Role of hepatic STAT3 in brain-insulin action on hepatic glucose production

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Summary

STAT3 regulates glucose homeostasis by suppressing the expression of gluconeogenic genes in the liver. The mechanism by which hepatic STAT3 is regulated by nutritional or hormonal status has remained unknown, however. Here, we show that an increase in the plasma insulin concentration, achieved either by glucose administration or by intravenous insulin infusion, stimulates tyrosine phosphorylation of STAT3 in the liver. This effect of insulin was mediated by the hormone’s effects in the brain, and the increase in hepatic IL-6 induced by the brain-insulin action is essential for the activation of STAT3. The inhibition of hepatic glucose production and of expression of gluconeogenic genes induced by intracerebral ventricular insulin infusion was impaired in mice with liver-specific STAT3 deficiency or in mice with IL-6 deficiency. These results thus indicate that IL-6-STAT3 signaling in the liver contributes to insulin action in the brain, leading to the suppression of hepatic glucose production.

Introduction

An increase in glucose production in the liver is largely responsible for the hyperglycemia in individuals with type 2 diabetes. Insulin controls hepatic glucose production through regulation of both glycogen metabolism and gluconeogenesis, with regulation of the latter being achieved predominantly at the level of expression of the genes for gluconeogenic enzymes including those for phosphoenolpyruvate carboxykinase (PCK1) and for glucose-6-phosphatase (G6PC; Radziuk and Pye, 2001). Insulin suppresses the expression of such gluconeogenic genes through activation of the insulin receptor (IR) and downstream signaling by phosphoinositide 3-kinase (PI3K) in the liver (Michael et al., 2000; Miyake et al., 2002). The transcription factor FoxO1 and the transcriptional coactivator CREB binding protein appear to be involved in insulin regulation of gluconeogenic gene expression in the liver (Nakae et al., 2002; Zhou et al., 2004).

Apart from the direct activation of the insulin receptor signaling in the liver, recent evidence has revealed that insulin action in the brain also contributes to the suppression of hepatic glucose production (Obici et al., 2002; Pocai et al., 2005a, 2005b). Intracerebral ventricular (ICV) infusion of insulin results in the inhibition of glucose production as well as of the expression of gluconeogenic genes in the liver (Obici et al., 2002; Pocai et al., 2005a). Moreover, ICV infusion of diazoxide, an opener of ATP-sensitive potassium (KATP) channel, mimicked this effect of insulin (Obici et al., 2002), and either ICV infusion of sulfonylureas, which selectively closes KATP channel, or the disruption of the gene for SUR1, a component of KATP channel expressed in the brain, prevented the insulin’s action in the brain (Pocai et al., 2005b). These results suggest that the brain-insulin action leading to the suppression of hepatic glucose production is mediated by KATP channel though the molecular effector in the liver responsible for this effect of insulin has remained unknown.

We recently demonstrated that mice lacking STAT3 specifically in the liver displayed insulin resistance associated with an increase in glucose production and the expression of gluconeogenic genes in the liver and forced expression of active STAT3 suppressed gluconeogenic genes both in cultured hepatocytes and in mouse liver (Inoue et al., 2004). Although these
findings indicate the contribution of hepatic STAT3 in the control of gluconeogenic genes in the liver, the mechanism by which the transcription factor is regulated by nutritional or hormonal status has remained unclear. In this study, we provide evidence that STAT3 is activated by the insulin action in the brain and acts as a hepatic effector of the brain-insulin action that leads to the suppression of glucose production in the liver.

**Results**

**Insulin induces hepatic STAT3 phosphorylation in vivo**

We found that tyrosine phosphorylation of STAT3, which is essential for the activity of this transcription factor, was induced in the liver of mice in response to feeding. Tyrosine phosphorylation of STAT3 was thus not apparent in the liver of mice deprived of food for 16 hr but was manifest 3 hr after the onset of subsequent feeding, before decreasing again to prefeeding levels by 6 hr (Figure 1A), indicating that hepatic STAT3 signaling is activated at postprandial states. The phosphorylation of STAT3 in the liver was also transiently induced by intraperitoneal administration of glucose, with maximal stimulation being apparent after 2 to 3 hr (Figure 1B). Such feeding or glucose loading also induced tyrosine phosphorylation of IR as well as serine and threonine phosphorylation of Akt and FoxO1 (Figures 1A and 1B), respectively, both of which are downstream mediators of IR signaling (Nakae et al., 2002; Lizcano and Alessi, 2002). The phosphorylation of IR and its downstream targets was more rapid than was that of STAT3, however.

The circulating concentrations of both glucose and insulin are increased after feeding. We thus investigated the contribution of each of these factors to STAT3 phosphorylation in the liver. During a hyperglycemic clamp, in which the blood glucose concentration was clamped at 350 to 400 mg/dl by continuous infusion of glucose, phosphorylation of STAT3 was apparent in the liver within 2 hr (Figure 1C). However, additional infusion of somatostatin, which inhibits endogenous insulin secretion, markedly attenuated the increase in STAT3 phosphorylation, whereas infusion of insulin together with somatostatin prevented this effect of the latter hormone. The pattern of changes in the level of Akt phosphorylation in the liver was similar to that of the changes in STAT3 phosphorylation (Figure 1C). Moreover, during a euglycemic-hyperinsulinemic clamp, in which the blood glucose concentration was clamped at 100 to 120 mg/dl while insulin was continuously infused, phosphorylation of STAT3 in the liver was induced in a time-dependent manner (Figure 1D). These results suggested that the postprandial increase in the circulating insulin concentration is responsible for the activation of STAT3 in the liver.

**Glucose intolerance in STAT3 and PDK1 deficiency in the liver**

The difference between the time courses of STAT3 and IR phosphorylation after glucose administration or feeding (Figures 1A and 1B), together with the inability of insulin to induce STAT3 phosphorylation in cultured hepatocytes (Inoue et al., 2004), suggested that insulin does not elicit STAT3 phosphorylation in the liver through direct activation of hepatic IR-PI3K signaling but rather that the circulating insulin level is coupled to hepatic STAT3 signaling by an unidentified mechanism. 3'-phosphoinositide-dependent kinase-1 (PDK1) plays a key role in PI3K signaling and is thought to be essential for the metabolic actions of insulin (Lizcano and Alessi, 2002). Indeed, mice that lack PDK1 specifically in the liver (L-Pdk1KO mice) exhibited hyperinsulinemia (Table 1) as well as an exaggerated increase in blood glucose concentration during a glucose-challenge test.
Statistically significant differences are indicated as follows: BM, body mass; BG, blood glucose.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>BM (g)</th>
<th>BG (mg dl⁻¹)</th>
<th>Plasma Insulin (ng ml⁻¹)</th>
<th>ALT (Karmen Unit)</th>
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<tbody>
<tr>
<td>C57/BL6</td>
<td>12</td>
<td>26.9 ± 0.4</td>
<td>157.5 ± 6.0</td>
<td>1.09 ± 0.20</td>
<td>15.5 ± 1.0</td>
</tr>
<tr>
<td>IL-6KO</td>
<td>8</td>
<td>26.1 ± 0.5</td>
<td>149.4 ± 6.0</td>
<td>1.32 ± 0.19</td>
<td>12.8 ± 0.4</td>
</tr>
<tr>
<td>Stat3-flox/flox</td>
<td>5</td>
<td>26.0 ± 0.6</td>
<td>140.6 ± 7.2</td>
<td>1.15 ± 0.21</td>
<td>16.2 ± 1.3</td>
</tr>
<tr>
<td>L-ST3KO</td>
<td>9</td>
<td>26.6 ± 0.5</td>
<td>126.2 ± 4.0</td>
<td>1.34 ± 0.13</td>
<td>14.6 ± 1.1</td>
</tr>
<tr>
<td>Pdk1-flox/flox</td>
<td>7</td>
<td>25.0 ± 1.2</td>
<td>120.4 ± 4.3</td>
<td>1.19 ± 0.25</td>
<td>23.2 ± 2.3</td>
</tr>
<tr>
<td>L-Pdk1KO</td>
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<td>27.3 ± 0.7</td>
<td>145.7 ± 3.7</td>
<td>10.42 ± 1.99*</td>
<td>21.9 ± 1.3</td>
</tr>
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<td>Pdk1-flox/flox; Stat3-flox/flox</td>
<td>15</td>
<td>25.9 ± 0.7</td>
<td>130.0 ± 4.6</td>
<td>1.32 ± 0.23</td>
<td>16.6 ± 1.8</td>
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<td>L-DKO</td>
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<td>143.3 ± 8.8</td>
<td>7.32 ± 1.48</td>
<td>25.1 ± 2.9</td>
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<tr>
<td>Insr-flox/flox</td>
<td>5</td>
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<td>128.6 ± 5.3</td>
<td>3.72 ± 0.71</td>
<td>16.0 ± 3.9</td>
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<td>NIKKO</td>
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<td>28.5 ± 1.2</td>
<td>139.3 ± 5.8</td>
<td>5.50 ± 1.44</td>
<td>15.4 ± 2.3</td>
</tr>
</tbody>
</table>

BM, body mass; BG, blood glucose. Statistically significant differences are indicated as follows:

* p < 0.05 versus Stat3-flox/flox, L-ST3KO, Pdk1-flox/flox, and Pdk1-flox/flox; Stat3-flox/flox.

IL-6 activates STAT3 phosphorylation, IL-6 activates STAT3 and inhibits the expression of gluconeogenic genes in cultured hepatocytes (Christ et al., 2000; Inoue et al., 2004), and transplantation of a tumor that produces this cytokine into mice resulted in inhibition of G6pc expression in the liver (Metzger et al., 1997). We thus investigated whether IL-6 is involved in phosphorylation of hepatic STAT3 induced by insulin action in the brain. Administration of antibodies to IL-6 prevented phosphorylation of STAT3 in the liver induced by a bolus injection of IL-6 (Figure 4A), indicating that these antibodies neutralize circulating IL-6 in mice. Administration of these neutralizing antibodies to IL-6 also prevented the induction of STAT3 phosphorylation in the liver either by a euglycemic-hyperinsulinemic clamp (Figure 4B) or by the IOV insulin infusion (Figure 4C). Moreover, phosphorylation of STAT3 in the liver induced by the IOV insulin infusion was inhibited in mice that lack IL-6 (IL-6KO mice; Figure 4D). These results indicate that IL-6 is essential for phosphorylation of hepatic STAT3 induced by the brain-insulin action. Inhibition of hepatic glucose production in response to the IOV insulin infusion was
attenuated in IL-6KO mice (Figure 4E), suggesting that IL-6 is involved in the brain-insulin action leading to the hepatic glucose production. Moreover, inhibition of hepatic glucose production assessed by a euglycemic hyperinsulinemic clamp was also attenuated in IL-6KO mice (Figure 4F), which is consistent with the notion that IL-6 contributes to the suppression of hepatic glucose production. Although IL-6KO mice have been shown to develop age-related obesity, the animals younger than 6 months old were not obese (Wallenius et al., 2002). Body mass of IL-6KO mice used in this study (2 to 3 months old) did not differ from that of control mice (Table 1), suggesting that the metabolic phenotypes of these animals observed in the present study are not likely attributable to secondary effects induced by obesity.

In response to intraperitoneal administration of lipopolysaccharide (LPS), known to induce various cytokines in living animals, the mRNA abundance of IL-6 as assessed with RT-PCR analysis was markedly increased in the liver of wild-type mice, whereas this treatment did not induce the signal in the liver of IL-6KO mice (Figure 5A). After the ICV insulin infusion, the mRNA abundance of IL-6 in wild-type mice was two times greater than that in mice after the ICV vehicle infusion, indicating that insulin action in the brain stimulates IL-6 production in the liver. In situ hybridization analysis revealed that spindle-shaped IL-6 mRNA-positive cells scattered in the liver lobules were induced in wild-type mice, but not in IL-6KO mice, in response to LPS treatment (Figure 5B). The positive cells were located...
adjacent to larger-sized cuboidal-shaped hepatocytes. In the liver after the ICV insulin infusion in wild-type mice, similar spindle-shaped IL-6 mRNA-positive cells were observed, though the number and the signal intensity were smaller than those in the liver of LPS-treated wild-type mice. Whereas the plasma concentration of IL-6 was below the detection limit (3 pg ml$^{-1}$) after the ICV infusion of insulin or vehicle in wild-type mice, the hepatic content of IL-6 after the ICV insulin infusion was higher than that after the ICV vehicle infusion (3.07 ± 0.96 versus <0.94 pg per milligram of protein; means ± SEM; n = 11 and 6, respectively). These results together with a finding that phosphorylation of STAT3 was not detected in organs other than the liver including skeletal muscle and adipose tissue after the ICV infusion of insulin (data not shown) suggest that IL-6 acts as a paracrine or autocrine factor.

**Discussion**

We previously showed that STAT3 regulates the expression of gluconeogenic genes and thereby controls glucose production...
in the liver (Inoue et al., 2004). Our present study revealed that hepatic STAT3 is activated by insulin action in the brain at post-prandial states. This notion was supported by the inhibition of hepatic STAT3 phosphorylation in NIRKO mice after glucose administration and by the induction of STAT3 phosphorylation in response to ICV infusion of insulin in normal mice. Moreover, the suppression of glucose production induced by ICV infusion of insulin was attenuated in mice lacking STAT3 specifically in the liver. These results indicate that STAT3 is a hepatic effector of the brain-insulin action that leads to the inhibition of hepatic glucose production. Phosphorylation of STAT3 in the liver of NIRKO mice was markedly but not completely inhibited after glucose administration, suggesting that a mechanism independent of the brain-insulin action also contributes to postprandial activation of STAT3 in the liver. Such mechanisms remain to be elucidated.

Consistent with a previous observation (Pocai et al., 2005a), the suppression of glucose production in response to the ICV infusion of insulin was associated with the decrease in the expression of gluconeogenic genes including Pck1 and G6pc. In L-ST3KO mice, both of these effects of insulin were attenuated, suggesting that the mechanism independent of the brain-insulin action also contributes to postprandial activation of STAT3 in the liver. Such mechanisms remain to be elucidated.

Figure 4. IL-6 mediates suppression of hepatic glucose production induced by insulin action in the brain.

A) C57/BL6 mice were injected intraperitoneally first with antibodies to IL-6 or control immunoglobulin G (IgG) and then, after 5 days, with IL-6 (100 ng) or vehicle. Phosphorylation of hepatic STAT3 was examined by immunoblot analysis 30 min after IL-6 injection.

B) Phosphorylation of STAT3 in the liver 2 hr after the initiation of a euglycemic-hyperinsulinemic clamp (+) or infusion of vehicle (−) in C57/BL6 mice injected intraperitoneally with neutralizing antibodies to IL-6 or with control IgG.

C and D) Phosphorylation of STAT3 in the liver 3 hr after the initiation of ICV infusion of insulin (or vehicle) either in C57/BL6 mice that had been administered antibodies to IL-6 or control IgG (C) or in IL-6KO and C57/BL6 mice (Cont) (D).

E) Inhibition of hepatic glucose production after a pancreatic insulin clamp with or without ICV infusion of insulin in IL-6KO and C57/BL6 mice (Cont).

F) The absolute values (left) and percent inhibition (right) of hepatic glucose production assessed with the use of a euglycemic-hyperinsulinemic clamp in IL-6KO and control C57/BL6 mice.

Data in (E) and (F) are means ± SEM (n = 4); *p < 0.05 (ANOVA).
to be increased in obese or insulin-resistant individuals (Pickup et al., 1997; Kern et al., 2001), and continuous intravenous infusion of substantial amounts of IL-6 (500 ng/hr) results in insulin resistance in mice (Kim et al., 2004), suggesting that this cytokine contributes to the development of insulin resistance. However, the plasma concentration of IL-6 after continuous intravenous infusion of IL-6 at a rate similar to that shown to induce insulin resistance in mice (400 ng/kg/min) was markedly higher than that after the ICV infusion of insulin (2450 ± 384 pg/ml versus <3 pg/ml; means ± SEM; n = 3). It is thus possible that IL-6 possesses bilateral functions in the regulation of glucose homeostasis, a pathophysiological function and a normal physiological function. It is likely that, although persistent and pronounced systemic increases in the concentration of IL-6 might trigger insulin resistance, transient and smaller local increases in the amount of this cytokine contribute to normal glucose homeostasis.

In conclusion, insulin proficiently regulates hepatic glucose production by two different mechanisms, activation of PI3K signaling through its direct effect in the liver and activation of IL-6/STAT3 signaling thorough the effect in the brain. Each signaling pathway is evoked in a different time course at postprandial states and appears to be important to maintain normal glucose homeostasis in the liver. Given that dysregulation of hepatic glucose production is an important pathophysiological feature of type 2 diabetes (Taylor, 1999), the mechanism uncovered in this study may serve as a therapeutic target for this increasingly prevalent condition.

Experimental procedures

Generation of mice with a floxed Pdk1 allele (Pdk1-flox mice)
The targeting vector was designed to delete exons 3 and 4 of the PDK1 gene. A LoxP sequence was inserted into the Hind III site of intron 4, and a
pMC1-neo cassette (Shibata et al., 1996) flanked by LoxP sequences was then inserted into the NdeI site of intron 2. A pMC1-DTA cassette (Shibata et al., 1996) was incorporated at the 5′ end of the region of homology. J1 embryonic stem (ES) cells were transfected with the targeting vector and then subjected to selection with G418. ES cell clones that had undergone homologous recombination with the targeting vector were identified by Southern blot analysis. The LoxP-flanked neo cassette was removed by transient transfection of the cells with a plasmid encoding Cre recombinase. Chimeric mice were obtained by injecting J1 cells that had undergone homologous recombination and removal of the neo cassette into C57BL/6 blastocysts. Male chimeras were then mated with C57BL/6 females to obtain mice with a floxed Pdk1 allele.

Other animals
C57/BL6 mice were studied unless indicated otherwise. All genetically engineered and C57/BL6 mice were male and 2 to 3 months of age. We generated IL-6KO mice by intercrossing IL-6−/− animals with a genetic background of C57/BL6 (Jackson Laboratory); C57/BL6 mice were used as controls. We generated NIRKO mice, which are homozygous for a floxed allele of the IR gene (Insr) and harbor a transgene for Cre recombinase under the control of the Nestin gene promoter, as described (Bruning et al., 2000). We generated L-ST3KO mice, L-Pdk1KO mice, and L-DKO mice, which harbor a transgene for Cre recombinase under the control of the albumin gene promoter and are homozygous for a floxed allele of Stat3, Pdk1, or both Pdk1 and Stat3, respectively, by crossing Alb-Cre mice (Yakar et al., 1999) with Stat3−/− mice (Takeda et al., 1998), Pdk1−/− mice, and Stat3−/−Pdk1−/− mice, respectively. We used Insr−/−floxed/floxed littermates (for NIRKO mice), Stat3−/−floxed/floxed littermates (for L-ST3KO mice), and Stat3−/−floxed/floxed Pdk1−/−floxed/floxed littermates (for L-Pdk1KO and L-DKO mice) as controls. Although mice with liver-specific deficiency of Pdk1 were previously shown to develop a severe condition characterized by prominent edema and premature death (Mora et al., 2005), the L-Pdk1KO mice generated in the present study appeared normal, with the exception of glucose intolerance, until at least 6 months of age. The metabolic phenotypes of L-Pdk1KO and L-DKO mice will be described elsewhere. The study was approved by the animal experimentation committee of Kobe University Graduate School of Medicine.

Immunoblot analysis of STAT3 phosphorylation and of other signaling molecules
The effects of feeding or glucose administration on STAT3 phosphorylation in the liver were investigated in mice that had been deprived of food for 16 hr. The animals were supplied with normal chow or injected intraperitoneally with glucose (4 g/kg), and liver homogenates prepared at the indicated times thereafter were subjected to immunoblot analysis.

Infusion of insulin (ICV), measurement of hepatic IL-6 content, and neutralizing antibodies to IL-6
Insulin (porcine, Sigma-Aldrich) dissolved in artificial cerebroventricular fluid was infused at a rate of 30 ng/kg/hr through an ICV catheter that had been implanted into the third cerebral ventricle 7 to 10 days previously, as described (Asakawa et al., 2001). Homogenates of mouse liver prepared 3 hr after the initiation of ICV infusion of insulin were centrifuged to remove debris, and the resulting supernatants were analyzed with an enzyme-linked immunosorbent assay kit for mouse IL-6 (R&D Systems). Liver extracts of IL-6KO mice supplemented with various amounts of mouse recombinant IL-6 were used as an assay standard. Neutralizing antibodies to IL-6 (MPS-20F3, R&D Systems) were injected intraperitoneally (0.1 mg per mouse) and their effects were evaluated 3 to 5 days later.

Glucose tolerance test and clamp analyses
A glucose tolerance test (2 g/kg of glucose loaded intraperitoneally) and a euglycemic-hyperinsulinemic clamp, in which blood glucose concentration was clamped at 100 to 120 mg/dl while insulin (2.5 mU/kg/min) was infused continuously, was performed as described (Shigeyoshi et al., 1997). Blood was sampled at time 0, and intravenous priming with [3-3H]glucose at a rate of 0.4 μCi/min was started after 1 hr. At 3 hr, the pancreatic insulin clamp, consisting of intravenous infusion of insulin (0.3 μl/kg/min), somatostatin (3 μg/kg/min), and glucagon, was begun and was maintained for 2 hr. The rate of glucose infusion was adjusted to maintain a blood glucose concentration of 100 to 120 mg/dl. Hepatic glucose production during the pancreatic insulin clamp was determined as described (Obici et al., 2002; Pocai et al., 2005a).

Hepatic gene expression
Hepatic gene expression 3 hr after intraperitoneal glucose administration (4 g/kg) or at the end of a pancreatic insulin clamp with or without ICV insulin infusion was evaluated by reverse transcription and polymerase chain reaction (RT-PCR) analysis, with 36B4 mRNA as the invariant control, as described (Miyake et al., 2002). The primers for G6pc were 5′-AGCTCTCGGAAA GTATCTCA-3′ and 5′-TCACCCCTGACCTTTTAGA-3′, for Il-6 were 5′-CCACGGCTTCTCCTACTTC-3′ and 5′-TGGAAGTGGTATCCTCCTGGA-3′, and those for Pck1 and 36B4 were as described (Miyake et al., 2002; Testigawara et al., 2005).

In situ hybridization
The liver was perfused for 24 hr at 4°C and placed in 0.1 M phosphate buffer (pH 7.4) with 20% sucrose for 4 hr. Antisense probes to mouse IL-6 cDNA (1-1793) was labeled with digoxigenin-UTP following standard protocols of cRNA synthesis. Liver sections (40 μm thick) were made by cryostat and processed for in situ hybridization as described previously (Shigeyoshi et al., 1997). Sections were processed for immunocytochemistry with the nuclear acid detection kit (Boehringer Mannheim, Germany) and visualized blue with nitroblue tetrazolium salt for 6 hr. Sense probes yielded no specific hybridization signals in the liver sections.

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