Pioglitazone does not improve insulin signaling in mice with GH over-expression

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Abstract

Type 2 diabetes and obesity are very serious health problems in both developed and developing countries. An increased level of GH is known to promote insulin resistance. Transgenic (Tg) mice over-expressing bovine GH are short-living and characterized, among other traits, by hyperinsulinemia and increased insulin resistance in comparison with normal (N) mice. Pioglitazone (PIO) is a member of the thiazolidinediones – a group of insulin-sensitizing drugs that are selective agonists of peroxisome proliferator-activated receptor gamma (PPARγ). The aim of the study was to analyze the effects of PIO on the insulin-signaling pathway in Tg and N mice. Plasma levels of insulin and glucose as well as hepatic levels of proteins involved in insulin signaling were analyzed by ELISA or western blot methods. Treatment with PIO decreased plasma level of glucose in N mice only. Similarly, PIO increased insulin sensitivity (expressed as the relative insulin sensitivity index; RISI) only in N mice. In the liver, PIO decreased the phosphorylation of insulin receptor substrate-1 (Ser307-pS-IRS1), which inhibits insulin action, and had a tendency to increase tyrosine phosphorylation of IRS2 (Tyr-pY-IRS2) only in N mice but did not affect either of these parameters in Tg mice. Levels of total and phosphorylated mammalian target of rapamycin were increased in Tg mice. Moreover, the level of AKT2 was decreased by PIO in N mice only. In conclusion, the lack of improvement of insulin sensitivity in insulin-resistant Tg mice during PIO treatment indicates that chronically elevated GH levels can inhibit the beneficial effects of PIO on insulin signaling.

Key Words
- pioglitazone
- insulin signaling
- growth hormone
- transgenic mice

Introduction

Diabetes, obesity, and other non-communicable chronic diseases are very serious health problems in both developed and developing countries, leading to increased morbidity and premature mortality (Abegunde et al. 2007). Obesity per se constitutes one of the main causes of insulin resistance and type 2 diabetes. Importantly, growth hormone (GH), which is a key regulator of growth and metabolism processes, may exert anti-insulinemic and diabetogenic actions. These effects are considered to be the key physiological effects of GH on carbohydrate and lipid metabolism.
metabolism (Davidson 1987). Increased GH levels are known to promote insulin resistance in humans and laboratory animals (Hansen et al. 1986, Kopchick et al. 1999, Bartke 2003, Wang et al. 2007). For this reason, we decided to use in our study transgenic (Tg) mice overexpressing bovine GH (bGH) with a sequence from the phosphoenolpyruvate carboxykinase (PEPCK) gene as a promoter (PEPCK-bGH mice). These giant mice are short-living and characterized, among other traits, by increased postnatal growth and adult body weight, organomegaly, reduced adiposity, various symptoms of accelerated aging, early onset of age-related changes in cognitive function, decreased plasma adiponectin, increased plasma resistin and cholesterol, elevated levels of tumor necrosis factor α (TNFα) and interleukin-6 (IL6) in adipocytes, hyperinsulinemia and increased insulin resistance (Bartke 2003, Wang et al. 2007), as well as depletion of very small embryonic-like stem cells from bone marrow (Kucia et al. 2013).

Pioglitazone (PIO) is an anti-diabetic drug that belongs to the thiazolidinedione (TZD) class – selective agonists of peroxisome proliferator-activated receptor gamma (PPARγ), which constitute a very important group of insulin-sensitizing drugs. It can exert beneficial antioxidant and anti-proliferative effects (Elte & Blickle 2007), as well as anti-tumor activity by inducing apoptosis, and may decrease the risk of cardiovascular events (Lincoff et al. 2007).

The aim of the study was to analyze the effects of PIO on the insulin-signaling pathway (hepatic levels of insulin receptor (IR), insulin receptor substrate-1 (IRS1) – total and phosphorylated at a serine(307) residue (Ser307-pS-IRS1) (phosphorylation that inhibits insulin action), IRS2 – phosphorylated at a tyrosine residue (Tyr-pY-IRS2)) in PEPCK-bGH Tg and normal mice. Moreover, plasma glucose and insulin levels were determined in these animals. Importantly, the influence of PIO on various components of the insulin-signaling pathway under chronically elevated GH levels has not yet been, to our knowledge, analyzed.

Additionally, hepatic total mammalian target of rapamycin (mTOR; FKBP12-rapamycin-associated protein, FRAP1), phosphorylated mTOR (mTOR-pY), and AKT2 levels were assessed. It is known that hormones (including insulin), growth factors, and other mitogens activate the PI3K/AKT/mTOR signaling cascade (Mamane et al. 2006). Furthermore, rapamycin – a natural macrolide – used in cancer therapy and as a immunosuppressant drug as well, which inhibits mTOR, may lead to increases in life span in various species (Bjedov et al. 2010, Anisimov et al. 2011, Miller et al. 2011, Selman & Partridge 2012, Wilkinson et al. 2012). Therefore, it was also of interest to assess the effects of PIO on mTOR signaling.

Materials and methods

Animals and assessment of blood chemistry

Approximately 6-month-old male mice over-expressing bGH (PEPCK-bGH; Tg) and age-matched normal (N) controls were randomly assigned to control or treatment groups. At the beginning of the study (‘before treatment’ groups), the mice were divided into four experimental groups: N (ten animals), normal assigned to PIO treatment (N-PIO) (ten animals), Tg (ten animals), and Tg assigned to PIO treatment (Tg-PIO) (ten animals). These group designations were used both before and after treatment, and thus, ‘after treatment’, N-PIO and Tg-PIO groups in which insulin, blood glucose, relative insulin sensitivity index (RISI), and adiponectin were assessed denote animals that had been receiving PIO treatment. Basal glucose, insulin and adiponectin levels, as well as RISI were measured at the start of the study. The RISI was calculated from the equation: \[100/\text{blood glucose level} \times \text{insulin level}.\] Then, the PIO treatment (20 mg/kg body weight per day for the period of 20 days) was started in groups: N-PIO and Tg-PIO. PIO was incorporated in bacon-flavored diet (pellets of modified LabDiet Laboratory Rodent Diet 5001 with 0.2% PIO; TestDiet, Richmond, IN, USA). Once-daily PIO-treated animals were provided with small piece of food containing drug (0.5 g for Tg and 0.3 g for N animals, corresponding to 20 mg PIO/kg of body weight on average per day). The mice in groups N and Tg (without PIO treatment) were fed unmodified Lab Diet 5001 chow (PMI Nutrition International, Richmond, IN, USA).

The PEPCK-bGH (Tg) mice were originally produced by microinjecting the bGH structural gene fused with the promoter of the rat Peck gene into the pronuclei of fertilized mouse eggs (McGrane et al. 1988). The hemizygous Tg mice used in this study were produced by mating Tg males with normal C57BL/6×C3H F1 hybrid females. The mice used in the study were housed under temperature- and light-controlled conditions (22 ± 2°C, 12 h light:12 h darkness cycle). At the end of the experiment, basal glucose, insulin and adiponectin levels, as well as RISI were measured once more (see below). Half of the animals from each experimental group were treated with insulin and the remaining animals were treated with saline before tissues were collected to assess stimulation of the
phosphorylation of IR and the downstream pathway of insulin action. All animal procedures were approved by the Laboratory Animal Care and Use Committee (LACUC) at the Southern Illinois University School of Medicine (Springfield, IL, USA). After 20 days of PIO treatment, the animals were fasted overnight and fasting glucose levels were measured in blood collected from the tail vein using OneTouch Ultra glucometer (Life Scan, Milpitas, CA, USA). Then, the animals were killed and plasma obtained from blood collected by cardiac puncture was used for assessment of insulin using Rat/Mouse Insulin ELISA kit (Linco Research, Inc., St Charles, MO, USA) following the manufacturer’s protocols. Adiponectin levels were assayed using mouse adiponectin ELISA kit (Linco Research). Total amount of IR in liver was assayed using an IR (Total) Human ELISA kit (Invitrogen). The assessment of the above-mentioned four parameters (glucose, insulin, adiponectin, and total IR), as well as RISI, was performed in all animals in each group (i.e., in ten mice per group). Phosphorylated IRS2 levels were assayed using PathScan Phospho-IRS-2 (panTyr) Sandwich ELISA kit (Cell Signaling Technology, Inc., Danvers, MA, USA). In this case, five animals per each subgroup (treated with saline or with insulin) were analyzed. Livers were rapidly collected, quickly frozen on dry ice, and stored at −80°C until processed.

**Protein extraction and western blotting**

Total proteins were obtained from tissue homogenates. Approximately 100 mg liver samples were homogenized in 1 ml ice-cold T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA), with Protease Inhibitor Cocktail kit (Pierce Biotechnology), Phosphatase Inhibitor Cocktail 1 (Sigma–Aldrich), and Phosphatase Inhibitor Cocktail 2 (Sigma–Aldrich). After mixing, homogenates were centrifuged at 16 000 g for 30 min. Protein concentrations were assessed using Pierce bichinonic acid (BCA) Protein Assay kit (Pierce Biotechnology) in accordance with the manufacturer’s protocol. The western blot procedure was performed using the respective primary antibodies: total IRS1, phospho-IRS-1 (Ser307), total mTOR, phospho-mTOR (Ser2448), Akt2 (all from Cell Signaling Technology, Inc.), and secondary goat anti-rabbit or anti-mouse antibodies (Calbiochem, La Jolla, CA, USA). Monoclonal anti-β-actin antibody (Sigma–Aldrich Corp.) was used, after stripping the membrane, as a control for protein loading.

Western blotting analysis was performed according to the method described previously (Gesing et al. 2011), and six animals per group were analyzed. Photos of blots were taken with Image Reader LAS-4000 (FujiFilm, Tokyo, Japan) and quantified for statistical analysis using GeneTools software (SynGene, Cambridge, UK).

**Statistical analysis**

The data are expressed as mean±S.E.M. To evaluate the effects of the genotype and pharmacological intervention, two-way ANOVA was used. Additionally, we used the Bonferroni test – post hoc test for analyzing differences between group means. A value of *P*<0.05 was considered significant. All statistical calculations were conducted using SPSS version 17.0 (SPSS) with *α*=0.05. All graphs were made using Prism 4.02 (GraphPad Software, San Diego, CA, USA).

**Results**

Before PIO treatment, plasma insulin level was increased in Tg mice, when compared with N animals (*P*=0.049; Fig. 1A). After the treatment period, no changes between these two kinds of animals, not treated with PIO, were detected, although the overall effect of genotype (i.e., between pooled N and N-PIO mice and pooled Tg and Tg-PIO animals) was statistically significant with higher insulin levels in pooled Tg and Tg-PIO mice (*P*=0.022; Fig. 1B). The PIO treatment decreased the plasma level of glucose in N mice only (*P*=0.048), with no effects in Tg animals (Fig. 1D). Before PIO treatment, no changes in blood glucose between N and Tg mice were observed (Fig. 1C). Similarly, no differences between these two experimental groups, not treated with PIO, were seen after the treatment period (Fig. 1D). Expectedly, insulin sensitivity (as indicated by the RISI) was decreased in Tg mice (pooled Tg and Tg-PIO) when compared with N animals (pooled N and N-PIO) (*P*=0.012; Fig. 2A). The RISI indicated that PIO increased insulin sensitivity in N mice only (*P*=0.033; Fig. 2B). Before PIO treatment, the plasma adiponectin level was decreased in Tg mice, when compared with N animals (*P*=0.0001; Fig. 3A). A similar pattern was observed after the treatment period (*P*=0.043; Fig. 3B). The PIO treatment increased adiponectin level in plasma in N mice (*P*=0.003), as well as in Tg animals, although approaching only borderline significance (*P*=0.053; Fig. 3B). No effects of PIO treatment on total IR level in the liver in N or Tg mice were detected (Fig. 4A). Importantly, the level of total IR was decreased in Tg mice in comparison with N animals (*P*=0.009; Fig. 4A). In the liver, PIO did not change the phosphorylation of Tyr-pY-IRS2 (although it had a tendency to increase this
changes in Tyr-pY-IRS2 level in N mice (without PIO treatment) treated with saline when compared with N animals treated with insulin were detected (Fig. 4B). Similarly, in Tg mice (without and after PIO treatment), there were no differences between saline- and insulin-treated subgroups (Fig. 4B). Interestingly, PIO decreased total IRS1 level in livers of N mice ($P=0.003$; Fig. 4C). Furthermore, an increasing tendency of this parameter in PIO-treated Tg mice has been shown ($P=0.14$; Fig. 4C). No differences in total mTOR level between N and N-PIO, as well as between Tg and Tg-PIO mice, were observed (Fig. 5A). However, the level of mTOR was increased in Tg mice (pooled Tg and Tg-PIO) when compared with N animals (pooled N and N-PIO) ($P=0.0001$; Fig. 5A). Similar to total mTOR, phosphorylated mTOR protein level was also increased in Tg mice (pooled Tg and Tg-PIO) in comparison with N animals (pooled N and N-PIO) ($P=0.001$; Fig. 5B). Additionally, PIO decreased hepatic AKT2 level in N mice ($P=0.011$), with no effects observed in Tg mice (Fig. 5C).

**Figure 1**
Plasma insulin level (mg/dl) before (A) and after (B) pioglitazone (PIO) treatment and blood glucose level (mg/dl) before (C) and after (D) PIO treatment in normal (N), and transgenic mice over-expressing bovine growth hormone (Tg). Values are means ± S.E.M. *a,b,c*Values that do not share the same letter in the superscript are significantly different ($P<0.05$).

**Figure 2**
The relative insulin sensitivity index (RISI) (100/√blood glucose level × insulin level) before (A) and after (B) pioglitazone (PIO) treatment in normal (N) and transgenic mice over-expressing bovine growth hormone (Tg). Values are means ± S.E.M. *a,b,c*Values that do not share the same letter in the superscript are significantly different ($P<0.05$).
Insulin resistance is a very serious health problem, which may lead to diabetes and obesity. One should emphasize that elevated GH as well as insulin-like growth factor 1 (IGF1) may lead to higher risk of mortality (Holdaway et al. 2004). Tg mice over-expressing GH are characterized, among other traits, by hyperinsulinemia and increased insulin resistance (Wang et al. 2007). Therefore, PEPCK-bGH mice constitute an excellent model system for studies of the control of insulin sensitivity and insulin resistance during anti-diabetic treatment.

Krag et al. (2009) have shown that one of the mechanisms that may be responsible for improvement of insulin sensitivity due to PIO treatment is the decrease in pro-inflammatory IL6 level. IL6 is a cytokine, produced in adipose tissue (Fried et al. 1998), as well as in skeletal muscles, being one of the important myokines (Pedersen & Febbraio 2008), involved in the regulation of insulin sensitivity. It has been previously reported that IL6 may inhibit the insulin-signaling pathway by upregulation of suppressors of cytokine signaling-3 (SOCS3) – a marker of the activation of IL6 signaling (reviewed in Coelho et al. (2013)). In turn, SOCS3, as well as SOCS1, may lead – among other effects – to impaired IRS1 and IRS2 tyrosine phosphorylation. Interestingly, Wan et al. (2012a) have shown that IL6 may be involved in the regulation of mitochondrial function in adipose tissue, being an activator of AMP-activated protein kinase – one of the key regulators of mitochondrial biogenesis. However, recent data have revealed that the cytokine in question is not necessary for regulation of mitochondrial content in adipose tissue (Wan et al. 2012b). Nevertheless, there is a growing amount of data showing a dual role of IL6 in the regulation of insulin sensitivity (e.g., Jiang et al. 2013).

The mechanisms of action of PIO, relying on the decrease in plasma levels of glucose, and the increase in the RISI in N mice only, as seen in our study, are consistent with the well-known insulin-sensitizing properties of this drug. Puddu et al. (2012) have recently shown that PIO protects pancreatic islet cells (line HIT-T15) from the detrimental effects of advanced glycation end-products (AGEs). Moreover, PIO is an effective drug in lowering HbA1c (Russell-Jones et al. 2012).

One should recall that TZDs may decrease GH and IGF1 synthesis and levels, and, as a consequence of this, attenuate anti-insulin activity (exerted by GH), which may lead to the improvement of the insulin-signaling pathway. Intriguingly, the doses of PIO, commonly used in the treatment of type 2 diabetes, did not improve GH and IGF1 levels in acromegalic patients (characterized by impaired insulin sensitivity) (Kim et al. 2012). These observations may be considered as consistent with our results showing lack of a beneficial effect of PIO treatment in Tg mice. Nevertheless, it seems that the role of the interactions between GH and PIO requires further analyses.

The effects of PIO, leading to increased plasma adiponectin levels in N mice, agree with the results of the studies by Yu et al. (2002), showing increased adiponectin levels after treatment with TZDs. PIO also increased serum adiponectin levels in 8-week high-fructose-diet-fed rats (Schaal 2012) and in obese men (Powell et al. 2012). Adiponectin level was also increased in Wistar rats, fed a high-fat insulin-resistance-inducing diet, treated with PIO (Gong et al. 2012). Moreover, PIO treatment in these animals, fed a diet inducing derangements in the insulin-signaling pathway, led to increased levels of adiponectin receptor type 2 and to decreased insulin resistance (Gong et al. 2012). The increase in adiponectin levels in Tg mice, as a result of PIO treatment, seems, to some degree, to be consistent with the results of

Figure 3
Plasma adiponectin level (mg/dl) before (A) and after (B) pioglitazone (PIO) treatment in normal (N) and transgenic mice over-expressing bovine growth hormone (Tg). Values are means ± S.E.M. a,b,c-Values that do not share the same letter in the superscript are significantly different (P<0.05).
the study performed by Krag et al. (2008), showing increased adiponectin levels in GH-deficient patients with GH replacement therapy, receiving PIO treatment. Concerning the well-known anti-atherogenic properties of adiponectin, one should also recall the data of Saremi et al. (2013), who have recently reported that PIO may retard atherosclerosis progression in people with prediabetes. Interestingly, adiponectin-deficient mice are unresponsive to the anti-diabetic effects of TZDs (Nawrocki et al. 2006). Furthermore, the suppressive effects of PIO on angiotensin II-induced cardiac hypertrophy, as were seen in wild-type mice, were diminished in adiponectin-deficient mice (Li et al. 2010).

Decreased phosphorylation of IRS1 (Ser^{307}-pS-IRS1) in the liver due to PIO treatment in N mice constitutes beneficial effect of this drug because that kind of phosphorylation leads to inhibition of insulin action. Also, a tendency to increase phosphorylation of IRS2 (Tyr-pY-IRS2) in N animals seems to be beneficial for proper insulin signaling as it is known that the opposite situation, i.e., inhibition of tyrosine phosphorylation of IRS proteins, caused by SOCS1 and SOCS3, may lead to insulin resistance (Ueki et al. 2004). Importantly, PIO may improve insulin sensitivity through the suppression of SOCS3 (Kanatani et al. 2007). Interestingly, SOCS1 knockout mice are characterized by increased liver IRS2 expression and IRS2 tyrosine phosphorylation that may lead to enhanced hepatic insulin sensitivity (Jameson et al. 2005).

Figure 4

(A) Hepatic total insulin receptor (IR) protein level (units/100 µg protein) in normal (N) and transgenic mice over-expressing bovine growth hormone (Tg) without pioglitazone (PIO) treatment and after PIO treatment in normal (N-PIO) and transgenic mice over-expressing bovine growth hormone (Tg-PIO); (B) hepatic insulin receptor substrate-2 (IRS2) phosphorylated at a tyrosine residue (IRS2-T pY) protein level (units/300 µg protein) in N mice treated with saline (N-sal) or insulin (N-ins) and Tg mice treated with saline (Tg-sal) or insulin (Tg-ins) without PIO treatment, and after PIO treatment in N mice treated with saline (N-PIO) or insulin (Tg-PIO); (C) hepatic total IRS1 protein level (using primary antibody from Cell Signaling Technology, Inc.) in N and Tg mice without PIO treatment, and after PIO treatment in N (N-PIO) and Tg mice (Tg-PIO); (D) hepatic IRS1 phosphorylated at a serine (307) residue (IRS1-ser-pY) protein level (using primary antibody from Cell Signaling Technology, Inc.) in N and Tg mice without PIO treatment and after PIO treatment in N (N-PIO) and Tg mice (Tg-PIO). Values are means ± S.E.M. a,b,cValues that do not share the same letter in the superscript are significantly different (P<0.05).
in N mice, due to PIO treatment, seems to be quite difficult to interpret. However, Taniguchi et al. (2006) have shown that liver-specific deletion of the p85α regulatory subunit of PI3K, constituting the next downstream step (after IRS1) in the insulin signaling pathway, may paradoxically improve the hepatic and peripheral insulin sensitivity. Importantly, the decreased level of total IR in Tg mice in comparison with N animals may be considered to be consistent with the well-known impaired insulin sensitivity in Tg mice over-expressing GH.

One should emphasize that mTOR may integrate and coordinate various extracellular signals (Mamane et al. 2006). Importantly, reduced expression of genes associated with the mTOR signaling pathway has been shown in individuals from long-lived families in the Leiden Longevity Study (Slagboom et al. 2011). Therefore, increased total mTOR levels in short-lived Tg mice, as observed by us, may suggest an important role of TOR signaling in lifespan regulation. Also, increased phosphorylated mTOR protein levels in Tg mice in comparison with N animals may confirm this relevant observation.

Presumably, the decrease in AKT2, an enzyme involved in PI3K/AKT/mTOR signaling, can also be viewed as beneficial. This hypothesis could be confirmed by the observation that AKT2 is the major isoform shown to be over-expressed in cancer in humans (reviewed by Hers et al. 2011). On the other hand, Garofalo et al. (2003) have unexpectedly shown that AKT2-deficient mice are insulin-resistant with the tendency to development of diabetes in males. Therefore, the role of AKT2 in insulin sensitivity regulation requires further analysis.

Interestingly, the differences in insulin levels were no longer present between normal and Tg mice without PIO treatment after the treatment period (when compared with the beginning of the study). This could have been caused by the method of blood collection used. Initially, the blood was collected by orbital bleeding after brief isoflurane anesthetic while the final collection was performed after ketamine/xylazine anesthesia with cardiac bleeding. Therefore, these two different methods of blood collection (differently stimulating the stress of the animals) could cause the difference in insulin values after the treatment period when comparing N and Tg animals.

In summary, absence of effects of PIO treatment in Tg mice over-expressing GH suggest that chronically increased GH level may inhibit the beneficial effects of PIO on the insulin signaling pathway. In contrast, PIO improved insulin signaling in animals with normal GH levels. Therefore, one might suggest that PIO may not be useful in the management of impaired glucose tolerance.

Figure 5

(A) Hepatic total mTOR protein level (using primary antibody from Cell Signaling Technology, Inc.) in normal (N) and transgenic mice over-expressing bovine growth hormone (Tg) without pioglitazone (PIO) treatment and after PIO treatment in normal (N-PIO) and transgenic mice over-expressing bovine growth hormone (Tg-PIO); (B) hepatic mTOR phosphorylated at a tyrosine residue (mTOR-pY) protein level (using primary antibody from Cell Signaling Technology, Inc.) in N and Tg mice without PIO treatment and after PIO treatment in N (N-PIO) and Tg mice (Tg-PIO); (C) hepatic AKT2 protein level (using primary antibody from Cell Signaling Technology, Inc.) in N and Tg mice without PIO treatment and after PIO treatment in N (N-PIO) and Tg mice (Tg-PIO). Values are means ± s.e.m. a,b,c Values that do not share the same letter in the superscript are significantly different (P < 0.05).
or type 2 diabetes in patients with elevated GH levels. Presumably, higher doses of PIO would be required to exert beneficial effects on insulin signaling under conditions of GH overproduction. Further studies are needed to determine the therapeutic possibilities of PIO and explain how this anti-diabetic drug may exert beneficial effects.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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