated in a 5% CO₂ atmosphere at 37 °C for 1 h to let the cells, mainly fibroblasts, to attach. The cells that remained suspended, mainly cardiomyocytes, were collected and plated. After 24 h, medium was replaced with DMEM-F12 containing 10% FBS for another 24 h period, then it was replaced with the same fresh medium with or without the addition of 1 μg/mL porcine GH (Zamira Life Sciences). After 24 h, cells were incubated in the absence of serum in DMEM-F12 with or without GH for 6 h.

For insulin stimulation experiments, after the 6 h incubation in the absence of serum, the medium was removed and cells were incubated in DMEM-F12 with or without 10⁻⁵ M porcine insulin (Sigma, St. Louis, MO, USA) for 10 min. Medium was removed and cold phosphate buffer saline was added for washing, which was then completely removed and the wells with the cells attached were stored at -70°C.

**Ethical approval**

All animal procedures were approved by the Laboratory Animal Care and Use Committee of the Southern Illinois University School of Medicine (GH-transgenic mice) or of the School of Pharmacy and Biochemistry and the School of Sciences of the University of Buenos Aires (LID and Swiss-Webster mice, and neonatal rats), and complied with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

**Acute insulin stimulation and tissue collection in animal models**
Mice were fasted for 6 h and anesthetized with ketamine/xylazine mixture. After anesthesia was induced, the inferior cava vein was exposed, and porcine insulin was injected at a dose of 10 IU per kg of body weight in saline solution (0.9% w/v NaCl). To evaluate basal conditions, control mice were exposed to the same procedure but received vehicle. Five min after injection, the heart was removed, frozen, and kept at −70°C until tissue homogenization for immunoblotting or ELISA assays. Additional mice were subjected to a similar procedure but instead of receiving the hormone, blood was extracted from the cava vein to analyse metabolic parameters, and the heart was removed and kept frozen at -70°C until processing for RNA purification, or the heart was cut from apex to base, fixed in 10% v/v formalin and embedded in paraffin for histological examination.

**Histological examination**

Tissue sections were deparaffinized, subjected to Masson’s trichrome staining, and examined for myocardial fibrosis using a light microscope (DM2000, Leica Microsystems, Wetzlar, Germany). The whole sections were observed, and representative photomicrographs were obtained by a Leica DFC400 digital camera and Leica Application Suite software (Leica Microsystems).

**Glucose, insulin, cholesterol, and triglycerides measurements**

Glucose levels were measured in blood using the hand-held glucometer Presto Blood Glucose Meter (AgaMatrix, Salem, NH, USA) for the GH-Tg mice or the ACCU-CHEK®Nano meter (Roche Diagnostics Corp., IN, USA) for the LID and Swiss-Webster mice. Serum insulin levels were determined using the Rat/mouse insulin ELISA kit cat# EZRMI-13K, EMD Millipore (Billerica, MA, USA). Circulating cholesterol and triglyceride concentrations were measured by enzymatic colorimetric assay kits from Pointe Scientific (Canton, MI, USA).

**Tissue and cell solubilization**
The entire heart was homogenized in cold solubilization buffer containing 1% w/v Triton X-100 together with protease and phosphatase inhibitors as described previously (Miquet et al. 2011). For cardiomyocyte experiments, cells were harvested with solubilization buffer and processed as previously described (Santos et al. 2014). Heart extracts and cell lysates were centrifuged to eliminate insoluble material, protein concentration in the supernatants was determined using the BCA protein assay (Thermo Scientific Pierce, Rockford, IL, USA), and samples were used for ELISA or an aliquot was diluted in Laemmli buffer, boiled for 5 min, and stored at −20 °C until immunoblotting.

**ELISA for IRS1 Ser<sup>307</sup> phosphorylation determination**

IRS1 phospho-Ser<sup>307</sup> levels were measured in fresh aliquots of tissue homogenates or cell lysates containing 35 µg of protein using the PathScan® Phospho-IRS-1 (Ser<sup>307</sup>) Sandwich ELISA Kit (7287S) from Cell Signaling Technology Inc. (Danvers, MA, USA).

**Immunoblotting**

Equal amounts of total protein in solubilized samples were subjected to SDS-PAGE and immunoblotting as previously described (Bacigalupo et al. 2019). Immunoreactive bands were detected by chemiluminiscence using Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA). Band intensities were quantified with Gel-Pro Analyzer 4.1 software (Media Cybernetics, Bethesda, MD, USA). Protein loading control was performed by relativizing protein content to β-actin, β-tubulin or Coomassie blue staining of PVDF membranes after blotting experiments (Welinder and Ekblad 2011; Bacigalupo et al. 2019).

The list of the antibodies used for immunoblotting is available in Table 1. The Supplementary Table 1 is a more detailed version with references for the antibodies used.

**Quantitative reverse transcriptase PCR (RT-qPCR)**
Total RNA was extracted from heart tissue using TRIzol Reagent (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA) and cDNA was obtained using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) following standard procedures (Bacigalupo et al. 2019). Primers were designed for specific amplification of Ghr, Igf1r, Igf1, Socs3 and Glut4 as target genes and Cyclophilin A, 18 S ribosomal RNA, Actin beta and beta-2 microglobulin as reference genes, following general recommendations and as described before (Piazza et al. 2017). Primer sequences are available in Table 2. qPCR reaction was performed in a StepOnePlus™ Real-Time PCR System using SYBR® Select Master Mix (Applied Biosystems™, Beverly, MA, USA), as previously described (Piazza et al. 2017; Bacigalupo et al. 2019). Relative gene expression levels were calculated by the comparative cycle threshold (Ct) method (Pfaffl 2001). Target gene relative expression levels were normalized by the geometric mean of the four housekeeping genes used (Vandesompele et al. 2002).

**Statistical analysis**

GraphPad Prism statistical program (GraphPad Software, San Diego, CA, USA) was used for graphs confection and statistical analysis. Experiments were performed analysing all groups of each experimental model in parallel. Results are expressed as mean ± SEM of the indicated number (n) of different individuals per group. In the case of cell culture, n refers the number of independent experiments. Two-way ANOVA followed by the Bonferroni post-test was used to assess differences between the four experimental groups corresponding to exposure or not to elevated GH levels and administration with insulin or saline. Unpaired Student’s t-test was used when two groups were analysed (exposed or not to elevated GH levels). Data were considered significantly different if P<0.05.
Supplementary Table 1 - List of antibodies used
<table>
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<tr>
<th>Antibody anti-target protein</th>
<th>Company</th>
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1) https://antibodyregistry.org/search.php?q=AB_631835;
2) https://antibodyregistry.org/search.php?q=AB_632138;
3) https://antibodyregistry.org/search.php?q=AB_2139810;
4) https://antibodyregistry.org/search.php?q=AB_2127885;
5) https://antibodyregistry.org/search.php?q=AB_629533;
6) https://antibodyregistry.org/search.php?q=AB_11211209;
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13) https://antibodyregistry.org/search.php?q=AB_915783;
20) https://antibodyregistry.org/search.php?q=AB_330339;
22) https://antibodyregistry.org/search.php?q=AB_10545274;
26) Tested in liver insulin receptor knock-out (LIRKO) mice (Miao et al. 2014).
27) Tested in p38 knockout mice (Kim et al. 2008).
28) Tested in cells transfected with specific siRNAs against p38 (Lee et al. 2017).
29) Tested in p38MAPK knockdown cells (Zhao et al. 2016).
30) Used in cells incubated with different insulin concentrations (Piro et al. 2010).
31) Used in T2DM patients with reduced IRS1 and IRS2 protein levels and phosphorylation (Folli et al. 2011).
32) Used in cells subjected to treatments that decreased or increased IRS1 protein expression and phosphorylation (Bailey-Downs et al. 2012).
33) Used to assess insulin-induced GLUT4 translocation in mouse skeletal muscle (Wei et al. 2020).
34) Used to determine hyperglycemia-mediated GLUT4 suppression (the protein levels determined by Western-blotting correlated with mRNA levels) (Xie et al. 2022).
35) Used to determine p85 protein content and insulin-induced coimmunoprecipitation with IRS1 in mouse heart (Miquet et al. 2011).
36) Tested in p50α/p55α null mice (the anti-pan-p85 antibody also detects p50α/p55α subunits) (Pensa et al. 2014).
38) Company validation of the antibody includes the use of cells transfected with IR and stimulated with insulin analyzed by Western blotting, pre-incubation with the blocking peptide corresponding to phospho-IR Tyr972 was also performed: [https://www.sigmaaldrich.com/AR/es/product/mm/07838](https://www.sigmaaldrich.com/AR/es/product/mm/07838)

39) Used to determine insulin-induced hepatic phosphorylation of IR on Tyr972 in rats (Muñoz et al. 2009).

40) Used to determine insulin-induced phosphorylation of IR on Tyr972 in skeletal muscle of rats (Muñoz et al. 2020).

41) Company validation of the antibody includes the use of cells transfected with IR and stimulated with insulin analyzed by Western blotting, pre-incubation with the blocking peptide immunogen was also performed: [https://www.sigmaaldrich.com/AR/es/product/mm/07841](https://www.sigmaaldrich.com/AR/es/product/mm/07841)

42) Used to determine insulin-induced IR phosphorylation in skeletal muscle of rats (Surapongchai et al. 2018).

43) Used to determine insulin-induced hepatic phosphorylation of IR on Tyr1158/Tyr1162/Tyr1163 in rats (Muñoz et al. 2009).

44) Company validation of the antibody includes Western blotting analysis using as a control blocking with a human beta-tubulin peptide: [https://www.abcam.com/beta-tubulin-antibody-loading-control-ab6046.html#lb.](https://www.abcam.com/beta-tubulin-antibody-loading-control-ab6046.html#lb.)

45) Used to determine total actin levels in flies heterozygous for an actin null allele (Fulga et al. 2007).

46) Tested in cells with AKT1 knockdown through siRNA transfection (Chiu et al. 2020).

47) Tested in cells where the T308 phosphorylation site was mutated to alanine (T308A) (Hart and Vogt 2011).

48) Tested in cells treated with activators and inhibitors of mTOR signaling, and in cells with mTOR knockdown (Zhang et al. 2019).

49) Tested in cells transfected with siRNA directed against ErK1/2 (Kim et al. 2021).

50) Tested in cells transfected with siRNA directed against ErK1/2 (Schroyer et al. 2018).

51) Tested in cells with silenced with shRNAs and siRNAs that targeted GSK3β (Cuartas-López and Gallego-Gómez 2020).

52) Tested in cells stably expressing GSK3β-specific siRNA (Ko et al. 2015).

53) Used in double IRS1 mutants created by replacing serine residues S636 and S639 with either aspartic acid (S636/639D) or alanine (S636/639A) (Tzatsos and Kandror 2006).

54) Used in cell experiments showing that knockdown of components of mTORC1 suppresses insulin-stimulated phosphorylation of IRS1 at S636/639 and stabilizes IRS1 after long term insulin stimulation (Tzatsos 2009).

55) Used in double IRS1 mutants created by replacing the S616 (human numbering, corresponding to 612 in mouse) with aspartic acid (S616D) (Tzatsos and Kandror 2006).

56) Tested in IRS1 knockdown cells (Groeneveld et al. 2016).


58) Tested in AS160 knocked down cells using siRNA (Kim et al. 2011).

**References**


Groeneveld MP, Brierley GV, Rocha NM, Siddle K & Semple RK 2016 Acute knockdown of the insulin receptor or its substrates Irs1 and 2 in 3T3-L1 adipocytes suppresses adiponectin production. Sci Rep 6 21105.


Tzatsos A 2009 Raptor binds the SAIN (Shc and IRS-1 NPXY binding) domain of insulin receptor substrate 1 (IRS-1) and regulates the phosphorylation of IRS-1 at Ser-636/639 by mTOR. *J Biol Chem* **284** 22525-22534.


Supplementary Figures

Supplementary Figure 1. Cardiac histology of GH-Tg and LID mice. Heart sections were stained with Masson's trichrome and examined for myocardial fibrosis using a light microscope. The whole cardiac sections were observed and representative photomicrographs of GH-Tg (A) and LID (B) female mice, and the corresponding normal controls, are shown (original magnification 100X and 400X). Six individuals of each experimental group were analysed in parallel. Blue-stained regions corresponding to cardiac extracellular-matrix content is evidenced in GH-Tg mice.
Supplementary Figure 2. Activation of insulin signaling in the heart of LID mice. Normal (N) and LID (L) male mice were injected with saline (−) or insulin (+) and the heart was removed after 5 min. Equal amounts of solubilized heart protein were subjected to immunoblotting using specific antibodies to detect AKT phosphorylation at S\(^{473}\) and T\(^{308}\) and protein content. Coomassie blue staining (CBS) of PVDF membranes was used to control protein loading. Data are the mean ± SEM of five to seven different individuals per group. Groups denoted by different letters are significantly different (\(P<0.05\)), assessed by two-way ANOVA followed by Bonferroni post-test. NS: not significant. Representative immunoblots are shown.
Supplementary Figure 3. p85 protein content in the heart of LID and GH-treated mice.

Protein content of p85 was assessed in the heart of normal (N) and LID (L) female mice (A) and of Swiss-Webster female mice treated with GH (2 mg/Kg/day in two daily subcutaneous injections) for four days, the corresponding control animals received saline (−) (B). Mice were injected with saline (−) or insulin (Ins) and the heart was removed after 5 min. Equal amounts of solubilized heart protein were subjected to immunoblotting using specific antibodies to detect the p85 subunit of PI3K. β-tubulin was used as to control protein loading. Data are the mean ± SEM of six to eleven different individuals per group. Two-way ANOVA followed by Bonferroni post-test was performed, a P value less than 0.05 was considered statistically significant. NS: not significant. Representative immunoblots are shown.
Supplementary Figure 4. Uncropped images from Western blots corresponding to heart extracts of GH-Tg mice.
Supplementary Figure 5. Uncropped images from Western blots corresponding to heart extracts of LID mice.
Supplementary Figure 6. Uncropped images from Western blots corresponding to heart extracts of GH-treated mice.
Supplementary Figure 7. Uncropped images from Western blots corresponding to cell lysates of primary culture of rat cardiomyocytes incubated with GH.