Comparison of In Vivo Effects of Insulin on SREBP-1c Activation and INSIG-1/2 in Rat Liver and Human and Rat Adipose Tissue

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Objective: The stimulatory effects of insulin on de novo lipogenesis (DNL) in the liver, where it is an important contributor to non-alcoholic fatty liver disease (NAFLD), hepatic and systemic insulin resistance, is strong and well established. In contrast, insulin plays only a minor role in DNL in adipose tissue. The reason why insulin stimulates DNL more in liver than in fat is not known but may be due to differential regulation of the transcription and post-translational activation of sterol regulatory element binding proteins (SREBPs). To test this hypothesis, we have examined effects of insulin on activation of SREBP-1c in liver of rats and in adipose tissue of rats and human subjects.

Design and Methods: Liver and epidydimal fat were obtained from alert rats and subcutaneous adipose tissue from human subjects in response to 4 h euglycemic-hyperinsulinemic clamps.

Results: Here we show that acutely raising plasma insulin levels in rats and humans increased SREBP-1 mRNA comparably 3-4 fold in rat liver and rat and human adipose tissue, but increased post-translational activation of SREBP-1c only in rat liver, while decreasing it in adipose tissue. These differential effects of insulin on SREBP-1c activation in liver and adipose tissue were associated with robust changes in the opposite direction of INSIG-1 and to a lesser extent of INSIG-2 mRNA and proteins.

Conclusions: We conclude that these findings support the hypothesis that insulin stimulated activation of SREBP-1c in the liver, at least in part, by suppressing INSIG-1 and -2, whereas in adipose tissue, an increase in INSIG-1 and -2 prevented SREBP-1c activation.

Introduction

A key action of insulin is regulation of de novo lipogenesis (DNL), that is, the synthesis of fatty acids from excess carbohydrates (CHO), which can neither be oxidized nor stored as glycogen (1,2). Although DNL has been demonstrated to be active in both liver and adipose tissue (1), levels of DNL activity in these two tissues are different. In the liver, DNL can be responsible for up to 30% of hepatic fat in obese subjects (3) and is an important contributor to the development of nonalcoholic fatty liver disease (NAFLD) and hepatic as well as systemic insulin resistance (4). However, in adipose tissue, DNL plays only a minor role and contributes little to adipose tissue triglyceride stores (5,6). The reason why insulin stimulates DNL more in liver than in fat is not known.

In the liver, insulin controls DNL by regulating the transcription and post-translational activation of sterol regulatory element binding proteins (SREBPs; reviewed in Ref. 7). SREBP-1c, the predominant isoform in liver and adipose tissue (8,9), is synthesized as a precursor protein and retained in an inactive form in the endoplasmic reticulum (ER), where it is bound to two other proteins, insulin-induced gene (INSIG) and SREBP cleavage-activating protein (SCAP; reviewed in Ref. 9). Activation of SREBP-1c requires dissociation from INSIG proteins and translocation of the SREBP/SCAP complex to the Golgi complex, where SREBP-1c undergoes a two-step proteolytic process, liberating the N-terminal, transcriptionally active (~68 kDa) form of SREBP-1c (9). Although the molecular details for this process are not clear, there is evidence for an important role of the INSIG proteins (7,10–12).

The large difference in insulin stimulation of DNL in liver and adipose tissue (5,6) suggests that there are probably differences in insulin stimulation of SREBP-1c activation between liver and fat. Surprisingly, however, in contrast to a large body of information in

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liver, there is little information concerning insulin regulation of SREBP-1c activation in adipose tissue. In fact, we are not aware of in vivo data in human or rat adipose tissue of insulin effects on either SREBP-1c activation or INSIG-1 and INSIG-2 expression.

The objective of this study was to explore possible reasons for the different insulin effects on DNL in liver and fat. To this end, we compared acute, in vivo effects of physiological hyperinsulinemia on transcription and translational and post-translational activation of SREBP-1c and on INSIG-1 and INSIG-2 mRNA and proteins in liver and epididymal fat of rats and in subcutaneous fat of human subjects.

Methods and Procedures

Male Sprague-Dawley rats (~300 g purchased from Charles River Laboratories, Wilmington, MA) were used, and procedures were as described (13). Briefly, a polyvinyl catheter was inserted into the internal jugular vein and extended to the right atrium. This catheter was used for infusions. A second catheter was advanced through the left carotid artery to the aortic arch. This catheter was used for blood collections.

Rat euglycemic-hyperinsulinemic clamp studies

Clamps were performed with awake, unrestrained rats. Insulin (28 pmol/kg min) was infused through the jugular vein catheter for 4 h. Glucose concentrations were clamped at euglycemic levels by a variable rate infusion of 25% glucose. Blood glucose levels were monitored with an Elite Glucometer (Bayer, Elkhart, IN), and glucose infusion rates (GIRs) were adjusted every 5-10 min as needed.

Rat saline controls

Saline was infused for 4 h, and plasma glucose concentrations were maintained at ~5.5 mmol/l by a variable rate infusion of glucose.

After the studies, the rats were killed by an overdose of carbon monoxide gas, and the livers and the epididymal fat were freeze clamped, excised, and kept frozen at −80°C until assayed.

All protocols were approved by the Temple University Institutional Animal Care and Use Committee.

Human studies

Study subjects (Table 1) were admitted to the Temple University Hospital Clinical Research Center on the day before the studies. At ~0800 h on the day of the study, venous blood samples were obtained, and an open subcutaneous fat biopsy was performed by a surgeon from the lateral aspect of one upper thigh as described (14). The excised fat (200-300 mg) was dropped immediately into isopentane kept at its freezing point by liquid nitrogen. Following the initial fat biopsy, a euglycemic-hyperinsulinemic clamp was performed. At the end of the clamp, another fat biopsy was obtained from the contralateral leg. The frozen fat was stored at −80°C until analyzed. Informed written consent was obtained from all subjects after explanation of the nature, purpose, and the potential risks of these studies. The study protocol was approved by the Institutional Review Board of Temple University Hospital.

Euglycemic-hyperinsulinemic clamping in humans

To obtain comparable serum insulin levels in humans and rats, regular human insulin was infused intravenously (IV) in humans at a rate of 14 pmol/kg min. Plasma glucose concentrations were clamped at ~5.5 mmol/l by a feedback-controlled variable glucose infusion. Glucose samples were obtained before and at hourly intervals during insulin infusions for the determination of plasma glucose and insulin levels.

Western blots

Lever and adipose tissues were extracted, and protein content was measured as described (13). Blots were probed with the following antibodies from Santa Cruz Biotechnology (Santa Cruz, CA): INSIG-1 (SC-25124-rat and human), INSIG-2 (SC-66936 rat and human), SREBP-1c (SC-8984 rat), and fatty acid synthase (FAS; SC-55580).

| TABLE 1 Study subjects | Mean (n = 6) | SEM |
|------------------------|-------------|-----|
| Age (years)            | 42          | 6   |
| Gender                 | M/F         |     |
| Height (cm)            | 176.8       | 5.2 |
| Weight (kg)            | 87.67       | 2.54|
| BMI (kg/m²)            | 28.72       | 1.09|

The SREBP primers do not differentiate between SREBP-1a and 1c. However, in human and mouse tissues, SREBP-1c is the predominant isoform with 1c to 1a ratios ranging from 10:1 in liver and 3:1 in adipose tissue (8).

| TABLE 2 | Rat | Human |
|---------|-----|-------|
| ACC     | Forward primer | gatggcttggaagaagcgtc |
|         | Reverse primer | tcaaccccctgtgcgag |
| FAS     | Forward primer | caaccgtgtgctccatcct |
|         | Reverse primer | ggaccaacttcgaaagct |
| INSIG1  | Forward primer | tgcgtatccagcgaaatgt |
|         | Reverse primer | cccggcgagaggaagatg |
| INSIG2  | Forward primer | gacggtatgctgtaagattctt |
|         | Reverse primer | tggacgtaagcagacaatgct |
| PECK-1  | Forward primer | acagagaaaccagctaggagac |
|         | Reverse primer | ctggattcctgtagcct |
| SREBP1  | Forward primer | gacgcacttgatgacatt |
|         | Reverse primer | aggagaaccctccagagagga |
| ChREBP  | Forward primer | gttccagatctttctcttg |
|         | Reverse primer | catggcaacataggcgttctg |
| XBP1S   | Forward primer | tggagaacccagagtgtaa |
|         | Reverse primer | ctcgccactcctgctctg |
| 18S     |              | Ambion catalog # 5103G |

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Real-time reverse transcriptase PCR
Total RNAs were isolated from frozen rat liver and fat, and real-time reverse transcriptase PCR was performed as described (13,14). Rat and human primers are shown in Table 2.

Statistical analysis
All data are expressed as mean ± SE. Statistical analyses were performed using the SigmaStat program (Aspire Software, Ashburn, VA). Group comparisons were made with one-way ANOVA or the Kruskal-Wallis ANOVA on Ranks when the samples were drawn from a non-normal population or did not have equal variances. Population normality was tested using the Kolmogorov-Smirnov test with Lilliefors’ correction, and equal variance was tested using the Levene Median test. Post hoc testing was done with the Student-Newman-Keuls Test.

Results
Glucose, insulin, FFA, and GIR during euglycem-hyperinsulinemic clamping
Glucose concentrations were clamped at 5.5 ± 0.5 mmol/l during saline and insulin infusions in rats and were 5.2 ± 0.2 and 5.5 ± 0.1 mmol/l during preinsulin and postinsulin in human subjects.

Insulin levels were 237 ± 35 pmol/l during saline and 1659 ± 56 pmol/l during insulin in rats and 39 ± 11 and 1501 ± 123 pmol/l during saline and insulin in humans.

FFA levels were 524 ± 13 μmol/l during saline and 175 ± 20 μmol/l during insulin in rats and 433 ± 39 and 50 ± 9 μmol/l during saline and insulin in humans.

In response to hyperinsulinemia, GIR increased from 0 to 218 ± 2 μmol/kg min in rats and from 0 to 46 ± 7 μmol/kg min in humans.

Insulin stimulated SREBP-1 mRNA similarly in rat liver and in rat and human fat
When compared with saline, insulin increased SREBP-1 mRNA 4.1-fold (11.8 ± 2.3 vs. 2.9 ± 0.3, P < 0.02) in rat liver, 4.4-fold (23.2 ± 4.1 vs. 5.3 ± 0.3, P < 0.001) in rat epididymal adipose tissue, and 3.1-fold (2.5 ± 0.7 vs. 0.84 ± 0.1, P < 0.001) in human subcutaneous adipose tissue (Figure 1).

As the similar insulin stimulation of SREBP-1c transcription did not explain the differences in DNL in liver and adipose tissue, we next examined insulin effects on post-translational activation of SREBP-1c in these tissues.

Insulin increased post-translational activation of SREBP-1c in liver but not in fat
In rat liver, insulin increased the abundance of the transcriptionally active (68 kDa) form of SREBP-1c 3.3-fold (0.73 ± 0.12 vs. 0.22 ± 0.01, P < 0.02), whereas insulin had no significant effect on the 125-kDa precursor form of SREBP-1c resulting in a 3.1-fold increase in the 68/125-kDa protein ratio (Figure 2). In contrast, in rat epididymal fat, insulin decreased by >50% both the 68-kDa form of SREBP-1c (0.32 ± 0.06 vs. 0.73 ± 0.06, P < 0.01) and the 125-kDa precursor form of SREBP-1c (0.32 ± 0.14 vs. 0.63 ± 0.2, P < 0.01). Unfortunately, we were unable to determine SREBP-1c protein in human adipose tissue due to its low concentration and the scarcity of available biopsy material. Similar problems have been reported by other investigators (6).

INSIG-1 is decreased by insulin in liver and increased in fat
To investigate possible causes for these differences in insulin-induced activation of SREBP-1c in liver and adipose tissue, we examined the effects of insulin on INSIG-1 and INSIG-2, both of which are considered to play key roles in post-translational SREBP-1c activation (Figure 3) (15,16).

In rat liver, insulin (when compared with saline) reduced INSIG-1 mRNA by >70% (7.5 ± 1.6 vs. 25.4 ± 3.1, P < 0.001) and INSIG-1 protein by >40% (36 ± 8 vs. 60 ± 1, P < 0.04).

In rat adipose tissue, insulin increased INSIG-1 mRNA to >30-fold (85 ± 21 vs. 2.6 ± 0.2, P < 0.01) and INSIG-1 protein to more than twofold (1.26 ± 0.26 vs. 0.55 ± 0.01, P < 0.01).
In human adipose tissue, insulin increased INSIG-1 mRNA to ninefold (7.8 ± 4.0 vs. 0.9 ± 0.3, P = 0.05) and INSIG-1 protein to 1.8-fold (1.2 ± 0.2 vs. 2.1 ± 0.5, P < 0.05).

Effects of insulin on INSIG-2
In rat liver, insulin (when compared with saline) reduced INSIG-2 mRNA by >40% (8.7 ± 1.7 vs. 15.4 ± 2.5, P < 0.03) and INSIG-2 protein by >50% (10.1 ± 2.4 vs. 4.3 ± 1.9, P < 0.05) (Figure 4).

In rat adipose tissue, INSIG-2 mRNA did not change in response to insulin (4.5 ± 1.3 vs. 3.8 ± 0.4, NS), but INSIG-2 protein increased 1.8-fold (8.1 ± 0.8 vs. 4.4 ± 1.1, P < 0.03).

In human adipose tissue, neither INSIG-2 mRNA nor protein changed significantly in response to insulin (2.4 ± 0.8 vs. 2.6 ± 0.9, NS and 1.0 ± 0.4 vs. 1.8 ± 0.5, NS, respectively).

Effects of insulin on SREBP-1c targets
An increase in SREBP-1c alone has been reported to be sufficient to promote an increase in the transcription of glucokinase (GK) (17) and to mediate the inhibitory effect of insulin on phosphoenolpyruvate (PEPCK; Figure 5) (18). In agreement, we found >15-fold increase in GK mRNA and >80% suppression of PEPCK mRNA associated with more than fourfold increase in the transcriptionally active 68-kDa form of SREBP-1c. On the other hand, SREBP-1c is known to need collaboration of several other transcription factors, including ChREBP (17,19) and X box protein-1 (XBP-1) (20) to promote expression of lipogenic enzymes. In this study, euglycemia-hyperinsulinemia was neither associated with significant changes in the transcription factors ChREBP and XBP-1 nor with changes in mRNAs of the two DNL-related enzymes, FAS and acetyl-CoA carboxylase (ACC).

Discussion
Our main objective was to compare the in vivo effects of insulin on transcription and post-transcriptional activation of SREBP-1c in rat liver and rat and human adipose tissue. The results showed that acutely raising plasma insulin to postprandial levels increased SREBP-1 mRNA comparably (threefold to fourfold) in livers and epididymal adipose tissue of alert, free moving rats and in subcutaneous adipose tissue of human subjects. These in vivo findings confirmed previous in vitro reports in rat hepatocytes (15,16,21) and further showed that insulin had similar effects in rat and human adipocytes. However, they did not explain the different in vivo insulin effects on DNL in liver and fat (5,6).
On the other hand, we also found that insulin action on the post-translational activation of SREBP-1c protein was distinctly different in liver and adipose tissue. In rat liver, physiologic hyperinsulinemia produced a more than threefold increase in the activated 68-kDa SREBP-1c protein, indicating strong post-translational activation and confirming previous reports in hepatocytes albeit obtained with superphysiological (100 nM) insulin doses (12,22). In contrast, to livers in rat adipose tissue, the 68-kDa SREBP-1c protein did not increase. This failure of physiological insulin doses to activate SREBP-1c in vivo in adipose tissue has not been previously reported and may be one reason why insulin stimulation of DNL in fat has been found to be relatively weak (5,6).

To explore the reason why insulin activated SREBP-1c protein in the liver but not in adipose tissue, we determined INSIG-1/2 responses to insulin. Both INSIG-1 and INSIG-2 have been shown to bind SREBP-1c and to prevent the ER to Golgi transfer of the SREBP-1c/SCAP complex and the proteolytic activation of SREBP-1c (reviewed in Ref. 23). Here, we found that in rat liver, the hyperinsulinemia-associated activation of SREBP-1c protein was accompanied by downregulation of both INSIG-1 and INSIG-2 mRNAs and proteins. In contrast, in rat adipose tissue, the 68-kDa SREBP-1c protein did not increase. This failure of physiological insulin doses to activate SREBP-1c in vivo in adipose tissue has not been previously reported and may be one reason why insulin stimulation of DNL in fat has been found to be relatively weak (5,6).

Our findings in rat liver confirm the reports in hepatocytes showing that activation of SREBP-1c was associated with reduced insulin levels of INSIG-2 mRNA and protein (12,24). These findings are also in agreement with the reports showing that hepatic overexpression of INSIG-1 (or INSIG-2) inhibited activation of SREBP-1c in rat liver (11,25,26) and that lowering of INSIG-1 in Chinese hamster ovary cells increased proteolytic activation of SREBP-1c, whereas overexpression of INSIG-1 decreased SREBP-1c activation (27). On the other hand, our findings seem to disagree with the results from a study that showed that INSIG-1 mRNA was decreased in livers of fasted rats and increased back to basal levels during refeeding (24). The reason for this apparent discrepancy, although not entirely clear, maybe related to differences in the experimental protocol. Yabe et al. (24) studied livers from mice after oral feeding, whereas we studied livers of rats after 4 h of IV insulin infusions. Thus, oral food ingestion and IV insulin administration may have resulted not

![FIGURE 4 Upper panels: Effects of 4-h infusions of saline or insulin (euglycemia-hyperinsulinemia) on INSIG-2 mRNA (left), two representative INSIG-2 Western blots (middle), and INSIG-2 protein (right) in rat liver and epididymal fat (saline, n = 4; insulin, n = 5). Lower panels: INSIG-2 mRNA (left), two representative INSIG-2 Western blots (middle), and INSIG-2 protein (right) before (pre) and after (post) insulin infusions (euglycemia-hyperinsulinemia) from six human studies (mean ± SE).](image)

![FIGURE 5 Effects of 4-h infusions of saline or insulin (euglycemia-hyperinsulinemia) on X box protein-1 s (XBP1s) mRNA, glucokinase (GK) mRNA, phosphoenolpyruvate carboxykinase (PEPCK) mRNA, carbohydrate regulatory element binding protein (ChREBP) protein, fatty acid synthase (FAS) mRNA, and acetyl-CoA carboxylase (ACC) mRNA in rat liver. Representative ChREBP Western blots are also shown. Saline studies n = 4, insulin studies n = 5 (mean ± SE).](image)
only in different portal venous insulin concentrations but also oral feeding was likely to have induced release of gastrointestinal factors that could have influenced INSIG-1 expression.

With respect to INSIG responses to insulin in adipocytes or fat, we found only one report (28) that showed strong increase in INSIG-1 mRNA in adipocytes of rats at the onset of high-fat-diet-induced obesity who presumably were hyperinsulinemic.

Altogether, our findings are compatible with the notion that the insulin-mediated activation of SREBP-1c in the liver was caused, at least in part, by the downregulation of INSIG-1 and to a lesser extent INSIG-2, whereas the lack of SREBP-1c activation in adipose tissue was due to upregulation of INSIG-1 and to a lesser extent INSIG-2.

Clearly, our findings neither proved causality nor excluded other causes. For instance, ER stress has been found to be associated with activation of SREBPs in various conditions and cell types including livers of genetic models of obesity (ob/ob mice and Zucker fa/fa rats) (29), homocysteine-induced ER stress (30), ethanol-fed rodents (31), and various cell types under ER stress (29,32,33). The linkage of ER stress to SREBP activation is an attractive concept as both the activation of ATF-6, one of the ER stress proximal sensors, and the activation of SREBP-1 are induced by the same proteases and in the same locations (32). In support of this concept, we have previously shown that 4 h of euglycemia-hyperinsulinemia produced a modest degree of ER stress in rat liver (34). However, it remains to be shown whether insulin-induced ER stress is also present in adipose tissue, and if so, how is it compared with ER stress in liver.

Insulin, via SREBP-1c, activates expression of many genes involved in DNL (9). Therefore, we have examined the effects of insulin on several SREBP-1c target genes in rat liver, including GK, the key enzyme in hepatic glucose uptake/phosphorylation, PECK, a key gluconeogenic enzyme, and ACC and FAS, two sequential enzymes in the DNL pathway. The observed insulin-induced increase in the active form of SREBP-1c protein in the liver was associated with >15-fold increase in GK mRNA and >80% decrease in PECK mRNA. These in vivo results expanded previous data in cultured hepatocytes, which had shown that insulin-induced activation of SREBP-1c potently increases GK gene expression (15) and strongly inhibited PECK gene expression (18). However, we found no effect of insulin on ACC or on FAS mRNA. Perhaps, our 4-h insulin infusions were too short to increase the expression of these enzymes. On the other hand, activation of these genes has been shown to depend not only on hyperinsulinemia and activation of SREBP-1c and other transcription factors (35) but also on hyperglycemia (17,19). The absence of hyperglycemia in our studies, therefore, may have prevented the upregulation of ACC and FAS. Supporting this notion, there was no effect of insulin on ChREBP, a transcription factor, which has emerged as a major mediator of glucose action on lipogenic gene expression and lipid synthesis (19), or on XBP-1s, a transcription factor, which has recently been shown to be induced by CHO feeding and to be required for hepatic DNL (20). Thus, our findings are in accordance with the concept that stimulation of DNL in the liver requires an excess of carbohydrates that need to be stored as fat in addition to insulin-induced activation of SREBP-1c.

In summary, our results showed that acutely raising plasma insulin levels in rats and humans increased SREBP-1 mRNA comparably in rat liver and rat and human adipose tissue, but increased post-translational activation of SREBP-1c only in rat liver, whereas decreasing it in adipose tissue. These differential effects of insulin on SREBP-1c activation in liver and adipose tissue were associated with changes in the opposite direction of INSIG proteins. Thus, the insulin-induced activation of SREBP-1c in the liver was accompanied by a decrease in INSIG-1 and INSIG-2 proteins, whereas in adipose tissue, the insulin-induced decrease in SREBP-1c activation was accompanied by an increase in INSIG-1 and INSIG-2 proteins. These findings support the hypothesis that insulin stimulated activation of SREBP-1c in the liver, at least in part, by suppressing INSIG-1 and INSIG-2, whereas in adipose tissue, an increase in INSIG-1 and INSIG-2 prevented SREBP-1c activation.

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