Insulin-Regulated Srebp-1c and Pck1 mRNA Expression in Primary Hepatocytes from Zucker Fatty but Not Lean Rats Is Affected by Feeding Conditions

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Abstract

Insulin regulates the transcription of genes for hepatic glucose and lipid metabolism. We hypothesized that this action may be impaired in hepatocytes from insulin resistant animals. Primary hepatocytes from insulin sensitive Zucker lean (ZL) and insulin resistant Zucker fatty (ZF) rats in ad libitum or after an overnight fasting were isolated, cultured and treated with insulin and other compounds for analysis of gene expression using real-time PCR. The mRNA levels of one insulin-induced (Srebp-1c) and one insulin-suppressed (Pck1) genes were measured. Additionally, the effects of insulin and T1317 on their levels in hepatocytes from ad libitum or fasted ZL or ZF rats were analyzed. Insulin's ability to regulate Srebp-1c and Pck1 expression was diminished in hepatocytes from ad libitum ZF, but not ZL rats. Glucagon or compactin suppressed Srebp-1c mRNA expression in lean, but not fatty hepatocytes. Glucagon induced Pck1 mRNA expression similarly in hepatocytes from ad libitum ZL and ZF rats. Insulin caused the same dose-dependent increase of Akt phosphorylation in hepatocytes from ZL and ZF rats. It synergized with T1317 to induce Srebp-1c, and suppressed Pck1 mRNA levels in hepatocytes from fasted, but not that from ad libitum ZF rats. These patterns of mRNA expression may explain insulin resistance and sensitivity and contribute to the development of metabolic dysfunction.

Introduction

The increased rate of metabolic diseases, such as obesity, diabetes and cardiovascular disease, has become a major public health concern [1,2]. The common characteristic of human obesity and type 2 diabetes is insulin resistance [3]. Liver plays a critical role in mediating glucose and lipid homeostasis regulated by hormones and nutrients. Factors derived from adipose tissues, such as free fatty acid [4], adipokines [5] and inflammatory cytokines [6] have been proposed to be responsible for the hepatic insulin resistance.

In liver and hepatocytes, insulin regulates the expression of a variety of genes responsible for glycolysis, glycogenesis and lipogenesis, and inhibits gluconeogenesis [7]. This insulin-regulated hepatic gene expression, at least in part, is responsible for glucose and lipid homeostasis [8,9]. When liver is insulin sensitive, insulin induces glycolysis, lipogenesis and suppresses gluconeogenesis in hepatocytes. For hepatic glucose metabolism, insulin increases the expression of glucokinase gene (Gck) [10,11], the enzyme responsible for the first step of hepatic glycolysis. It suppresses the expression of the cytosolic form of phosphoenolpyruvate carboxykinase (Pck1) [12] and glucose 6-phosphatase catalytic subunit (G6pc) [7], the first and last steps of gluconeogenesis, respectively. For hepatic lipid metabolism, insulin increases the expression levels of sterol regulatory element binding protein 1c gene (Srebp-1c) [13], a member of sterol regulatory element-binding proteins (SREBPs) which are critical transcription activators for hepatic cholesterol and fatty acid biosynthesis, and their homeostasis [14]. In liver of SREBP-1c deleted mice [15], the fasting-refeeding cycle no longer appropriately regulated the expression levels of critical lipogenic genes such as fatty acid synthase (Fas) and stearoyl-CoA desaturase 1 (Scd1) [16,17]. When liver is insulin resistant, insulin no longer suppresses gluconeogenesis, but still stimulates lipogenesis, creating a vicious cycle that aggravates insulin resistance and ultimately contributes to the onset of overt diabetes. The co-existence of hepatic insulin resistance (elevated gluconeogenesis) and sensitivity (elevated lipogenesis) at gene expression level has been observed in rodent diabetic models [3,8]. However, the mechanism of this co-existence of insulin sensitivity and resistance has not been revealed [18].

The binding of insulin to its receptor initiates a cascade of signal transduction events that lead to metabolic changes in its target...
tissues [19]. The two well studied pathways activated by insulin stimulation are the phosphatidylinositol 3-kinase (PI3K)-AKT/ protein kinase B (PKB) pathway [20] and mitogen-activated protein kinase (MAPK) pathway [21]. Recently, insulin regulated Srebp-1c expression has been shown to be mediated by atypical protein kinase C (PKC) [22,23] and mammalian target of rapamycin complex (mTORC) 1 [24]. Elevated activity of PKCs in Goto-Kakizaki type 2 diabetic rats has been attributed to the excessive expression of hepatic Srebp-1c [25]. However, how these signaling components cause the transcriptional changes remains to be elucidated.

Zucker fatty (ZF) rats [26] and its sub strain Zucker diabetic fatty rats [27] have been widely used as rat models for the development of metabolic diseases [28,29] due to a missense mutation in the extracellular domain of all leptin receptor isoforms [30–32]. Insulin resistance in Zucker fatty or diabetic fatty rats has been associated with higher basal insulin secretion caused by increased fuel metabolism in pancreatic beta cells [33,34]. The defects in pancreatic beta cell gene expression in Zucker diabetic fatty rats [35,36], but not in obese ZF rats who have normal glycemia [37], have been attributed to the development of diabetes. However, the insulin-regulated gene expression in hepatocytes from these insulin resistant animals has not been studied.

Recently, in an attempt to understand how insulin induces transcription of its responsive gene, we identified insulin responsive elements in the Srebp-1c promoter as two liver X receptor (LXR) binding sites and one sterol regulatory element [38]. This suggests that insulin regulates the expression of its responsive genes after it stimulates the synthesis of endogenous agonists for nuclear receptor activation. It has been reported that the hepatic expression of Srebp-1c was elevated in liver of Zucker diabetic fatty rats [39]. We hypothesize that insulin-regulated expression of genes involved in glucose and lipid metabolism may be altered in liver of insulin resistant animals. To focus on insulin resistance and obesity, but not diabetes, we analyzed insulin-regulated gene expression in hepatocytes from ZF rats, which have hyperlipidemia, but normal glycemia [37]. Herein, we report the regulation of the mRNA levels of Srebp-1c and Pck1, two representative insulin-regulated genes, in hepatocytes isolated from Zucker lean (ZL) and ZF rats.

Materials and Methods

Reagents

The reagents for primary hepatocyte isolation and culture have been published [40]. Reagents for cDNA synthesis and real-time PCR were obtained from Applied Biosystems (Foster city, CA). Antibodies to phospho-Akt (Thr473) phospho-Akt (Ser473), and total Akt, were obtained from Cell Signaling Technologies (Danvers, MA). All other compounds were purchased from Sigma (Saint Louis, MO) unless described otherwise.

Animals

Male ZL and ZF rats were bred at UTK or purchased from Harlan Breeders (Indianapolis, IN). Rats were housed in colony cages, and fed a standard rodent diet before isolation of primary hepatocytes. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee at Knoxville (Protocol number 1642).

Hepatocyte isolation and treatments

For primary hepatocyte isolation, ZL or ZF rats in ad libitum or fasted overnight as indicated in the figure legends were euthanized with carbon dioxide. A catheter was inserted into portal vein and connected to a peristaltic pump with liver perfusion medium and liver digestive buffer (Invitrogen). The inferior vena cava was cut open to allow the outflow of the media at flow rate of 10 ml/min. After completion of the digestion, livers were excised from the rat and put into a tissue culture plate containing liver digest buffer for removing connection tissues and allowing the release of hepatocytes. Medium containing hepatocytes were filtered through a cell strainer and spun at 50 g for 3 minutes. The cell pellets were washed twice with DMEM containing 5% fetal bovine serum, 100 units/ml sodium penicillin, and 100 μg/ml streptomycin sulfate. After wash, the isolated hepatocytes were plated onto 60-mm collagen type 1 coated dishes (2 to 3 million cells/dish) and incubated in 4 ml of the same medium at 37°C and 5% CO2. After incubation for 3–4 hours, the attached cells were washed once with 4 ml of PBS, and incubated in medium A (medium 199 with 100 nM dexamethasone, 100 nM 3,3',5-triiodo-L-thyronine (T3), 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate) containing 1 nM insulin for 14–16 hours until being used for the indicated experiments. For the treatments, primary hepatocytes were washed once with 3 ml of PBS and then incubated in 2 ml of medium A containing indicated reagents for indicated time as shown in the figure legends.

RNA extraction and Quantitative Real-Time PCR

Methods for preparation and analysis of RNA were described previously [41]. The real time PCR primer sets for detecting Fas (from Dr. Bruce Spigelman’s group), Gck, Pek1, and Srebp-1c [41] have been published. The primer sets for Insr (forward 5'-CTGGAGAATCCTGCTGGGCTATT-3', and reverse 5'-GGGCT- ATAGACACGGAAAAGAAG-3'), and Sod1 (forward 5'-AAGA-TATCCACGACCCCCCTA-3', and reverse 5'-TCCGACGAGGCGCATGA-3') were designed using Primer Express software (Applied Biosystems). The gene expression level was normalized to that of 36B4 unless described otherwise. Data were presented as either the fold induction calculated from the ΔΔCt values [40] or the difference of the cycle threshold (ΔCt) numbers between the experimental gene and 36B4, the invariant control gene [41].

Immunoblot analysis

After indicated treatments in figure legends, primary hepatocytes in a 60 mm dish were washed once with 3 ml PBS and scrapped from the dish in 400 μl of whole-cell lysis buffer (1% Triton X-100, 10% glycerol, 1.0% IGEPAL CA-630, 50 mM Hepes, 100 mM NaF, 10 mM EDTA, 5 mM Sodium orthovanadate, 1.9 mg/ml aprotinin, 5 μg/ml leupeptin, 1 mM Benzamid, 2.5 mM DMSF, pH 8.0). The lysates were allowed to sit on ice for at least 20 minutes before subjected to 20000 g centrifugation for 20 minutes. The protein content in the supernatant was determined with PIERCE BCA protein assay kit (Rockford, IL). Proteins (30 μg/lane) in whole cell lysates were separated on SDS/PAGE, transferred to BIO-RAD Immun-Blot PVDF membrane (Hercules, CA), and detected with primary antibodies according to the protocols provided by the manufacturers. Bound primary antibodies were visualized by chemiluminescence (ECL Western Blotting Substrate; Thermo Scientific) using a 1:5,000 dilution of goat anti-rabbit IgG (Upstate) conjugated to horseradish peroxidase. Filters were exposed to X-ray films (Phenix Research Products, Candler, NC) for protein band detection.

Statistics

Data were presented as means ± SD. The number of experiments represented the independent experiments using
hepatocytes isolated from different animals on different days. Levene’s test was used to determine homogeneity of variance among groups using SPSS 19.0 statistical software and where necessary natural log transformation was performed before analysis. Multiple comparisons were analyzed by one-way ANOVA. The Independent-Samples T-Test was used to compare two conditions. Differences were considered statistically significant at \( P<0.05 \).

**Results**

**Elevated mRNA levels of Srebp-1c, Fas, Scd1, but not Insr, Gck and Pck1 in isolated primary hepatocytes from ad libitum ZF rats**

To analyze the mRNA levels of hepatic insulin-regulated genes, primary hepatocytes were isolated from ZL and ZF rats in *ad libitum* condition. The mRNA levels of representative control and insulin-regulated genes involved in hepatic glucose and lipid metabolism were subjected to real-time PCR analysis as shown in Figure 1. The mRNA level of insulin receptor gene (Fig. 1A, *Insr*) in freshly isolated primary hepatocytes of ZF rats was not significantly different from that of ZL rats. The mRNA levels of three lipogenic genes, *Srebp-1c* (Fig. 1B), *Fas* (Fig. 1C) and *Scd1* (Fig. 1D) in the freshly isolated primary hepatocytes of ZF rats were significantly higher than those of ZL rats. On the other hand, the mRNA levels of two genes for glucose metabolism, *Gck* (Fig. 1E) and *Pck1* (Fig. 1F), in the freshly isolated primary hepatocytes of ZF rats were similar to those of ZL rats. All these results demonstrated that ZF rat hepatocytes had significantly higher mRNA levels of *Srebp-1c*, *Fas*, and *Scd1* than ZL rat hepatocytes did, but not that of *Insr*, *Gck*, and *Pck1*.

**The changes of Srebp-1c and Pck1 mRNA levels in response to insulin stimulation were diminished in hepatocytes from ad libitum ZF rats**

To investigate insulin effects on gene expression in hepatocytes from ZL and ZF rats, we examined the regulation of the mRNA levels of two representative genes, one insulin-induced gene (*Srebp-1c*) and one insulin-suppressed (*Pck1*) gene, which are robustly regulated by insulin at transcriptional level in liver and primary hepatocytes [24]. Figure 2A shows the −ΔCt numbers of *Srebp-1c* and *Pck1* of the control groups in lean and fatty hepatocytes after the overnight pretreatment. The *Srebp-1c* mRNA level in fatty hepatocytes was higher than that in lean hepatocytes (−5.2±1.5 vs −8.1±1.4). However, *Pck1* mRNA in lean hepatocytes was not significantly different from that in fatty hepatocytes (−2.3±1.3 vs −1.7±0.7). These results demonstrated that the primary lean and fatty hepatocytes after overnight pretreatment in culture still maintained similar expression patterns as the freshly isolated hepatocytes did as shown in Figure 1.

To compare the mRNA levels of *Srebp-1c* and *Pck1* in lean and fatty hepatocytes after insulin stimulation, their expression levels in hepatocytes incubated in increasing concentrations of insulin were determined as shown in Figure 2 B and C. Figure 2B shows that the *Srebp-1c* mRNA level in hepatocytes from *ad libitum* ZL rats was significantly induced by insulin at the lowest concentration tested (0.1 nM). However, insulin failed to induce *Srebp-1c* mRNA expression in hepatocytes from *ad libitum* fatty rats. Figure 2C shows that insulin as low as 0.1 nM was sufficient to significantly reduce the *Pck1* mRNA level in lean hepatocytes (0.4±0.23-fold) with further suppression at higher insulin concentrations (Fig. 2C). In contrast, no significant reduction of *Pck1* expression can be observed until insulin reached 100 nM in fatty hepatocytes. The

![Image](58x134)
mRNA expression to insulin-mediated suppression in fatty hepatocytes, glucagon dramatically induced its expression by 27.5 ± 5.2- and 10.2 ± 2.3-fold of the control value in the absence or presence of insulin, respectively. The induction folds of Pck1 mRNA mediated by glucagon treatment in fatty hepatocytes were not significantly different from those in lean hepatocytes without or with insulin. These results again demonstrated impairment of insulin-regulated Srebp-1c and Pck1 mRNA expression in hepatocytes from ZF rats in ad libitum. However, only the glucagon-mediated reduction of Srebp1-c mRNA expression, but not induction of Pck1, was diminished in the same cells. In addition, the presence of insulin significantly attenuated glucagon-induced Pck1 mRNA expression in hepatocytes derived from either lean or fatty rats in ad libitum.

Blocking de novo cholesterol biosynthesis suppressed basal and insulin-induced Srebp-1c mRNA expression in hepatocytes from ZL, but not ZF rats in ad libitum

To assess the effects of endogenous cholesterol synthesis on the Srebp-1c mRNA expression, hepatocytes from ZL and ZF rats in ad libitum were treated without or with insulin in the absence or presence of compactin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase, which has been shown...
to suppress the synthesis of endogenous ligands for LXR activation [43]. Figure 4A shows that insulin induced Srebp-1c mRNA expression in lean, but not fatty hepatocytes, supporting results shown in Figures 2 and 3. Compactin at 50 μM was sufficient to significantly suppress both basal and insulin-induced Srebp-1c mRNA expression in lean hepatocytes to 0.6±0.1- and 1.1±0.5-fold of the control value, respectively. However, it did not significantly affect Srebp-1c mRNA expression in fatty hepatocytes without or with insulin. Compactin did not affect Pck1 mRNA expression in hepatocytes from either lean or fatty ad libitum rats in the absence or presence of insulin. These results demonstrated that compactin treatment only inhibited basal and insulin-induced Srebp-1c mRNA expression in lean, but not that of fatty hepatocytes.

Insulin-induced phosphorylation of Akt was not altered in hepatocytes from ZF rats in ad libitum

As the first step to investigate insulin signal transduction pathway in hepatocytes from ZL and ZF rats in ad libitum, the levels of phosphorylated and total Akt in them after insulin stimulation for 10 minutes was compared. As shown in Figure 5A, insulin started to induce noticeable phosphorylation of Akt on Threonine 308 (Thr308) and Serine 473 (Ser473) as low as 0.01 and 0.001 nM in hepatocytes from either ad libitum ZL or ZF rats, respectively. The phosphorylation of Thr308 and Ser473 in both types of hepatocytes reached plateau at 10 and 1 nM insulin, respectively. There was no obvious difference of Akt phosphorylation on these two sites upon insulin stimulation for 10 minutes in hepatocytes from ZL and ZF rats in ad libitum. To exclude the variations of Immunoblot performed in different days, the phosphorylation of Akt on Ser473 in three control groups or 1 nM insulin treatment groups of hepatocytes from either ZL or ZF rats in ad libitum were compared side by side. As shown in Figure 5B, the levels of Akt phosphorylation on Ser473 in lean hepatocytes were similar to that in fatty hepatocytes from ad libitum rats. There was no difference of total Akt protein levels in hepatocytes from lean and fatty rats without or with insulin. These results indicated that insulin-induced phosphorylation of Akt on Thr308 or Ser473 was not impaired in hepatocytes from ad libitum ZF rats.

Fasting restored the induction of Srebp-1c, and suppression of Pck1 mRNA expression mediated by insulin and T1317 in hepatocytes from ZF rats

To explore the effects of feeding condition on the insulin-regulated gene expression, the mRNA levels of Srebp-1c and Pck1 in response to insulin stimulation was analyzed in hepatocytes from ZL and ZF rats in ad libitum after an overnight fasting. Hepatocytes were treated with insulin, T1317 and insulin+T1317, and the expression levels of Srebp-1c and Pck1 mRNA were examined as shown in Figure 6. Figure 6A shows that insulin or

![Figure 4. The effects of compactin and insulin on the mRNA levels of Srebp-1c (A) and Pck1 (B) in hepatocytes from ad libitum Zucker lean and fatty rats. Primary hepatocytes after pretreatment as described in Materials and Methods were incubated in medium A without or with 50 μM compactin in the absence or presence of 1 nM insulin for 6 hours. Total RNA was extracted and subjected to real-time PCR analysis. The expression level of the indicated transcripts in the control hepatocytes (lean or fatty) was arbitrarily assigned a value of one for its corresponding cell type. Results were plotted as fold induction and presented as means ± SD of four independent hepatocyte isolations for both lean and fatty rats (n = 4 for hepatocyte isolations; for Srebp-1c, b>a>c, b>d and b>3 b’, using one-way ANOVA; for Pck1, e/g>f, and f’>f, using one-way ANOVA; all P<0.05). doi:10.1371/journal.pone.0021342.g004](Image 58x24 to 76x41)

![Figure 5. Immunoblot analysis of phospho-Akt and total Akt levels in primary hepatocytes treated with increasing concentrations (A) or 1 nM (B) of insulin. After overnight pretreatment, primary hepatocytes from ad libitum ZL and ZF rats were incubated in medium A containing indicated concentrations of insulin for 10 minutes. After which, hepatocytes were washed once with 3 ml PBS and lysed as described in Materials and Methods. Total protein (30 μg/lane) was separated on 8% SDS/PAGE gels, detected by specific primary antibodies as indicated, and visualized by chemiluminescence. A. The levels of phospho-Akt(Thr308), phospho-Akt(Ser473), and total Akt in ad libitum lean and fatty hepatocytes treated with increasing concentration of insulin (0 to 100 nM). Graph was the representative of three independent experiments with similar results using hepatocytes isolated from three different ad libitum ZL or ZF rats in different days. B. The levels of phospho-Akt(Ser473) and total Akt in ad libitum lean and fatty hepatocytes treated without or with 1 nM insulin. Graph represented three independent isolations of hepatocytes from different ad libitum ZL or ZF rats in different days, which were run side by side. doi:10.1371/journal.pone.0021342.g005](Image 315x239 to 554x432)
T1317 alone induced Srebp-1c mRNA expression in hepatocytes from either ad libitum or fasted ZL rats. T1317 synergized with insulin to induce Srebp-1c mRNA expression dramatically. The induction folds of Srebp-1c mRNA mediated by T1317, but not T1317+Insulin, were significantly higher in hepatocytes isolated from fasted lean rats than that from ad libitum lean rats (37.2±5.1- vs 12.7±2.6-fold). In hepatocytes isolated from ad libitum fatty rats, only insulin+T1317, but not insulin or T1317 significantly induced Srebp-1c mRNA expression. In hepatocytes isolated from fasted fatty rats, insulin, T1317 and insulin+T1317 significantly induced Srebp-1c mRNA expression to 14.9±8.2-, 8.7±4.1-, and 80±16.5-fold of the control value, respectively. These numbers were significantly higher than those of the corresponding treatments in hepatocytes from ad libitum rats. The induction folds mediated by T1317 and T1317+insulin in hepatocytes from fasted ZF rats were still lower than those of the corresponding treatments in hepatocytes from fasted ZL rats. These results indicated that overnight fasting partially corrected the impairment of insulin-induced Srebp-1c mRNA expression in hepatocytes from ZF rats in ad libitum.

Figure 6B shows the Pck1 mRNA expression levels in hepatocytes from ZL or ZF rats in ad libitum or an overnight fasting treated without or with T1317 in the absence or presence of insulin. T1317 at 1 μM did not affect the Pck1 mRNA expression in hepatocytes from either ad libitum or fasted lean or fatty rats in the absence or presence of insulin. Inulin at 1 μM dramatically reduced the expression of Pck1 mRNA in hepatocytes from ad libitum lean rats to the same extent as that in hepatocytes from fasted lean rats. However, insulin only suppressed Pck1 mRNA expression in hepatocytes from fasted fatty rats, but not that from ad libitum fatty rats. Insulin+T1317 significantly reduced the Pck1 mRNA expression levels in hepatocytes from ad libitum lean, fasted lean and fatty rats, but not at that from ad libitum fatty rats. Insulin+T1317 significantly reduced the Pck1 mRNA expression levels in hepatocytes from fasted fatty rats to 0.17±0.08- and 0.15±0.08-fold of the control group value, which are still respectively higher than 0.04±0.008- and 0.04±0.015-fold (the corresponding groups) in hepatocytes from fasted lean rats, indicating partial improvement of insulin action in hepatocytes from fasted fatty rats. These results demonstrated that an overnight fasting of ZF rats restored the insulin-mediated suppression of Pck1 mRNA expression in their hepatocytes.

Discussion

In the current study, we observed that the mRNA levels of Srebp-1c, Fas and Scd1, but not that of Insr, Gck and Pck1, were higher in freshly isolated hepatocytes from ad libitum ZF rats than those from ad libitum ZL rats. It has been reported that ZF rats had hyperinsulinemia, and elevated free fatty acid levels, but normal plasma glucose levels in basal stage or after a glucose load [44]. Elevated expression of hepatic lipogenic genes have been observed in ZF rats [45] and Zucker diabetic fatty rats [39,46]. Our results matched these original observations. It has been shown that hepatocytes isolated from rats in different nutritional conditions retained their differences in glycolgen deposition which is influenced by the nutritional state [47]. The hepatocytes from ad libitum fatty rats still retained the expression patterns of Srebp-1c and Pck1 after overnight pre-treatment (Figure 2A). These results indicated that our current experimental settings retained the characteristics of the hepatocytes in vivo.

We have shown here that insulin induced Srebp-1c and suppressed Pck1 mRNA expression in hepatocytes from ad libitum lean rats. In hepatocytes from ad libitum fatty rats, insulin no longer induced Srebp-1c mRNA expression at all the concentrations tested. Insulin at 100 nM suppressed Pck1 mRNA expression in fatty hepatocytes. However, the degree of reduction in fatty hepatocytes was not comparable to that in lean hepatocytes (Figure 2). This indicates that insulin-suppressed Pck1 mRNA expression was not completely diminished in fatty hepatocytes, not as insulin-induced Srebp-1c was. It has been shown that hepatocytes from the hyperinsulinemic ZF rats had insulin binding equivalent to that of lean littermates and had no reduction of insulin receptor at 10 weeks of age [48]. Therefore, low insulin-binding or receptor expression may not be the reason for the diminished insulin-regulated Srebp-1c and Pck1 mRNA expression. This conclusion is supported by the insulin-mediated Akt phosphorylation, which is similar in hepatocytes isolated from ad libitum lean and fatty rats (Figure 5).

Insulin regulates the expression of hepatic genes involved in glucose and lipid metabolism [7,8]. It induces Gck and suppresses Pck1 [7], both involved in hepatic glucose metabolism. It also induces the expression of Srebp-1c mRNA and in turn, increases hepatic fatty acid biosynthesis [13]. If the hyperinsulinemia resulted in the elevated mRNA levels of Srebp-1c, Fas and Scd1 in hepatocytes from ad libitum fatty rats, the question becomes why it did not cause elevation of Gck and reduction of Pck1 mRNA expression. It seems...
that the common insulin signaling pathways branched at some points that will specifically determine the responsiveness of a gene to insulin in a certain condition. Indeed, bifurcation of insulin pathway at mTORC1 step has been observed in rat liver which separated the insulin-derived signals responsible for elevation of Srebp-1c mRNA from those for reduction of Pck1 mRNA [24]. Whether any branching point is responsible for the phenomenon observed here remains to be investigated.

The diminished regulation of mRNA levels of Srebp-1c and Pck1 in response to insulin suggested that hepatocytes from ad libitum fatty rats might have lost responses to other hormones. Glucagon, a pancreatic hormone antagonizing insulin action [42], has been shown to inhibit Srebp-1c and induce Pck1 mRNA expression in hepatocytes in the absence or presence of insulin [49]. Therefore, glucagon was used to test hepatocytes from ad libitum lean or fatty rats. Glucagon inhibited basal and insulin-induced Srebp-1c mRNA expression in lean, but not in fatty hepatocytes from ad libitum rats. When the Pck1 mRNA expression was analyzed, glucagon induced its expression in both lean and fatty hepatocytes to the same extent without or with insulin. These results demonstrated that in fatty hepatocytes, Pck1 mRNA expression was still responsive to glucagon stimulation. It is noteworthy that insulin attenuated glucagon-mediated induction of Pck1 mRNA expression in fatty hepatocytes to the same degree as that in lean hepatocytes (Figure 3B). It appears that part of insulin signaling system responsible for regulation of Srebp-1c and Pck1 mRNA expression was impaired in fatty hepatocytes, whereas other parts responsible for attenuation of glucagon action probably remained unchanged. The results obtained from insulin dose-response curves of Akt phosphorylation (Figure 5) supported this conclusion. Insulin dose-dependently phosphorylated Akt on Thr308 and Ser473 to the same extent as that in lean hepatocytes (Figure 3A) suggests that the transcription of Pck1 mRNA expression to insulin in fatty hepatocytes. It has been shown that elevation of PKCζ activity contributed to the increased hepatic Srebp-1c expression in type 2 diabetic rats [22,23] [25]. In addition, insulin-induced Srebp-1c mRNA expression in primary rat hepatocytes requires mTORC1 [24]. Whether any of these plays a role in the impairment of insulin-mediated induction of Srebp-1c mRNA in primary hepatocytes from ad libitum fatty rats remains to be investigated.

As insulin induces Srebp-1c transcription via activation of liver X receptor, a nuclear receptor activated by cholesterol derivatives [50], the elevation of its mRNA expression in fatty hepatocytes could be caused by the excessive synthesis of endogenous agonists of LXR activation. However, when the endogenous cholesterol biosynthesis was inhibited by HMG CoA reductase inhibitor compactin [43], the Srebp-1c mRNA expression in fatty hepatocytes was not suppressed by it as it was in lean hepatocytes (Figure 4). This suggests that the excessive synthesis of endogenous ligands for LXR activation may not be the reason for unresponsiveness of Srebp-1c mRNA expression to insulin in fatty hepatocytes. Alternatively, another ligand not derived from cholesterol might have activated LXR. The fact that T1317 alone, a synthetic ligand for LXR activation [51], could not significantly induce Srebp-1c mRNA expression in hepatocytes from ad libitum fatty rats (Figure 5A) suggests that the transcription complex at Srebp-1c promoter in fatty hepatocytes was not sensitive to changes of ligand for LXR activation as it was in lean hepatocytes. It seems that Srebp-1c mRNA expression was locked in a stage which no dynamic change was allowed. This might have caused the unresponsiveness of Srebp-1c mRNA in fatty hepatocytes to positive and negative regulatory signals derived from insulin and glucagon, respectively. Whether there is any change of the activities of those transcription complexes on Srebp-1c and Pck1 promoter in fatty hepatocytes deserves further investigation.

As caloric restriction has been shown to increase insulin sensitivity in human and animals [52–54], the ZL and ZF rats were fasted for overnight and their hepatocytes were isolated for measurement of their responses to insulin and T1317. In hepatocytes from ad libitum or fasted lean rats, T1317 synergized with insulin to induce Srebp-1c mRNA expression (Figure 6). Only T1317 alone caused higher induction of Srebp-1c mRNA expression in hepatocytes from fasted lean rats than that from ad libitum lean rats, demonstrating limited effects of fasting on insulin-regulated gene expression in hepatocytes from lean rats. However, in hepatocytes from fasted fatty rats, insulin induced Srebp-1c and suppressed Pck1 mRNA expression as it did in hepatocytes from lean rats. In addition, T1317 also induced Srebp-1c mRNA expression in hepatocytes from fasted fatty rats. The Srebp-1c mRNA levels induced by insulin+T1317 in hepatocytes from fasted fatty rats were similar to those from ad libitum lean rats, significantly lower than those from fasted lean rats, and significantly higher than those from ad libitum fatty rats. These results demonstrated the partial restoration of insulin-regulated gene expression in hepatocytes from fasted fatty rats. It has been shown that an overnight fast was sufficient to mobilize fatty acid from adipose tissues of fatty rats [37]. In addition, fasting has been shown to partially restore insulin actions on adipocytes from insulin resistant ZF rats [55]. The restoration of insulin-regulated Srebp-1c and Pck1 mRNA expression in hepatocytes from fasting fatty rat indicates that short term fasting was sufficient to modify insulin action on hepatocytes from fatty rats. The molecular mechanisms that led to the restoration of insulin action in fatty hepatocytes deserve further investigation.

In summary, we have demonstrated that insulin-regulated Srebp-1c and Pck1 mRNA expression was diminished in hepatocytes from ad libitum fatty rats. This impairment was not due to any change of Akt phosphorylation in insulin in hepatocytes from ZF rats. This is the first time that the impairment of insulin action was shown at the regulation of mRNA levels in ZF hepatocytes. The fact that a simple overnight fasting partially restored insulin-regulated gene expression in fatty hepatocytes indicates the existence of potential pathway which can reverse the insulin resistance in hepatocytes from Zucker fatty rats. The understanding of the underlying molecular mechanisms will help us to combat metabolic diseases.

**Author Contributions**

Conceived and performed the experiments: GC. Performed the experiments: GC. Wrote the paper: GC.

**References**

1. Moladad AH, Serdula MK, Dietz WH, Bowman BA, Marks JS, et al. (1999) The Spread of the Obesity Epidemic in the United States, 1991–1996. JAMA 282: 1519–1522.

2. Must A, Spadano J, Coakley EH, Field AE, Colditz G, et al. (1999) The Disease Burden Associated With Overweight and Obesity. JAMA 282: 1523–1529.

3. McGarry JD (2002) Banting Lecture 2001: Dysregulation of Fatty Acid Metabolism in the Etiology of Type 2 Diabetes. Diabetes 51: 7–18.

4. Kabir M, Catalano KJ, Ananthnarayan S, Kim SP, Van Citters GW, et al. (2005) Molecular evidence supporting the portal theory: a causative link between visceral adiposity and hepatic insulin resistance. Am J Physiol Endocrinol Metab 288: E654–E661.
5. Banerjee RR, Rangavala SM, Shapiro JS, Rich AS, Rhoade S, et al. (2004) Regulation of Fasted Blood Glucose by Resistin. Science 303: 1195–1198.

6. Shoelson SE, Lee J, Goldfine AB (2006) Inflammation and insulin resistance. J Clin Invest 116: 1793–1801.

7. O'Brien RM, Granner DK (1996) Regulation of gene expression by insulin. Phys Rev 76: 1109–1161.

8. Shimomura I, Matsuda M, Hammer RE, Bashmakov Y, Brown MS, et al. (2005) Decreased IRS-2 and Increased SREBP-1c Lead to Mixed Insulin Resistance and Sensitivity in Livers of Lipodystrophic and ob/ob Mice. Molecular Cell 25: 77–86.

Spiegelman BM, Flier JS (2001) Obesity and the Regulation of Energy Balance. Cell 104: 513–534.

9. Magnuson MA, Andreade TL, Printz RL, Koch S, Granner DK (1989) Rat Glucokinase Gene: Structure and Regulation by Insulin. PNAS 86: 4838–4842.

10. Hanson RW, Patel YM (1997) Regulation of Phosphoenolpyruvate Carboxykinase (GTP) Gene expression. Annual Review of Biochemistry 66: 581–611.

11. Shimomura I, Bashmakov Y, Ikemoto S, Horton JD, Brown MS, et al. (1999) Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. PNAS 96: 13656–13661.

12. Manning BD, Cantley LC (2007) AKT/PKB Signaling: Navigating Downstream. 30. Iida M, Murakami T, Ishida K, Mizuno A, Kuwajima M, et al. (1996) Glucokinase Gene: Structure and Regulation by Insulin. PNAS 86: 4838–4842.

13. Hanson RW, Patel YM (1997) Regulation of Phosphoenolpyruvate Carboxykinase (GTP) Gene expression. Annual Review of Biochemistry 66: 581–611.

14. Horton JD, Goldstein JL, Brown MS (2002) SREBP: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109: 1125–1131.

15. Liang G, Yang J, Horton JD, Hammer RE, Goldstein JL, et al. (2002) Diminished Hepatic Response to Fasting/Refeeding and Liver X Receptor Agonists in Mice with Selective Deficiency of Sterol Regulatory Element-binding Protein-1c. J Biol Chem 277: 9520–9528.

16. Horton JD, Brown MS, Goldstein JL (1988) SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. PNAS 96: 13656–13661.

17. Unger RH (1997) How obesity causes diabetes in Zucker diabetic fatty rats. J Biol Chem 270: 1295–1299.

18. Zhou YP, Cockburn IN, Pugh W, Polonsky KS (1999) Basal insulin hypersecretion in insulin-resistant Zucker diabetic and Zucker fatty rats: Role of enhanced fuel metabolism. Metabolism 48: 857–864.

19. Garnett KE, Chapman P, Chambers JA, Waddell ID, Boam DS (2005) Differential gene expression between Zucker Fatty rats and Zucker Diabetic Fatty rats: a potential role for the immediate-early gene Egr1 in regulation of beta cell proliferation. J Mol Endocrinol 35: 13–25.

20. Griffen SC, Wang J, German MS (2003) A Genetic Defect in b-Cell Gene Expression Segregates Independently From the fa Locus in the ZDF Rat. Diabetes 52: 63–68.

21. Zucker LM (1972) Fat mobilization in vitro and in vivo in the genetically obese Zucker rat “fatty.” J Lipid Res 13: 234–243.

22. Liang G, Li, Ou J, Goldstein JL, Brown MS (2004) Central role for liver X receptor in insulin-mediated activation of Srebp-1c transcription and stimulation of fatty acid synthesis in liver. PNAS 101: 11245–11250.

23. Taniguchi CM, Emanuelli B, Kahn CR (2006) Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol 7: 85–96.

24. Zhou YP, Cockburn IN, Pugh W, Polonsky KS (1999) Basal insulin hypersecretion in insulin-resistant Zucker diabetic and Zucker fatty rats: Role of enhanced fuel metabolism. Metabolism 48: 857–864.

25. Garnett KE, Chapman P, Chambers JA, Waddell ID, Boam DS (2005) Differential gene expression between Zucker Fatty rats and Zucker Diabetic Fatty rats: a potential role for the immediate-early gene Egr1 in regulation of beta cell proliferation. J Mol Endocrinol 35: 13–25.

26. Griffith SC, Wang J, German MS (2003) A Genetic Defect in b-Cell Gene Expression Segregates Independently From the fa Locus in the ZDF Rat. Diabetes 52: 63–68.

27. Zucker LM (1972) Fat mobilization in vitro and in vivo in the genetically obese Zucker rat “fatty.” J Lipid Res 13: 234–243.

28. Liang G, Li, Ou J, Goldstein JL, Brown MS (2004) Central role for liver X receptor in insulin-mediated activation of Srebp-1c transcription and stimulation of fatty acid synthesis in liver. PNAS 101: 11245–11250.

29. Kamaka T, Lee Y, Higa M, Wang ZW, Pan W, et al. (2000) Leptin, troglitazone, and the expression of sterol regulatory element binding proteins in liver and pancreatic islets. Proceedings of the National Academy of Sciences of the United States of America 97: 8536–8541.

30. Chen G (2007) Liver lipid molecules induce PEPCK-C gene transcription and attenuate insulin action. Biochemical and Biophysical Research Communications 361: 805–810.

31. Chen G, Zhang Y, Lu D, Li N, Ross AC (2009) Retinoids synergize with insulin to induce hepatic Gck expression. Biochem J 419: 643–653.

32. Starke A, Imanura T, Unger RH (1987) Relationship of glucagon suppression by insulin and somatostatin to the ambient glucose concentration. J Clin Invest 79: 20–24.

33. DeBose-Boyd RA, Ou J, Goldstein JL, Brown MS (2001) Expression of sterol regulatory element-binding protein-1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. Proceedings of the National Academy of Sciences 98: 1477–1482.

34. Zucker LM, Antoniades HN (1972) Insulin and Obesity in the Zucker Genetically Obese Rat “Fatty.” Endocrinology 90: 1320–1330.

35. Burque N, Martinez MJ, Cao A, Miquelina-Colina ME, et al. (2010) A subset of dysregulated metabolic and survival genes is associated with severity of hepatic steatosis in obese Zucker rats. J Lipid Res 51: 500–513.

36. Chitaei DV, Collins HL, Ganell J, Sparks JD, Sparks CE (2004) Altered triglyceride-rich lipoprotein production in Zucker diabetic fatty rats. American Journal of Physiology - Endocrinology And Metabolism 287: E42–E49.

37. Igusa I, Maci K, Alberti KG (1996) Regulation of glycoegen synthesis from glucose and gluconeogenic precursors by insulin in periporal and perivenous rat hepatocytes. Biochem J 296: 91–102.

38. Clark JB, lark CM (1982) Age-Related Changes in Insulin Receptor Regulation in Liver Membranes from Zucker Fatty Rats. Endocrinology 111: 964–969.

39. Forre B, Cao C, Duggal I, Lenarchand P, Guichard C, et al. (1999) ADD1/ SREBP-1c Is Required in the Activation of Hepatic Lipogenic Gene Expression by Glucose. Mol Cell Biol 19: 3760–3768.

40. Janowski BA, Willy PJ, Devi TR, Falk JR, Mangelsdorf DJ (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR[alpha]. Nature 383: 729–731.

41. Clark JB, lark CM (1982) Age-Related Changes in Insulin Receptor Regulation in Liver Membranes from Zucker Fatty Rats. Endocrinology 111: 964–969.

42. Forre B, Cao C, Duggal I, Lenarchand P, Guichard C, et al. (1999) ADD1/ SREBP-1c Is Required in the Activation of Hepatic Lipogenic Gene Expression by Glucose. Mol Cell Biol 19: 3760–3768.

43. Janowski BA, Willy PJ, Devi TR, Falk JR, Mangelsdorf DJ (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR[alpha]. Nature 383: 729–731.

44. Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, et al. (2000) Regulation of mouse sterol regulatory element-binding protein-1c (SREBP-1c) by oxysterol receptors, LXR[alpha] and LXR[beta]. Genes & Development 14: 2819–2830.

45. Henry RR, Scheffer L, Olefsky JM (1985) Glycemic Effects of Intensive Caloric Restriction and Isoenergetic Refeeding in Non-insulin-Dependent Diabetes Mellitus. J Clin Endocrinol Metab 61: 917–925.

46. Okachiku N, Mizuno A, Yoshimoto S, Zhu M, Sano T, et al. (1995) Is caloric restriction effective in preventing diabetes mellitus in the Onaka Long Evans Tokushima Fatty rat, a model of spontaneous non-insulin-dependent diabetes mellitus? Diabetes Research and Clinical Practice 27: 97–106.

47. Reaven GM (2005) The Insulin Resistance Syndrome: Definition and Dietary Approaches to Treatment. Annu Rev Nutr 25: 391–406.

48. Stewart J, Green M, Kaiser D, Pohl S (1981) Insulin resistance in adipocytes from fed and fasted obese rats: Dissociation of two insulin actions. Molecular and Cellular Biochemistry 37: 177–183.