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Prolonged exposure to GH impairs insulin signaling in the heart

J G Miquet,

Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Instituto de Química y Físicoquímica Biológicas (UBA-CONICET), Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

J F Giani,

Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Instituto de Química y Físicoquímica Biológicas (UBA-CONICET), Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

C S Martinez,

Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Instituto de Química y Físicoquímica Biológicas (UBA-CONICET), Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

M C Muñoz,

Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Instituto de Química y Físicoquímica Biológicas (UBA-CONICET), Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

L González,

Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Instituto de Química y Físicoquímica Biológicas (UBA-CONICET), Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

A I Sotelo,

Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Instituto de Química y Físicoquímica Biológicas (UBA-CONICET), Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

R K Boparai¹, M M Masternak^{1,2}, A Bartke¹, F P Dominici, and

Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Instituto de Química y Físicoquímica Biológicas (UBA-CONICET), Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

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Correspondence should be addressed to J G Miquet; jmiquet@qb.ffyb.uba.ar.

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D Turyn

Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Instituto de Química y Físicoquímica Biológicas (UBA-CONICET), Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

¹Geriatrics Research, Departments of Internal Medicine and Physiology, School of Medicine, Southern Illinois University, Springfield, Illinois 62794, USA ²Institute of Human Genetics, Polish Academy of Sciences, 60-479 Poznan, Poland

Abstract

Acromegaly is associated with cardiac hypertrophy, which is believed to be a direct consequence of chronically elevated GH and IGF1. Given that insulin is important for cardiac growth and function, and considering that GH excess induces hyperinsulinemia, insulin resistance, and cardiac alterations, it is of interest to study insulin sensitivity in this tissue under chronic conditions of elevated GH. Transgenic mice overexpressing GH present cardiomegaly and perivascular and interstitial fibrosis in the heart. Mice received an insulin injection, the heart was removed after 2 min, and immunoblotting assays of tissue extracts were performed to evaluate the activation and abundance of insulin-signaling mediators. Insulin-induced tyrosine phosphorylation of the insulin receptor (IR) was conserved in transgenic mice, but the phosphorylation of IR substrate 1 (IRS1), its association with the regulatory subunit of the phosphatidylinositol 3-kinase (PI3K), and the phosphorylation of AKT were decreased. In addition, total content of the glucose transporter GLUT4 was reduced in transgenic mice. Insulin failed to induce the phosphorylation of the mammalian target of rapamycin (mTOR). However, transgenic mice displayed increased basal activation of the IR/IRS1/PI3K/AKT/mTOR and p38 signaling pathways along with higher serine phosphorylation of IRS1, which is recognized as an inhibitory modification. We conclude that GH-overexpressing mice exhibit basal activation of insulin signaling but decreased sensitivity to acute insulin stimulation at several signaling steps downstream of the IR in the heart. These alterations may be associated with the cardiac pathology observed in these animals.

Introduction

GH and its principal mediator, insulin-like growth factor 1 (IGF1), play an important role in cardiac development and in the maintenance of the structure and functionality of the heart (Colao 2008, DeBosch & Muslin 2008). As cardiac myocytes express receptors for GH as well as for IGF1, GH may act in the heart directly or through the induction of local or systemic IGF1. The cardiac expression of IGF1 and of receptors for GH and IGF1 is influenced by hemodynamic changes, and both hormones are believed to exert stimulatory effects on myocardial contractility (Volterrani *et al.* 2000).

Both GH deficiency and excess are important risk factors for cardiovascular disease. In acromegalic patients, chronic GH and IGF1 overproduction leads to the development of biventricular concentric cardiac hypertrophy, characterized by myocardial hypertrophy with interstitial fibrosis and lymphomononuclear infiltration. This cardiac pathology is associated with diastolic dysfunction and, if hormone overload is not controlled, it may progress to systolic dysfunction and heart failure (Saccà *et al.* 2003, Climent *et al.* 2007, Colao 2008). Although acromegaly is frequently associated with hypertension, diabetes, and dyslipidemia, which are important risk factors for cardiovascular disease, the excess of GH and IGF1 *per se* is believed to be a major contributor to the specific cardiomyopathy observed in acromegalic patients (Ciulla *et al.* 1999, Saccà *et al.* 2003).

The main physiological role of insulin in the heart is the regulation of substrate utilization; however, insulin signaling influences several processes within the heart, such as cardiomyocyte growth, contractility, and apoptosis. In addition, insulin acts as a vasodilator in the coronary vasculature, therefore augmenting myocardial perfusion, and participates in the coupling of metabolic and hemodynamic homeostasis in cardiac tissue (Belke *et al.* 2002, Muniyappa *et al.* 2007, Bertrand *et al.* 2008, DeBosch & Muslin 2008). Insulin exerts its cellular effects by binding to its membrane receptor (IR), which leads to the autophosphorylation of the receptor on several tyrosine residues. Once activated, IR binds and phosphorylates downstream mediators, including insulin receptor substrates (IRS) and Shc, activating two main signaling cascades: the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and the mitogen-activated protein kinases (MAPK) pathway respectively (Dominici *et al.* 2005, Muniyappa *et al.* 2007, Bertrand *et al.* 2008, DeBosch & Muslin 2008, Xu & Messina 2009). Several Ser/Thr kinases activated by insulin, including mammalian target of rapamycin (mTOR) and the MAPKs Erk1/2, may phosphorylate IRS1 on specific serine residues inhibiting its functions, therefore acting as part of a negative feedback mechanism induced by insulin to terminate its action (Gual *et al.* 2003). Persistent stimulation of PI3K/AKT and/or MAPK pathways has been implicated in myocardial hypertrophy (Proud 2004, Bertrand *et al.* 2008). These signaling pathways are also activated by several growth factors, including IGF1 and GH (Dominici *et al.* 2005, Xu & Messina 2009).

Chronically, GH actions oppose insulin effects. Acromegalic patients are hyperinsulinemic and insulin resistant, and about half of these patients develop diabetes mellitus (Møller & Jørgensen 2009). The anti-insulin effects observed on prolonged exposure to GH are believed to be a consequence, at least in part, of the ability of GH to interfere with insulin signaling (Dominici *et al.* 2005, Xu & Messina 2009). An adequate sensitivity of the myocardium to insulin is essential to maintain normal cardiovascular function (Yue *et al.* 2005, Muniyappa *et al.* 2007). Mice overexpressing GH have been extensively used as a model to study the effects of GH excess on the heart. These animals present cardiomegaly and develop concentric cardiac hypertrophy and alterations in cardiac functionality that may progress to heart failure, similar to that observed in acromegalic patients (Bollano *et al.* 2000, Colligan *et al.* 2002, Izzard *et al.* 2009). Considering the importance of insulin action on cardiac function, and taking into account that persistent elevated levels of GH are associated with hyperinsulinemia, insulin resistance, and anatomical and functional alterations in the heart, it is important to evaluate the sensitivity of the heart to insulin in conditions of chronic GH excess, as occurs in acromegaly.

Previous studies from our group have demonstrated impaired insulin signaling in the liver and skeletal muscle of GH transgenic mice in response to acute insulin stimulation (Dominici *et al.* 1999a,b). We hypothesize that chronic GH excess is associated with insulin resistance in the heart, which may be due to impaired insulin signaling in cardiac tissue. Therefore, the objective of this study was to establish the effect that prolonged exposure to high GH levels exerts on the activation of insulin signaling in the heart. For this purpose, GH-overexpressing transgenic mice were used as a model of chronic exposure to elevated GH levels. Mice received an acute insulin stimulus and the activation of the principal signaling pathways activated by this hormone was evaluated.

Materials and methods

Animals

Transgenic mice (*Mus musculus*) containing the bovine GH (*bGH*) gene fused to control sequences of the rat phosphoenolpyruvate carboxykinase (*PEPCK*) gene have been described previously (McGrane *et al.* 1990). The hemizygous transgenic mice were derived from a founder male generously provided by Dr Thomas Wagner and were produced by

mating transgenic males with normal C57BL/6× C3H F1 hybrid females purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mating produced approximately equal numbers of transgenic and normal progeny. Normal siblings of transgenic mice were used as controls.

Transgenic animals had markedly accelerated post-weaning growth, leading to a significant increase in body weight. The mice were housed three to five per cage in a room with controlled light (12 h light/day) and temperature (22 ± 2 °C). The animals had free access to food (Lab Diet Formula 5001 containing a minimum of 23% protein, 4-5% fat, and a maximum of 6% crude fiber, from Purina Mills, Inc., St Louis, MO, USA) and tap water. The total number of animals used was 26 (13 transgenic and 13 normal littermates). The experimental procedure was in compliance with federal and local laws and approved by the Southern Illinois University Animal Care and Use Committee.

Materials and reagents

The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad. The anti-phospho-tyrosine (PY99), anti-IR β subunit (anti-IR, C-19), anti-glucose transporter 1 (GLUT1; H-43), anti-p38 α MAPK (C-20), anti-p38 MAPK phosphorylated at Thr¹⁸⁰/Tyr¹⁸², anti-rabbit IgG conjugated with HRP, and anti-mouse IgG-HRP antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The anti-IRS1, anti-GLUT4, and anti-p85 subunit of PI3K antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). The anti-phospho-AKT (Ser⁴⁷³) antibody; anti-AKT antibody that detects endogenous levels of total AKT1, AKT2, and AKT3 proteins; anti-phospho-Erk1/2 antibody that detects Erk1 and Erk2 (p44 and p42 MAPK respectively) when phosphorylated at Thr²⁰² and Tyr²⁰⁴; the anti-Erk1/2 antibody; the anti-mTOR antibody; the anti-phospho-mTOR antibody that detects mTOR when phosphorylated at Ser²⁴⁴⁸; the anti-IRS1; anti-phospho-IRS1 Ser⁶¹²; and anti-phospho-IRS1 Ser^{636/639} antibodies were purchased from Cell Signaling (Beverly, MA, USA). The remaining reagents were purchased from Sigma-Aldrich.

Acute insulin stimulation and tissue collection

Female adult animals (7–8 months old) were used. Mice were starved overnight and anesthetized with keta-mine/xylazine mixture. After anesthesia was induced, the inferior vena cava was exposed and 10 IU porcine insulin per kilogram of body weight in normal saline (0.9% w/v NaCl) were injected. Additional mice were injected with saline to evaluate basal conditions. Two minutes after injection, the heart was removed, frozen, and kept at -80 °C until analysis. Ten normal and ten transgenic mice were used, five individuals per group received insulin and the others received saline.

Histological examination

Additional mice (three normal and three transgenic) were used for histological analysis. The whole hearts were cut from apex to base, fixed in 10% v/v formalin and embedded in paraffin. Four- μ m tissue sections were deparaffinized, stained with Masson's trichrome staining, and examined for myocardial fibrosis, using an Olympus BX-51 microscope equipped with a digital camera Olympus QColor 3 (Olympus, Tokyo, Japan). In ten randomly selected high power fields, the degree of fibrosis was estimated by the calculation of the percentage of blue-stained area corresponding to fibrosis in relation to the total heart area using Image-Pro Plus 4.5 software (Media Cybernetics, Inc., Silver Spring, MD, USA).

Glycemia and insulinemia determination

The fasting glycemia and insulinemia levels were measured in blood using the blood One Touch Ultra 2 (Lifescan, Inc., Milpitas, CA, USA) and the insulin ultrasensitive mouse-specific ELISA (Crystal Chem, Downers Grove, IL, USA) respectively. The homeostatic model of insulin resistance (HOMA-IR) was used to assess whole-body insulin resistance using the calculation (fasting glycemia (mg/dl)×fasting insulinemia (μIU/ml)/405), which was originally described for humans (Matthews *et al.* 1985), but is also used for rodents (Vieira *et al.* 2009).

Tissue solubilization and immunoprecipitation

The entire heart was homogenized in solubilization buffer containing 1% w/v Triton together with phosphatase and protease inhibitors as described previously (Giani *et al.* 2008). Heart extracts were centrifuged at 100 000 g for 1 h at 4 °C to eliminate insoluble material, and protein concentration in the supernatants was measured using the BCA assay (BCA Protein Assay Reagent, Thermo Scientific Pierce, Rockford, IL, USA). An aliquot of solubilized heart was diluted in Laemmli buffer, boiled for 5 min, and stored at –20 °C until immunoblotting.

For immunoprecipitation, equal amounts of solubilized heart protein (2 mg total protein in a final volume of 0.2 ml) were incubated at 4 °C overnight with anti-IR or anti-IRS1 antibodies at a final concentration of 4 μg/ml. After incubation, 15 μl protein A-Sepharose 50% v/v (Sigma Chemical Co.) were added to the mixture. Additional samples were incubated in the absence of immunoprecipitating antibody in order to corroborate that the precipitated proteins were specifically recognized by the immunoprecipitating antibody and not by protein A-Sepharose. The preparation was further incubated with constant rocking for 2 h at 4 °C and then centrifuged at 1000 g for 30 s. The supernatant was discarded and the precipitate was washed three times with buffer containing 0.05 mol/l Tris, 0.01 mol/l sodium vanadate, and 1% w/v Triton X-100 (pH 7.4). The final pellet was resuspended in 50 μl Laemmli buffer, boiled for 5 min, and stored at –20 °C until electrophoresis.

Western blotting analysis

Samples were subjected to electrophoresis in SDS-polyacrylamide gels using Bio-Rad Mini Protean apparatus (Bio-Rad Laboratories). Equal amount of total protein was loaded in each lane. Electrotransference of proteins from gel to polyvinylidene difluoride membranes was performed for 1 h at 100 mA per transferred membrane (constant current) using the V20-SDB semi-dry blotting apparatus (Scie-Plas, Cambridge, UK) in 0.025 mol/l Tris, 0.192 mol/l glycine, 20% (v/v) methanol, and 0.03% (w/v) SDS (pH 8.3). To reduce non-specific antibody binding, membranes were incubated for 1 h at room temperature in T-TBS buffer (0.01 mol/l Tris-HCl, 0.15 mol/l NaCl, and 0.02% w/v Tween 20, pH 7.6) containing 3% w/v BSA. The membranes were then incubated overnight at 4 °C with the primary antibody. After washing with T-TBS, the membranes were incubated with a secondary antibody conjugated with HRP for 1 h at room temperature and washed in T-TBS. Immunoreactive proteins were revealed by enhanced chemi-luminescence (ECL-Plus, Amersham Biosciences). Band intensities were quantified using Gel-Pro Analyzer 4.1 software (Media Cybernetics). To reprobe with other antibodies, the membranes were washed with acetonitrile for 10 min and then incubated in stripping buffer (2% w/v SDS, 0.1 mol/l 2-mercaptoethanol, 0.0625 mol/l Tris-HCl, pH 6.7) for 40 min at 50 °C while shaking, washed with deionized water, and blocked with BSA.

To determine the tyrosine phosphorylation of IR and IRS1, the corresponding immunoprecipitates were analyzed by western blotting using anti-phospho-Tyr antibody (1:1000). The serine phosphorylation of IRS1 was determined by western blotting with anti-

phospho-IRS1 Ser⁶¹² or Ser^{636/639} antibodies (1:1000) of samples previously immunoprecipitated with anti-IRS1 antibody. Membranes were reblotted with anti-IR (1:4000) or anti-IRS1 (1:1000) to determine the total abundance of these signaling proteins. To determine the amount of the p85 subunit of PI3K associated with IRS1, membranes corresponding to anti-IRS1 immunoprecipitates were incubated with anti-p85 antibody (1:2000).

To determine the phosphorylation levels of AKT, mTOR, MAPKs p38, and Erk1/2, and the total protein abundance of GLUT4 and GLUT1, equal amounts of solubilized proteins in Laemmli buffer were resolved by SDS-PAGE and subjected to immunoblotting with anti-phospho-AKT, anti-phospho-mTOR, anti-phospho-Erk1/2, anti-phospho-p38 MAPK, anti-GLUT4, or anti-GLUT1 (1:1000 dilutions for all antibodies). Cardiac AKT, mTOR, Erk1/2, and p38 MAPK protein abundance was detected by reprobing the corresponding membranes with the specific antibodies (1:1000).

To better detect the basal phosphorylation levels of IR, IRS1, and AKT and the basal association between IRS1 and p85, it was necessary to run separate experiments with twofold protein loading.

Statistical analysis

Experiments were performed analyzing all groups of animals in parallel, n representing the number of different individuals used in each group. Results are presented as mean \pm S.E.M. of the number of samples indicated. Statistical analyses were performed by two-way ANOVA followed by the Bonferroni post-test using the GraphPad Prism 4 statistical program by the GraphPad Software, Inc. (San Diego, CA, USA). Student's t -test was used when the values of two groups were analyzed. Data were considered significantly different if $P < 0.05$.

Results

Animal characteristics

The characteristics of GH overexpressing transgenic mice and their normal littermates are shown in Table 1. As expected, basal hyperinsulinemia with normoglycemia was corroborated in transgenic mice, in line with previous reports (Dominici *et al.* 1998,1999a). In addition, HOMA-IR score was significantly increased in GH transgenic mice, indicating whole-body insulin resistance.

In accordance with other publications (Bollano *et al.* 2000, Colligan *et al.* 2002), GH transgenic mice exhibited an increase in both body and heart weight compared with non-transgenic mice, but the heart weight remained significantly increased even after normalization to body weight, indicating hypertrophy and therefore a state of cardiomegaly.

The degree of fibrosis was estimated by the determination of the extracellular matrix content using Masson's trichrome staining showing fibrosis with blue color. Transgenic mice developed perivascular and interstitial myocardial fibrosis, while normal extracellular matrix content was observed in non-transgenic mice (Fig. 1A–D). Image quantification indicated a significant increase in the myocardial fibrosis in transgenic mice (Fig. 1E). Increased interstitial fibrosis in GH transgenic mice had been previously reported (Bollano *et al.* 2000).

Tyrosine phosphorylation and protein levels of IR

Insulin binding to its membrane receptor triggers the autophosphorylation of the β subunits of the IR in tyrosine residues, which results in further activation of its tyrosine kinase

activity and in the creation of docking sites for intracellular signaling mediators. Insulin stimulation induced a marked increase in the tyrosine phosphorylation of the IR in the heart, both in normal and in transgenic animals (Fig. 2A). No differences in the insulin-induced phosphorylation of the IR (Fig. 2A) or in the protein content (Fig. 2B) were observed between transgenic and normal mice. To better discriminate the basal phosphorylation levels of this protein, solubilized hearts were run in separate experiments with twofold protein loading. As shown in Fig. 2C, transgenic mice exhibited a significant increase in IR basal phosphorylation compared with normal mice, probably as a result of the continuous exposure to hyperinsulinemia.

Tyrosine phosphorylation and protein levels of IRS1

The activated IR phosphorylates different signaling mediators, including the IRS. IRS1 is believed to be the most important IRS involved in insulin stimulation of cardiac growth (DeBosch & Muslin 2008). The phosphorylation of IRS1 on tyrosine residues is required for insulin-induced responses. Insulin stimulation clearly induced IRS1 tyrosine phosphorylation in normal and transgenic mice. However, transgenic mice exhibited a significant reduction (~25% decrease) in the response to insulin compared with non-transgenic animals (Fig. 2D). There were no significant differences in the IRS1 protein content between normal and transgenic animals (Fig. 2E), indicating that the diminished tyrosine phosphorylation levels of IRS1 are not a consequence of a decrease in the protein abundance of this intracellular mediator. As described for the IR, transgenic mice exhibited higher basal IRS1 tyrosine phosphorylation level than normal controls (Fig. 2F).

IRS1-associated p85 regulatory subunit of PI3K

Once phosphorylated on tyrosine residues, IRS1 associates with the regulatory subunit of PI3K (p85), inducing the activation of the p110 catalytic subunit of the enzyme. Insulin stimulation induced a significant increase in the association of p85 with IRS1, but this association was 50% lower in transgenic mice compared with normal animals (Fig. 3A). The total protein content of p85 in heart extracts was similar in normal and transgenic mice (Fig. 3B). In line with the results obtained for IRS1 tyrosine phosphorylation, its basal association with p85 was significantly increased in transgenic mice (Fig. 3C).

AKT serine phosphorylation and protein levels

AKT, also known as protein kinase B (PKB), is activated by insulin via the PI3K signaling pathway. The phosphorylation of AKT at Ser⁴⁷³, an activating residue, was induced by insulin stimulation in normal and transgenic mice, albeit a 40% decrease was evident in transgenic mice (Fig. 3D). The protein levels of AKT were not altered by either genotype or insulin stimulation (Fig. 3E). As shown in Fig. 3F, transgenic mice exhibited a twofold increase in AKT basal phosphorylation compared with normal mice.

mTOR phosphorylation and protein levels

Many growth factors and hormones, including insulin, activate mTOR by phosphorylation at Ser²⁴⁴⁸ via the PI3K/AKT signaling pathway (Dann *et al.* 2007). As shown in Fig. 4A, no significant differences in mTOR phosphorylation were detected after acute insulin stimulation under the experimental conditions used. Transgenic mice exhibited augmented mTOR basal phosphorylation (Fig. 4A) and higher mTOR protein content than normal siblings (Fig. 4B); moreover, the ratio of mTOR phosphorylation to mTOR protein abundance was significantly increased in transgenic mice (Fig. 4C).

GLUT1 and GLUT4 protein levels

The uptake of extracellular glucose by cardiomyocytes is regulated by the content and activity of two GLUTs at the plasma membrane, namely, GLUT1 and GLUT4, of which GLUT4 is the most abundant (Coort *et al.* 2007, Muniyappa *et al.* 2007). The total amount of these GLUTs was analyzed in solubilized heart extracts. While no significant differences were detected for GLUT1 (Fig. 5A), GLUT4 protein content was ~40% decreased in transgenic mice compared with normal controls (Fig. 5B).

MAPKs phosphorylation and protein levels

The MAPKs are involved in cell growth, mitogenesis, and differentiation (DeBosch & Muslin 2008). Erk1 and Erk2 (44 and 42 kDa respectively) are activated by phosphorylation at Thr²⁰²/Tyr²⁰⁴. The p38 MAPKs, a subfamily of stress-activated protein kinases, are activated by phosphorylation at Thr¹⁸⁰/Tyr¹⁸² in response to various extracellular stimuli, including inflammatory cytokines and growth factors (Wang 2007). Insulin was reported to induce the activation of these MAPKs but with a slower kinetic than that of the IRS1/PI3K signaling pathway, normally requiring longer times to detect an increase in the phosphorylation of the MAPKs on activating residues (Carvalho *et al.* 2005). We therefore determined the basal phosphorylation of the MAPK Erk1/2 and p38 in normal and GH transgenic mice not stimulated with insulin. The basal phosphorylation of Erk1/2 was not significantly altered in transgenic mice compared with normal littermates (Fig. 6A). However, basal p38 MAPK phosphorylation was elevated in transgenic mice (Fig. 6C). No differences in the protein content of the MAPKs in the study were detected between normal and transgenic mice (Fig. 6B and D).

Phosphorylation of IRS1 at inhibitory serine residues

Ser/Thr phosphorylation of IRS proteins may interfere with IRS binding to the IR, induce IRS degradation, or inhibit the association with downstream effectors, depending on the localization of the phosphorylated residue on IRS (Draznin 2006, Boura-Halfon & Zick 2009). To address whether this could be a mechanism involved in the negative modulation of insulin signaling in GH transgenic mice, the levels of IRS1 phosphorylation at serine inhibitory residues were determined. As shown in Fig. 7, transgenic mice displayed enhanced phosphorylation of IRS1 at Ser⁶¹² and Ser^{636/639} compared with normal littermates.

Discussion

The mechanisms implicated in the cardiac dysfunction observed in acromegaly are not completely elucidated. Numerous experimental and clinical findings indicate that chronic elevation in circulating GH exerts anti-insulin effects. Insulin regulates various aspects of metabolism and cardiovascular function, including glucose and fatty acid metabolism, protein translation, cell growth, and vascular tone (Muniyappa *et al.* 2007, Bertrand *et al.* 2008, DeBosch & Muslin 2008). The main signaling cascades activated by insulin are the MAPK and the PI3K/AKT signaling pathways. The MAPK pathway plays a prominent role in the regulation of transcriptional and mitogenic processes, such as growth, proliferation, differentiation, and gene expression, while the PI3K pathway is involved in the metabolic actions of insulin as well as in cardiac growth (Coort *et al.* 2007, Bertrand *et al.* 2008, Xu & Messina 2009). GH seems to modulate insulin sensitivity by several mechanisms that have not yet been completely elucidated and may act in concert to induce insulin resistance. A mechanism that may participate in the insulin resistance observed under chronic GH excess is the ability of GH to interfere with post-receptor insulin signaling. These two hormones can directly interact by signaling cross talk, and prolonged exposure to GH was shown to

alter the abundance and activity of several insulin-signaling mediators (Dominici *et al.* 2005, Xu & Messina 2009).

Rodent models of chronic GH excess are useful tools to investigate the mechanisms of GH-induced insulin resistance. Mice overexpressing GH constitute a model of gigantism/acromegaly, presenting high continuous GH levels since birth, with a consequent increase in body size and organomegaly, including cardiomegaly. These animals also have metabolic alterations including basal hyperinsulinemia with normoglycemia, a reflection of an insulin-resistant state (Valera *et al.* 1993, Kopchick *et al.* 1999, Bartke 2003). Recent findings showed that male GH transgenic mice exhibit a reduced conversion of pyruvate to glucose as well as an improved glucose and insulin tolerance, in comparison to their normal littermates (Boparai *et al.* 2010). On the contrary, the female GH transgenic mice from the strain used in the current work displayed similar whole-body sensitivity to insulin as normal controls, as assayed by insulin tolerance test, concomitant with a slightly better glucose disposal after a glucose load (Boparai *et al.* 2010). The apparent improvement in glucose metabolism could be attributable to a marked compensatory increase in insulin secretion during the glucose tolerance test and to a favorable body composition (i.e. increased lean body mass and decreased body fat mass). Thus, even though GH transgenic mice may display improved glucose tolerance, this occurs under a scenery of hyperinsulinemia possibly due to enhanced β -cell function generating the capacity of releasing more insulin in the face of a glucose challenge. The good performance in the insulin tolerance test could be related to the higher percentage of muscle mass that transgenic mice exhibit, which may overcome the peripheral resistance to insulin.

Our group has previously reported alterations in several components of the insulin-signaling system in liver and skeletal muscle of female GH transgenic mice from the line used in the current study. In the liver, GH excess was associated with a decrease in the content of IR with a simultaneous increase in its basal activity, along with basal activation of the IR/IRS1/PI3K pathway, which was unresponsive to further stimulation by exogenous insulin in transgenic mice (Dominici *et al.* 1998, 1999a). In skeletal muscle, GH transgenic mice displayed decreased IR protein levels and a decline in insulin-induced activation of IR/IRS1/PI3K signaling pathway, along with increased protein content of p85, which was postulated to play a negative role in insulin signaling (Dominici *et al.* 1999b, 2005, Barbour *et al.* 2005). It was therefore of interest to evaluate whether insulin signaling was also impaired in the heart of mice overexpressing GH given the reported deleterious effects of GH excess in the cardiovascular system.

Our current results indicate that GH transgenic mice exhibit impaired insulin signaling in the heart. In response to acute exogenous insulin stimulation, the activation of IRS1/PI3K/AKT signaling cascade was significantly decreased compared with normal controls. The impaired insulin signal was not caused by a reduction in the protein levels of IR or by alterations in its insulin-induced tyrosine phosphorylation in the cardiac tissue, suggesting that mechanisms downstream of the IR were involved. Although no differences in the protein content levels of IRS1, p85, and AKT were detected, acute insulin stimulation of these mediators was impaired. Moreover, basal activation of IRS1/PI3K/AKT and p38 MAPK signaling pathways was detected in transgenic mice heart. Therefore, as previously reported in the liver and skeletal muscle of GH transgenic mice (3- to 5-month-old females; Dominici *et al.* 1999a,b), insulin signaling is impaired in the heart of the GH transgenic mice used in the current study (7- to 8-month-old females), but the molecular alterations observed are different, which may be a reflection of distinct underlying mechanisms in each tissue. However, we cannot rule out that the different age of the animals used in the previous studies on liver and muscle and in this study in heart may be a factor that influences the molecular alterations observed.

Insulin resistance has been mainly associated with defects at the post-receptor level of insulin signaling, with IR levels either normal or slightly diminished in patients and animal models (Draznin 2006). One of the molecular mechanisms described is the disruption of the balance between PI3K subunits (i.e. p85, the regulatory subunit and p110, the catalytic subunit) due to increased expression of p85 α , leading to an excess of free p85 α subunit that competes with the p85–p110 complex for binding sites on tyrosine-phosphorylated IRS1. GH excess is one of the possible causes of imbalance between PI3K subunits (Dominici *et al.* 1999b, 2005, Barbour *et al.* 2005, Draznin 2006); however, this would apparently not be the case in the heart of GH transgenic mice, as no differences in the total protein levels of p85 were detected.

Chronic hyperinsulinemia has been associated with insulin resistance, both as a compensatory mechanism to the peripheral insulin resistance and as an etiological mechanism *per se* (Rizza *et al.* 1985, Ueno *et al.* 2005). Prolonged insulin stimulation and other stimuli related to insulin resistance activate Ser/Thr kinases that phosphorylate IRS proteins, inhibiting their function and interfering with insulin signaling (Draznin 2006, Boura-Halfon & Zick 2009). mTOR is one of the kinases downstream of the PI3K/AKT pathway that is activated on prolonged insulin stimulation and has been implicated in the downregulation of insulin signaling, acting as a negative feedback control mechanism (Ueno *et al.* 2005, Draznin 2006, Bertrand *et al.* 2008, Boura-Halfon & Zick 2009). Increased activity and protein expression of mTOR has been related to impaired insulin signaling and serine phosphorylation of IRS proteins in hyperinsulinemic rats (Ueno *et al.* 2005). GH transgenic mice are hyperinsulinemic and exhibit cardiac basal activation of IRS1/PI3K/AKT pathway along with mTOR upregulation, which may be implicated in the insulin desensitization observed in these mice. In fact, increased basal phosphorylation levels of IRS1 at Ser⁶¹² and Ser^{636/639} were detected in GH transgenic mice heart. These serine residues are located at the carboxy-terminal tail of IRS1 and their phosphorylation would inhibit the ability of downstream effectors to bind to specific tyrosine residues in IRS1, including p85 (Boura-Halfon & Zick 2009). Although the MAPKs were reported to play a major role in the phosphorylation of these serine residues after short-term insulin treatment, mTOR seems to have a prominent role in their phosphorylation following prolonged insulin stimulation (Gual *et al.* 2003). However, the participation of other kinases in the IRS1 serine phosphorylation observed cannot be ruled out, as several Ser/Thr kinases may phosphorylate the same residues (Boura-Halfon & Zick 2009).

Hyperinsulinemia is believed to facilitate cardiac hypertrophy by over-activating insulin signaling in the heart (Bertrand *et al.* 2008, Yu *et al.* 2010). Chronic stimulation of the MAPK and PI3K/AKT signaling pathways has been associated with the establishment of myocardial hypertrophy (Proud 2004, Bertrand *et al.* 2008). In particular, the AKT/mTOR pathway has been demonstrated to play a role in insulin-mediated cardiac hypertrophy by promoting protein synthesis (Proud 2004, Patterson *et al.* 2009). Therefore, the basal activation of IRS1/PI3K/AKT pathway and the high levels of activated mTOR found in the heart of mice overexpressing GH may be involved in the hypertrophy observed in this animal model, while Erk1/2 would not be directly implicated, as no increase in their basal phosphorylation or protein levels were detected. Activation of p38 MAPK cascade in cardiac tissue believed to promote cardiac fibrosis and would not be directly involved in the promotion of cardiac hypertrophy (Muslin 2008). The fibrosis detected in the heart of GH transgenic mice could, therefore, be associated with the increased activation of p38 MAPK they exhibit.

Insulin stimulation of glucose uptake in cardiac myocytes is mainly mediated by GLUT4, while GLUT1 is the principal mediator of basal glucose uptake (Coort *et al.* 2007, Muniyappa *et al.* 2007). Diabetes has been associated with GLUT4 downregulation, which

was proposed to contribute to the myocardial dysfunction observed in this disease (Sliker *et al.* 1992, Burcelin *et al.* 1993, Garvey *et al.* 1993, Coort *et al.* 2007). Moreover, deficiency of GLUT4 was reported to result in cardiac hypertrophy (Katz *et al.* 1995, Abel *et al.* 1999, Zisman *et al.* 2000, DeBosch & Muslin 2008). In contrast, myocardial upregulation of GLUT1 associated with increased basal glucose uptake has been described in models of cardiac hypertrophy (Abel *et al.* 1999). Considering this evidence, and taking into account that GH transgenic mice exhibited a reduction in the total protein content of GLUT4, but no significant alterations in the abundance of GLUT1 in the heart, the present results point to GLUT4 downregulation as potential mechanism involved in the cardiac pathology observed in transgenic mice overexpressing GH.

Disruption of GH receptor in GHR-knockout (GHR-KO) mice produces GH resistance, extremely low levels of circulating IGF1, severe growth retardation, and dwarfism (Zhou *et al.* 1997, Coschigano *et al.* 2000). These mice present increased insulin sensitivity, as they exhibit hypoinsulinemia and hypoglycemia associated with an increment in the abundance and activity of the IRs in hepatocytes (Dominici *et al.* 2000, Liu *et al.* 2004). Previous studies from our group demonstrated that GHR-KO mice exhibit a marked increase in the activation of insulin signaling in the heart (Giani *et al.* 2008). The fact that mice exposed to chronically elevated GH levels exhibit cardiac insulin resistance, while mice in which the receptor of GH has been disrupted display enhanced cardiac insulin signaling, reflects the anti-insulin actions of GH in this tissue, and could be associated with the cardiac alterations observed in conditions of GH deficiency or excess. Interestingly, GHR-KO mice exhibit increased longevity, while the life span is significantly shortened in GH-overexpressing transgenic mice (Coschigano *et al.* 2000, Bartke 2003, 2005).

In summary, chronic exposure to elevated circulating GH levels in mice was associated with impaired insulin signaling in the heart. This was evident from the defects at the post-receptor level, including decreased activation on acute insulin stimulation through the IRS1/PI3K/AKT pathways. Higher basal activation of the IR, IRS1, PI3K, as well as of the Ser/Thr kinases AKT, mTOR, and p38 was observed in GH transgenic mice, along with protein upregulation of mTOR and increased levels of serine IRS1 phosphorylation, which is regarded as an inhibitory modification. Whether these alterations are a direct consequence of the elevated GH and IGF1 levels or secondary to the resulting hyperinsulinemia or other disorders that may arise in a context of chronic GH exposure remains to be elucidated.

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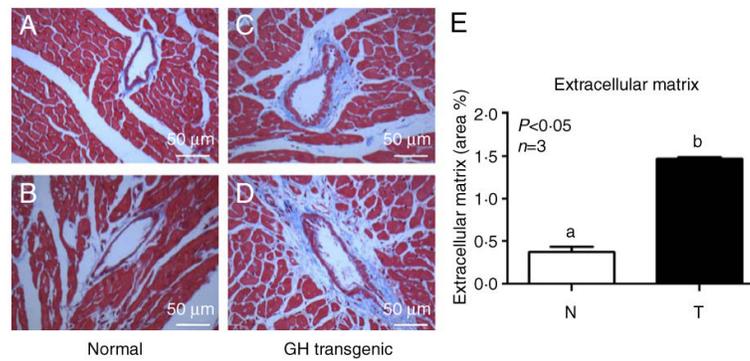


Figure 1.

Myocardial fibrosis evaluation by Masson's trichrome staining. Representative histological sections showing in blue staining the cardiac extracellular matrix content in normal (N) (A and B) and GH transgenic (T) (C and D) mice (400 \times). The percentage of blue-stained area corresponding to fibrosis in relation to the total heart area is shown in panel E. Data are the mean \pm S.E.M., three different individuals of each group were analyzed in parallel. Groups denoted by different letters are significantly different, $P < 0.05$. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0066>.

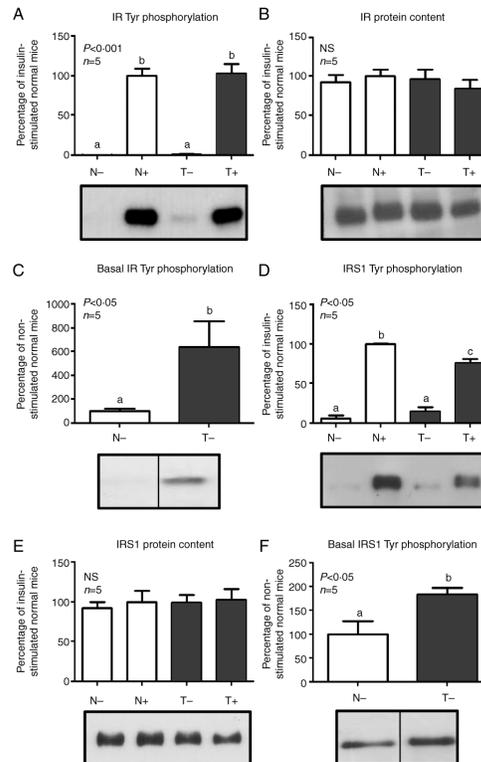


Figure 2.

Insulin receptor (IR) and insulin receptor substrate 1 (IRS1) tyrosine phosphorylation and protein content. Normal (N) and GH transgenic (T) mice were injected with saline (–) or insulin (+) and the heart was removed after 2 min. Equal amounts of solubilized heart protein were immunoprecipitated with an antibody to the β -subunit of the IR (anti-IR) and subjected to immunoblot analysis using an anti-phospho-Tyr (A and C) or anti-IR (B) antibodies or were immunoprecipitated with anti-IRS1 antibody and subjected to immunoblot analysis using an anti-phospho-Tyr (D and F) or anti-IRS1 (E) antibodies. When basal and insulin-stimulated samples were run together, the high intensity of the band corresponding to insulin-stimulated phosphorylation blunted the signal corresponding to basal phosphorylation in non-stimulated mice, rendering it almost undetectable (A and D). To better discriminate differences observed in basal tyrosine phosphorylation levels, separate experiments with twofold protein loading were performed (C and F). Data are the mean \pm S.E.M. of five subsets of different individuals. Groups denoted by different letters are significantly different; NS, not significant. Representative immunoblots are shown.

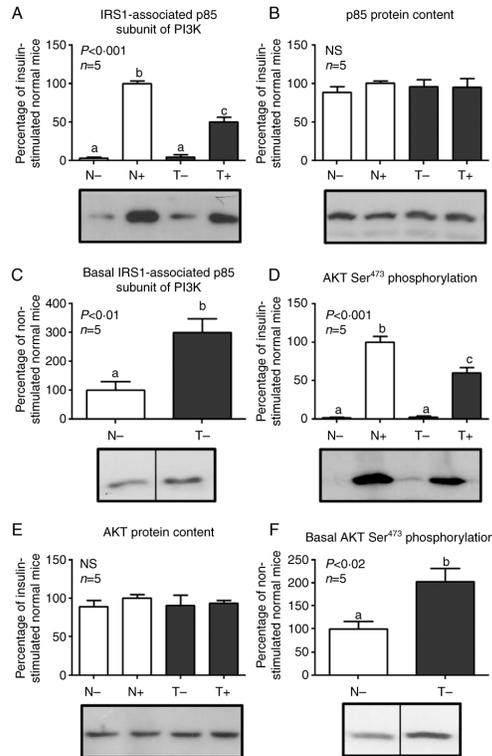


Figure 3.

Association of insulin receptor substrate 1 (IRS1) with the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and serine phosphorylation of AKT. Normal (N) and GH transgenic (T) mice were injected with saline (–) or insulin (+) and the heart was removed after 2 min. Equal amounts of solubilized heart protein were immunoprecipitated with anti-IRS1 antibody and subjected to immunoblot analysis using an anti-p85 antibody (A and C). To evaluate p85 protein content, solubilized hearts were directly subjected to immunoblotting with anti-p85 antibody (B). To analyze AKT, equal amounts of solubilized heart protein were directly subjected to immunoblotting using an anti-phospho-AKT Ser⁴⁷³ (D and F) or anti-AKT (E) antibodies. To better discriminate differences observed in basal association between IRS1 and p85 or basal AKT phosphorylation, separate experiments with twofold protein loading were performed (C and F). Data are the mean \pm S.E.M. of five subsets of different individuals. Groups denoted by different letters are significantly different; NS, not significant. Representative immunoblots are shown.

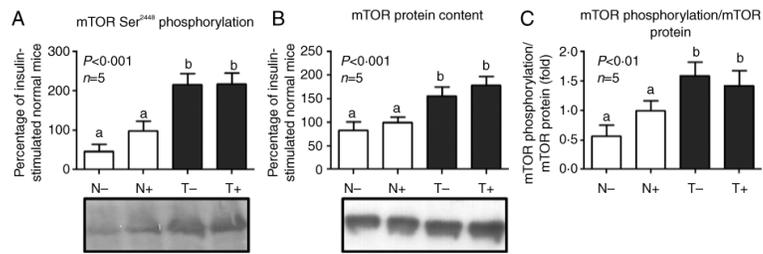


Figure 4.

mTOR phosphorylation and protein content. Normal (N) and GH transgenic (T) mice were injected with saline (-) or insulin (+) and the heart was removed after 2 min. Equal amounts of solubilized heart protein were subjected to immunoblot analysis using an anti-phospho-mTOR Ser²⁴⁴⁸ (A and C) or anti-mTOR (B) antibodies. The ratio of phospho-mTOR to mTOR protein content is shown in panel C. Data are the mean \pm S.E.M. of five subsets of different individuals, run in two separate experiments. Groups denoted by different letters are significantly different. Representative immunoblots are shown.

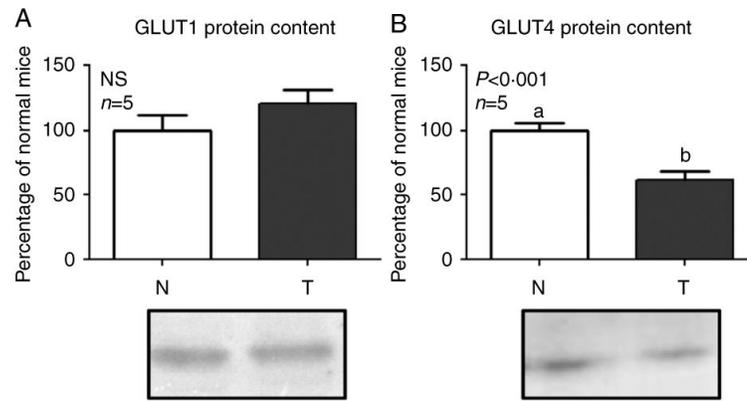


Figure 5.

Glucose transporters (GLUT) 1 and 4 protein content. Equal amounts of solubilized heart from normal (N) and GH transgenic (T) mice were subjected to immunoblot analysis using an anti-GLUT1 (A) or anti-GLUT4 (B) antibodies. Data are the mean \pm S.E.M. of five subsets of different individuals, run in two separate experiments. Groups denoted by different letters are significantly different; NS, not significant. Representative immunoblots are shown.

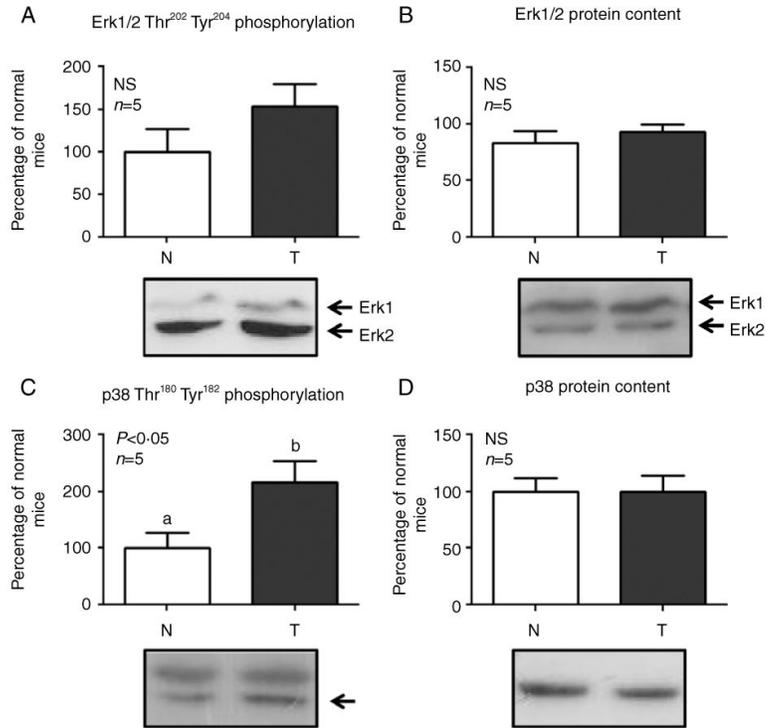


Figure 6.

Erk1/2 and p38 MAPKs phosphorylation and protein content. Equal amounts of solubilized heart from normal (N) and GH transgenic (T) mice were subjected to immunoblot analysis using an anti-phospho-Erk1/2 Thr²⁰² Tyr²⁰⁴ (A), anti-Erk1/2 (B), anti-phospho-p38 Thr¹⁸⁰ Tyr¹⁸² (C), or anti-p38 MAPK antibodies. In panels A and B, the bands corresponding to Erk1 and Erk2 were analyzed together. In panel C, the arrow indicates the analyzed phosphorylation band, which corresponds to the p38 protein band detected in panel D. Data are the mean \pm S.E.M. of five subsets of different individuals run in two separate experiments. Groups denoted by different letters are significantly different; NS, not significant. Representative immunoblots are shown.

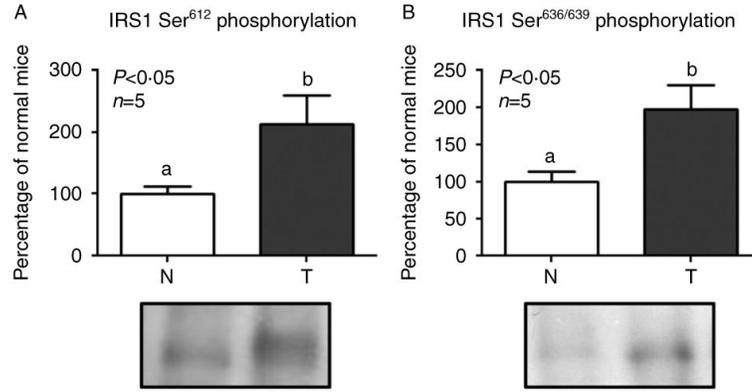


Figure 7. Insulin receptor substrate 1 (IRS1) serine phosphorylation. Equal amounts of solubilized heart from normal (N) and GH transgenic (T) mice were immunoprecipitated with anti-IRS1 antibody and subjected to immunoblot analysis using anti-phospho-IRS1 Ser⁶¹² (A) or anti-phospho-IRS1 Ser^{636/639} (B) antibodies. Data are the mean \pm S.E.M. of five subsets of different individuals run in two separate experiments. Groups denoted by different letters are significantly different. Representative immunoblots are shown.

Table 1

Characteristics of mice overexpressing GH and normal controls. Data are presented as mean value \pm S.E.M. ($n=12$)

	<u>Normal</u>	<u>GH transgenic</u>
Glycemia (mg/dl)	126 \pm 6 ^a	135 \pm 5 ^a
Insulinemia (μ IU/ml)	16 \pm 4 ^a	95 \pm 33 ^b
HOMA-IR	5.0 \pm 0.4 ^a	31.7 \pm 3.5 ^b
Body weight (g)	34 \pm 2 ^a	48 \pm 2 ^b
Heart weight (mg)	114 \pm 4 ^a	240 \pm 9 ^b
Heart wt/body wt (%)	0.34 \pm 0.01 ^a	0.51 \pm 0.01 ^b

HOMA-IR, homeostatic model of insulin resistance. Different letters (a and b) denote significant differences at $P<0.001$ versus normal mice.