An essential role of the JIP1 scaffold protein for JNK activation in adipose tissue

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The c-Jun NH2-terminal kinase (JNK) is activated during obesity. One consequence of obesity is that JNK phosphorylates the adapter protein insulin receptor substrate 1 (IRS-1) on Ser 307 and inhibits signaling by the insulin receptor. JNK can therefore cause peripheral insulin resistance during obesity and may contribute to the development of type 2 diabetes. Here we report that the JNK-interacting protein 1 (JIP1) scaffold protein, which binds components of the JNK signaling module, is essential for JNK activation in the adipose tissue of obese mice. These data identify JIP1 as a novel molecular target for therapeutic intervention in the development of obesity.

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The prevalence of obesity and diabetes in the USA has increased dramatically in recent years, with almost 20% of adults classified as obese and >7% having type 2 diabetes [Mokdad et al. 2001]. The molecular mechanisms by which obesity leads to peripheral insulin resistance, impaired pancreatic β-cell function, and, eventually, diabetes are poorly understood. Recent studies, particularly those of mice deficient for key signal transduction molecules, are beginning to provide insight and potential models for studying the role of obesity in the development of type 2 diabetes. Such studies have implicated the c-Jun NH2-terminal kinase (JNK) signaling pathway in diabetes [Bennett et al. 2003; Manning and Davis 2003]. JNK can inhibit insulin signaling by phosphorylating the adapter protein insulin receptor substrate 1 (IRS-1) on Ser 307 [Aguirre et al. 2000, 2002; Lee et al. 2003] and is strongly activated by inflammatory cytokines and free fatty acids—molecules that can contribute to the development of type 2 diabetes [Boden 1997; Uysal et al. 1997]. Furthermore, Jnk1−/− mice exhibit decreased adiposity, significantly improved insulin sensitivity, and enhanced insulin receptor signaling capacity [Hirosumi et al. 2002].

Recent studies have established an important role for scaffold proteins in the regulation of mitogen-activated protein (MAP) kinases, including JNK [Morrison and Davis 2003]. Thus, the JNK-interacting protein 1 (JIP1) scaffold binds multiple components of the JNK signaling pathway, including mixed-lineage kinases, MAP kinase 7, and JNK [Whitmarsh et al. 1998]. JIP1 is highly expressed in the brain and in the β cells of the islets of Langerhans [Dickens et al. 1997; Bonny et al. 1998]. Studies of viable Jip1−/− mice provided no evidence of a nonredundant role of JIP1 in β cells, but JIP1-deficiency was found to suppress neurotoxicity and JNK activation in response to ischemia [Whitmarsh et al. 2001; Im et al. 2003]. Genetic evidence for a role of JIP1 in responses to other stimuli or in other adult tissues remains to be established.

It has been demonstrated that JIP1 is a candidate type 2 diabetes gene in humans [Wacher et al. 2000]. This observation suggests that JIP1 may be relevant to JNK regulation associated with insulin resistance and obesity. The purpose of this study was to test this hypothesis.

Results and Discussion

Jip1 mRNA is highly expressed in the brain [Fig. 1A]. However, we also found Jip1 mRNA in white adipose tissue, and a low level of Jip1 mRNA was observed in muscle [Fig. 1A]. No expression of Jip1 mRNA was detected in liver. The expression of JIP1 in adipose tissue was confirmed by immunoblot analysis [Fig. 1B].

To examine the role of JIP1 in adipose tissue, we investigated the effect of Jip1 gene disruption. We have previously reported that Jip1−/− mice are viable and fertile [Whitmarsh et al. 2001], and similar observations have been reported for an independently created line of Jip1−/− mice [Im et al. 2003]. In contrast, a third independently created JIP1 mutant mouse line was found to have a preimplantation defect that caused early embryonic lethality [Thompson et al. 2001]. It is most likely that the lethality observed in one of the three reported Jip1 gene disruption studies is related to the mouse strain background. The studies reported here were performed using viable Jip1−/− mice that were back-crossed onto the C57BL/6J strain.

No differences between the adipose tissue of wild-type and Jip1−/− mice were detected by analysis of histology or gross morphology [data not shown]. We therefore investigated whether differences in the adipose tissue response to hormonal challenge could be detected. Male mice were treated with insulin, and the epididymal fat pads were isolated. Cytoplasmic extracts were examined by immunoblot analysis. Increased tyrosine phosphorylation of the adapter protein IRS-1, a direct target of the insulin receptor tyrosine kinase, was observed in response to treatment of mice with insulin [Fig. 1C]. This tyrosine phosphorylation of IRS-1 was markedly increased in JIP1-deficient mice compared with wild-type mice [Fig. 1C]. Interestingly, the increased tyrosine phosphorylation correlated with reduced inhibitory phosphorylation of IRS-1 on the JNK site Ser 307 [Fig. 1C] and also correlated with increased insulin-induced phosphorylation of AKT [a downstream target of insulin signaling; Fig. 1D] in the adipose tissue of Jip1−/− mice. Together, these data provide biochemical evidence for increased insulin sensitivity in the adipose tissue of Jip1−/− mice in vivo.
JIP1-deficient mice are resistant to diet-induced obesity

The observation that insulin signaling is altered in the adipose tissue of Jip1−/− mice indicates that JIP1-deficient mice should exhibit metabolic defects. To test this hypothesis, we investigated the effect of feeding the mice a high-fat/high-caloric (HF) diet. After 16 wk, the Jip1−/− mice gained 40% less weight than wild-type mice (P < 0.05). The difference in weight gain was associated with defects in the accumulation of adipose tissue in the Jip1−/− mice. For example, the epididymal fat pad mass of wild-type and Jip1−/− mice fed a standard diet was similar, but the Jip1−/− mice exhibited markedly decreased fat mass compared with wild-type mice when fed the HF diet (Fig. 2A). The difference in fat mass was primarily accounted for by cell size. Sections of adipose tissue from HF-diet-fed Jip1−/− mice indicated the presence of smaller adipocytes relative to wild-type mice (Fig. 2B). Decreased fat accumulation was also detected in the liver of Jip1−/− mice (Fig. 2C). The liver of the HF-diet-fed wild-type mice was paler in color and vacuolized because of an accumulation of fat droplets compared with Jip1−/− mice. The decreased adiposity of Jip1−/− mice may be caused by altered lipid metabolism, food intake, or core body temperature. However, no significant differences between Jip1−/− and wild-type mice were observed in plasma glycerol and free fatty acid concentrations, food intake, or core body temperature [data not shown].

Adipose tissue secretes several bioactive substances known as adipocytokines (Matsuzawa et al. 2003). Dysregulated production of adipocytokines, including leptin, tumor necrosis factor, and adiponectin/ACRP30, has been implicated in the pathophysiology of obesity-related insulin resistance (Matsuzawa et al. 1999). The reduction of adipocytokine mass in HF-diet-fed Jip1−/− mice compared with wild-type mice was associated with reduced concentrations of plasma leptin (Fig. 2D) and markedly increased concentrations of plasma adiponectin/ACRP30 (Fig. 2E). It has been established that adiponectin/ACRP30 can increase fatty acid oxidation and improve hepatic insulin sensitivity [Berg et al. 2001; Yamauchi et al. 2001]. Thus, the increased plasma adiponectin/ACRP30 concentrations in Jip1−/− mice may contribute to altered systemic insulin sensitivity and increased fat catabolism.

Diet-induced insulin resistance is reduced in JIP1-deficient mice

Increased adiposity has been implicated in the etiology of insulin resistance. We therefore investigated glucose homeostasis in wild-type and Jip1−/− mice. Fasting plasma insulin levels increased when these mice were...
fed an HF diet. However, the kinetics and severity of the hyperinsulinemia were reduced in $jip1^{-/-}$ mice (Fig. 3A). The increased plasma insulin in the HF-diet-fed mice was associated with an increase in the size of the islets in both wild-type and $jip1^{-/-}$ mice. However, consistent with the reduced severity of the hyperinsulinemia, the mean size of islets from HF-diet-fed $jip1^{-/-}$ mice was 30% less than wild-type mice (data not shown). Histological analysis did not detect obvious signs of β-cell degranulation in pancreas sections stained for insulin (data not shown). The HF-diet-fed wild-type and $jip1^{-/-}$ mice showed a similar amount of acute insulin secretion following a glucose challenge (Fig. 3C), and these mice developed mild fasting hyperglycemia (Fig. 3B). Glucose-tolerance tests of lean wild-type and $jip1^{-/-}$ mice performed by intraperitoneal injection of glucose demonstrated a similar rate of blood glucose clearance (data not shown). In contrast, HF-diet-fed wild-type mice were severely glucose intolerant and cleared blood glucose at a slower rate than HF-diet-fed $jip1^{-/-}$ mice (data not shown). We performed insulin-tolerance tests to examine the effect of insulin on blood glucose concentrations. Intraperitoneal injection of insulin caused reduced blood glucose concentrations in both wild-type and $jip1^{-/-}$ mice when fed a standard diet (Fig. 3D). As expected, HF-diet-fed wild-type mice showed marked insulin resistance in this assay. However, the HF-diet-fed $jip1^{-/-}$ mice showed increased insulin sensitivity compared with wild-type mice, as indicated by the increased rate of glucose clearing on insulin challenge (Fig. 3D). Together, these data demonstrate that JIP1 deficiency protected mice against the development of obesity and insulin resistance.

**JIP1 is required for obesity-induced JNK activation**

To investigate the role of JIP1 in obesity-induced insulin resistance, we examined JNK phosphorylation by immunoblot analysis and JNK activity using an in vitro kinase assay. Obesity caused increased JNK activation in the fat, muscle, and liver of wild-type mice (Fig. 4). Similarly, the HF diet caused increased JNK activation in the liver of $jip1^{-/-}$ mice, but no JNK activation in fat or muscle was detected (Fig. 4). The selective effect of JIP1 deficiency to attenuate JNK activation in fat and muscle, but not liver, correlates with the pattern of JIP1 expression in wild-type mice (Fig. 1A), indicating that JIP1 is essential for obesity-induced activation of JNK in fat and muscle. The mechanism of obesity-induced JNK activation in the liver of $jip1^{-/-}$ mice is unclear, but it is possible that a different member of the JIP group of scaffold proteins is required for hepatic JNK activation (Morrison and Davis 2003). Thus, the major JIP isoforms expressed in adipose tissue and liver are JIP1 and JIP4, respectively.

**JIP1 is required for obesity-induced phosphorylation of IRS-1 on Ser 307**

What molecular mechanisms might contribute to the protection against obesity and improved insulin sensitivity in $jip1^{-/-}$ mice? JNK can mediate an inhibitory feedback on insulin signaling by phosphorylation of IRS-1 on Ser 307 (Aguirre et al. 2000, 2002; Lee et al. 2003). Indeed, IRS-1 phosphorylation on Ser 307 was significantly increased in the fat, muscle, and liver of wild-type mice fed an HF diet (Fig. 5). However, increased Ser 307 phosphorylation was only detected in the liver of $jip1^{-/-}$ mice and was not observed in fat or muscle. These changes in IRS-1 phosphorylation on Ser 307 (Fig. 5) correlate with the level of JNK activation (Fig. 4) and JIP1 expression (Fig. 1A) in these tissues.
**JIP1 is essential for obesity-induced JNK activation in adipose tissue**

The results of this study demonstrate that the JIP1 scaffold protein is essential for JNK activation caused by obesity in adipose tissue and muscle, but not in liver. The defect in JNK activation in fat results in decreased inhibitory phosphorylation of IRS-1 on Ser 307 and consequently increased insulin sensitivity. The observation that JNK is activated in the liver of Jip1−/− mice demonstrates that the reduced JNK activation in fat and muscle is not a result of reduced obesity.

The absence of a defect in JNK activation in the liver of Jip1−/− mice may account for the incomplete protection against insulin resistance observed in insulin-tolerant mice maintained on a standard diet (L) and on an HF diet (O) for 16 wk. The expression of Tubulin, JNK, and phospho-JNK was examined by immunoblot analysis. JNK activity was measured in a kinase assay (KA) using c-Jun as the substrate and was quantitated by PhosphorImager analysis.

**Figure 4.** JIP1 is essential for obesity-induced JNK activation in adipose tissue. Extracts were prepared from the fat, muscle and liver of Jip1−/− and wild-type male mice maintained on a standard diet ([L] lean) and on an HF diet ([O] obese) for 16 wk. The expression of Tubulin, JNK, and phospho-JNK was examined by immunoblot analysis. JNK activity was measured in a kinase assay (KA) using c-Jun as the substrate and was quantitated by PhosphorImager analysis.

It is likely that the adipose tissue defects of Jip1−/− mice contribute to the metabolic phenotype of these animals. However, metabolic phenotypes usually involve complex interactions between multiple tissues. It is therefore likely that some of the metabolic phenotype of the Jip1−/− mice is accounted for by altered function of other tissues, including the hypothalamus and pancreatic β cells. Nevertheless, previous studies have failed to identify central nervous system or β-cell defects in non-stressed Jip1−/− mice, possibly as a result of complementation by the related gene Jip2 (Whitmarsh et al. 2001). Significantly, Jip2 is selectively expressed in a limited number of tissues, including neurons and β cells, whereas Jip1 is more broadly expressed in multiple tissues (Dickens et al. 1997; Yasuda et al. 1999). An important goal for further studies will therefore be the characterization of Jip2−/− mice and compound mutant Jip1−/− Jip2−/− mice.

Previous studies have demonstrated that the JIP1 scaffold protein is required for sustained JNK activation in neurons caused by ischemia but is not required for JNK activation caused by other stimuli (Whitmarsh et al. 2001; Im et al. 2003). Here we demonstrate that JIP1 is also essential for obesity-induced JNK activation in fat and muscle. This finding provides a new example of a MAP kinase signaling module that is regulated by a scaffold protein in vivo. JIP1-dependent JNK activation in fat contributes to the regulation of insulin sensitivity and adipose tissue mass. Drugs that inhibit JNK may be useful for the treatment of obesity and diabetes, but this approach may be limited by potential toxicity (Bennett et al. 2003; Manning and Davis 2003). The identification of an essential role for the JIP1 scaffold protein is therefore important because JIP1 represents a possible target for the design of therapeutic interventions in obesity. Inhibition of JIP1-dependent JNK activation is likely to provide greater therapeutic specificity than a general inhibitor of JNK activity.

In summary, we provide evidence for a role of the JIP1 scaffold protein in the regulation of adipose mass and JNK activation associated with obesity and insulin resistance.

**Materials and methods**

**Mice**

Jip1−/− mice (Whitmarsh et al. 2001) were back-crossed 10 generations to the C57Bl/6 strain (Jackson Laboratories) and were housed in a facility accredited by the American Association for Laboratory Animal Care (AAALAC). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts. Male mice (7 wk old) were fed an HF diet ad libitum (Diet F3282, Bio-Serv) or a standard diet for 16 wk. Their body mass was recorded weekly. Blood samples were collected from the tail vein after an overnight fast or

**Figure 5.** JIP1-deiciency prevents inhibitory phosphorylation of IRS-1 on Ser 307. IRS-1 expression and phosphorylation on Ser 307 in white adipose tissue, liver, and muscle of Jip1−/− and wild-type male mice maintained on a standard diet ([L] lean) and on an HF diet ([O] obese) for 16 wk was examined by immunoblot analysis.
from the retro-orbital sinus after 6, 12, and 16 wk on the diet. Blood glucose concentrations were measured by using a Dex-Glucometer (Bayer), and plasma insulin was measured by ELISA kit for rat insulin (Crystal Chem). Insulin-tolerance tests were performed on mice fed ad libitum by intraperitoneal administration of insulin (0.75 U/kg body weight) and measurement of blood glucose at 0, 15, 30, and 60 min after injection. Plasma leptin was determined using a mouse leptin ELISA kit (Crystal Chem). Adiponectin was measured with a mouse/rat adiponectin ELISA kit (B-Ridge International). Histology was performed using adipose tissue, pancreas, and liver fixed in 10% formalin for 24 h, dehydrated, and embedded in paraffin. Sections (5 μm) were cut and stained with hematoxylin and cosin.

Biochemical assays
Tissues were removed and rapidly frozen in liquid nitrogen. RNA was prepared (RNAeasy kit, Qiagen) and used as the template for PCR with a Titan one tube RT-PCR kit (Roche) using amplifiers for JIP1 (CTC GAGGCGGCACATCTGGC and AAGCACAAGTGGCAGGACGCG) and GAPDH (ACCAACGTCTCATGCATCACC and TCAACCCACCGTTT GCTGTA). JIP1 expression in adipose tissue was examined by immunoprecipitation with a monoclonal JIP1 antibody (Transduction Laboratories) and immunoblotting using a polyclonal JIP1 antibody (Yasuda et al. 1999). JNK expression and phosphorylation were examined by immunoblot analysis with antibodies to JNK (PharMingen) and phospho-JNK (Cell Signaling). JNK activity was measured in an in vitro kinase assay using c-Jun as the substrate (Whittmarsh and Davis 2001). Immunoblot analysis was also performed using antibodies purchased from Santa Cruz Biotechnology (anti-phosphotyrosine), Upstate Biotechnology (anti-IRS-1 and anti-pSer 307), and Cell Signaling (Akt and anti-pSer 473).

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