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Caroline Morel
University of Massachusetts Medical School

Et al.

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Requirement of JIP1-Mediated c-Jun N-Terminal Kinase Activation for Obesity-Induced Insulin Resistance

Caroline Morel, Claire L. Standen, Dae Young Jung, Susan Gray, Helena Ong, Richard A. Flavell, Jason K. Kim, and Roger J. Davis

Howard Hughes Medical Institute, Program in Molecular Medicine, Department of Medicine, Division of Endocrinology, Metabolism and Diabetes, University of Massachusetts Medical School, Worcester, Massachusetts 01605, and Howard Hughes Medical Institute and Department of Immunobiology, Yale University School of Medicine, New Haven, Connecticut 06520

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The c-Jun NH₂-terminal kinase (JNK) interacting protein 1 (JIP1) has been proposed to act as a scaffold protein that mediates JNK activation. However, recent studies have implicated JIP1 in multiple biochemical processes. Physiological roles of JIP1 that are related to the JNK scaffold function of JIP1 are therefore unclear. To test the role of JIP1 in JNK activation, we created mice with a germ line point mutation in the Jip1 gene (Thr¹⁰³ replaced with Ala) that selectively blocks JIP1-mediated JNK activation. These mutant mice exhibit a severe defect in JNK activation caused by feeding of a high-fat diet. The loss of JIP1-mediated JNK activation protected the mutant mice against obesity-induced insulin resistance. We conclude that JIP1-mediated JNK activation plays a critical role in metabolic stress regulation of the JNK signaling pathway.

Diet-induced obesity causes insulin resistance and metabolic syndrome, which can lead to β-cell dysfunction and type 2 diabetes (15). It is established that feeding mice a high-fat diet (HFD) causes activation of c-Jun NH₂-terminal kinase 1 (Jnk1) (10). Moreover, Jnk¹⁻/⁻ mice are protected against the effects of HFD-induced insulin resistance (10). Together, these observations indicate that JNK1 plays a critical role in the metabolic stress response. However, the mechanism that accounts for HFD-induced JNK1 activation is unclear. Recent studies have implicated the JIP1 scaffold protein in the JNK activation caused by metabolic stress (23, 39).

JIP1 can assemble a functional JNK activation module composed of a mitogen-activated protein kinase (MAPK) kinase kinase (a member of the mixed-lineage protein kinase [MLK] group), the MAPK kinase MKK7, and JNK (40, 42). This complex may be relevant to JNK activation caused by metabolic stress (23, 39). Indeed, MLK-deficient mice (14) and JIP1-deficient mice (13) exhibit defects in HFD-induced JNK activation and insulin resistance.

The protection of Jip¹⁻/⁻ mice against the effects of being fed an HFD may be mediated by loss of the JNK scaffold function of JIP1. However, JIP1 has also been reported to mediate other biochemical processes that would also be disrupted in Jip¹⁻/⁻ mice. For example, JIP1 interacts with AKT and has been implicated in the mechanism of AKT activation (8, 17, 18, 34). Moreover, JIP1 interacts with members of the Src and Abl tyrosine kinase families (4, 16, 24), the lipid phosphatase SHIP2 (44), the MAPK phosphatase MKP7 (43), β-amyloid precursor protein (20, 31), the small GTPase regulatory proteins Ras-GRF1, p190-RhoGEF, RalGDS, and Tiam1 (2, 8, 21), ankyrin G (35), molecular chaperones (35), and the low-density-lipoprotein-related receptors LRP1, LRP2, and LRP8 (7, 37). JIP1 also interacts with other scaffold proteins, including the insulin receptor substrate proteins IRS1 and IRS2 (35). Finally, JIP1 may act as an adapter protein for kinesin-mediated (11, 12, 16, 38, 42) and dynein-mediated (35) trafficking on microtubules. The JNK scaffold properties of JIP1 therefore represent only one of the possible biochemical functions of JIP1 that are disrupted in Jip¹⁻/⁻ mice.

The purpose of this study was to test the role of JIP1 as a JNK scaffold protein in the response of mice to being fed an HFD. Our approach was to examine the effect of a point mutation that selectively prevents JIP1-induced JNK activation. It is established that phosphorylation of JIP1 on Thr¹⁰³ is required for JIP1-mediated JNK activation by the MLK pathway (25). Consequently, the phosphorylation-defective Thr¹⁰³Ala JIP1 protein does not activate JNK (25). Here we describe the analysis of mice with a point mutation in the Jip1 gene that replaces the JIP1 phosphorylation site Thr¹⁰³ with Ala. We show that this mutation suppresses HFD-induced JNK activation and insulin resistance. These data demonstrate that JNK activation mediated by the JIP1 scaffold complex contributes to the response of mice to an HFD.

MATERIALS AND METHODS

Mice. Mice with a point mutation in JIP1 (Thr¹⁰³Ala) were constructed by homologous recombination in embryonic stem (ES) cells using standard methods (see Fig. 1). Briefly, a targeting vector was constructed (see Fig. 1). This targeting vector was designed to introduce point mutations in exon 3 of the Jip1 gene that create the Thr¹⁰³Ala mutation and also a novel XmaI restriction site (see Fig. 1). TC1 embryonic stem cells (strain 129svEv) were electroporated with this vector and selected with 200 µg/ml G418 (Invitrogen) and 2 µM ganciclovir (Syntex). Correctly targeted ES cell clones were identified by Southern blot analysis and were injected into C57BL/6J blastocysts to create chimeric mice that were bred...
FIG. 1. Creation of mice with a germ line knock-in mutation in the Jip1 gene. (A) Schematic illustration of the gene targeting strategy. A floxed NeoR cassette was inserted into intron 3 of the Jip1 gene by homologous recombination. Point mutations were introduced into exon 3. The NeoR cassette was excised using Cre recombinase. HindIII restriction sites are indicated (H). (B) The DNA sequence of the region of exon 3 of the Jip1 gene that surrounds codon 103 is presented. The mutated allele replaces codon 103, which encodes Thr (ACT) with Ala (GCC). Translationally silent mutations were also introduced to create an XmaI restriction site in the mutated Jip1 allele (Jip1TA). (C) Genomic DNA was prepared from wild-type (Jip1+/+), heterozygous (Jip1+/TA), and homozygous (Jip1TA/TA) mice. Genotype analysis was performed by PCR. (D) Extracts prepared from the cerebellum of Jip1+/+, Jip1+/+TA, and Jip1TA/TA mice were examined by immunoblot analysis by probing with antibodies to JIP1, JIP2, and β-tubulin. (E) The expression of Jip1 mRNA in white adipose tissue (WAT), brown adipose tissue (BAT), quadriceps muscle, and liver of C57BL/6J mice was measured by quantitative RT-PCR analysis of mRNA. The relative mRNA expression was calculated by normalization of the data to the amount of Gapdh mRNA in each sample (mean ± SE; n = 8).
to obtain germ line transmission of the mutated Jip1 allele. The floxed Neo<sup>c</sup> cassette was excised using Cre recombinase. The mice used in this study were backcrossed (10 generations) to the C57BL/6J strain (Jackson Laboratories). Male mice (8 to 10 weeks old) were fed a chow diet or an HFD. The mice were housed in a facility accredited by the American Association for Laboratory Animal Care (AALAC). The Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts approved all studies using animals.

**Genotyping.** The Jip<sup>TA</sup> allele was detected using two different assays. First, Southern blot analysis of HindIII-restricted genomic DNA was performed by probing with a 500-bp fragment of the Jip1 gene that was isolated by PCR using the primers 5′-CACATCTTGTTGTGCTCAATCCG-3′ and 5′-GTTCCTGGCCT CTGATACTGAACCC-3′. The Jip<sup>Wt</sup> and Jip<sup>TA</sup> alleles were detected at 11.9-kb and 6.6-kb bands, respectively. No significant difference between Jip<sup>Wt</sup> and Jip<sup>TA</sup> mice was detected ($P > 0.05$). Second, a PCR assay was employed using the primers 5′-CCAAGTTGTGTGTGGAGAGCTTG-3′ and 5′-GCAGATGTTG GAAGAAGCAC-3′. The Jip<sup>Wt</sup> and Jip<sup>TA</sup> alleles were amplified to yield 400- and 450-bp DNA fragments, respectively (see Fig. 1C).

**RNA analysis.** The expression of mRNA was examined by quantitative PCR analysis using a 7500 Fast real-time PCR machine. TaqMan assays were used to quantitate Foxo1 (Mm00490672_m1), G6p (Mm00839363_m1), Pck1 (Mm00440636_m1), Jip1 (Mm00501836_g1), Pgc1α (Mm00447183_m1), Gck...
germ line mutation in exon 3 of the JIP1-mediated JNK activation (25). We created mice with matings resulted in wild-type, heterozygous, and homozygous mutant mice in the expected Mendelian ratios. The homozygous mutant (JIP1<sup>TA</sup>) mice were viable and fertile. Comparison of wild-type (JIP1<sup>WT</sup>) and JIP1<sup>TA</sup> mice demonstrated that the Thr103Ala mutation did not affect the amount of JIP1 protein detected by immunoblot analysis (Fig. 1).

**HFD-induced JNK1 activation in JIP1<sup>WT</sup> and JIP1<sup>TA</sup> mice.** We examined the effect of feeding an HFD to JIP1<sup>WT</sup> and JIP1<sup>TA</sup> mice. The Thr103Ala point mutation caused no change in HFD-induced weight gain (Fig. 2A). Measurement of whole-body lean and fat mass by <sup>1</sup>H-MRS confirmed that the...
wild-type and mutant mice exhibited HFD-induced obesity (Fig. 2B). Importantly, the HFD-induced weight gain by JIP1WT and JIP1TA mice indicates that these mice mount responses to a similar stress-related insult when fed an HFD.

Measurement of JNK1 activity in epididymal adipose tissue demonstrated that HFD-induced JNK1 activation in JIP1 WT mice was markedly suppressed in JIP1 TA mice (Fig. 2C). To test whether the reduced HFD-induced JNK1 activation was physiologically significant, we examined the phosphorylation of the JNK1 substrate IRS1. We found that JNK1-mediated phosphorylation of IRS1 on Ser307 was reduced in HFD-fed JIP1TA mice compared with that in HFD-fed JIP1 WT mice (Fig. 2E). These observations demonstrate that JNK activation caused by feeding an HFD is mediated by the JIP1 scaffold protein pathway.

JIP1TA mice exhibit increased insulin sensitivity. The hyperglycemia and hyperinsulinemia found in HFD-fed JIP1 WT mice were significantly reduced in HFD-fed JIP1 TA mice (Fig. 2D and F). We performed glucose tolerance tests (GTT) to compare the responses of JIP1WT and JIP1TA mice to a glucose challenge. We found that feeding an HFD caused similar glucose intolerance in JIP1WT and JIP1TA mice (Fig. 3A). This glucose intolerance was caused in part by decreased glucose-induced insulin release. Indeed, no significant differences in glucose-induced insulin release between HFD-fed JIP1WT mice and HFD-fed JIP1TA mice were detected (Fig. 3B). In contrast, insulin tolerance tests (ITT) demonstrated that insu...
lin-induced disposal of blood glucose was significantly increased in HFD-fed JIP1TA mice over that in HFD-fed JIP1WT mice (Fig. 3C). These data suggest that the Thr103Ala mutation in JIP1 increased insulin sensitivity in HFD-fed mice.

To confirm the initial observation that JIP1TA mice are more insulin sensitive, we conducted a hyperinsulinemic-euglycemic clamp study in conscious mice. The steady-state glucose infusion rates during the clamp were significantly greater in JIP1TA mice than in JIP1WT mice (Fig. 4A), indicating that JIP1TA mice were more insulin sensitive than JIP1WT mice. Indeed,
hepatic glucose production (HGP) during the clamp was significantly decreased in JIP1TA mice compared with that in JIP1WT mice (Fig. 4B). Basal HGP was not significantly altered between the groups. These data demonstrate that JIP1TA mice exhibit increased hepatic insulin action compared with that of JIP1WT mice (Fig. 4C). Feeding an HFD caused hepatic insulin resistance, but HFD-fed JIP1TA mice exhibited increased insulin sensitivity compared with HFD-fed JIP1WT mice (Fig. 4C). No significant differences between JIP1WT mice and JIP1TA mice in insulin-stimulated whole-body glucose turnover and glucose metabolic fluxes (glycolysis and glycogen plus lipid synthesis) were detected (Fig. 4D to F). Together, these data demonstrate that JIP1TA mice are more insulin sensitive than JIP1WT mice.

To obtain biochemical evidence for insulin sensitivity, we examined insulin-stimulated AKT activation in adipose tissue of JIP1WT and JIP1TA mice (Fig. 5). Insulin treatment of chow-fed JIP1WT and JIP1TA mice caused increased AKT activation. Feeding an HFD suppressed insulin-stimulated AKT activation in JIP1WT mice (Fig. 5). In contrast, the HFD did not suppress insulin-stimulated AKT activation in JIP1TA mice (Fig. 5). These data support the conclusion that JIP1TA mice exhibit protection against HFD-induced hepatic steatosis.

JIP1TA mice are protected against HFD-induced hepatic steatosis. Feeding an HFD to JIP1WT mice caused morphological changes in the liver, including an increase in mass and accumulation of triglyceride (Fig. 6A to C). These changes were markedly suppressed in JIP1TA mice (Fig. 6A to C). The reduced accumulation of hepatic lipid in HFD-fed JIP1TA mice compared with that in HFD-fed JIP1WT mice was associated with decreased expression of lipogenic transcription factors (C/ebpα, C/ebpβ, and Srebp1c) (Fig. 6E to G). These observations are consistent with a reduction in triglyceride accumulation in the liver (Fig. 6A and C) and blood (Fig. 6D). Together, these data demonstrate that JIP1TA mice exhibit protection against HFD-induced hepatic steatosis.

We examined JIP1WT and JIP1TA mice in metabolic cages to investigate possible mechanisms that might contribute to the reduced steatosis of JIP1TA mice compared with that in JIP1WT mice (Fig. 7). The daily food intake by chow-fed JIP1WT and JIP1TA mice was similar, but a moderate decrease in food intake by HFD-fed JIP1TA mice compared with that by HFD-fed JIP1WT mice was detected (Fig. 7B). No significant differences between JIP1WT and JIP1TA mice in O2 consumption, CO2 production, energy expenditure, and physical activity were detected (Fig. 7C to F). However, the respiratory exchange quotient (RQ) ([VCO2]/[VO2]) of JIP1TA mice was significantly decreased compared with that of JIP1WT mice (Fig. 7G). These data indicate that fat oxidation by JIP1TA mice is increased over that by JIP1WT mice. Together, the decreased food intake and increased fat oxidation may contribute to the suppression of hepatic steatosis in JIP1TA mice compared with JIP1WT mice.

**Mechanism of protection against HFD-induced hepatic insulin resistance in JIP1TA mice.** The protection of adipose tissue in JIP1TA mice against HFD-induced insulin resistance (Fig. 5) may result from reduced HFD-induced JNK1 activation (Fig. 2C and E). This conclusion is consistent with the finding that adipose tissue-specific JNK1 activation contributes to the suppression of hepatic steatosis in JIP1TA mice compared with JIP1WT mice.
HFD-fed JIP1\(^{TA}\) mice against HFD-induced hepatic insulin resistance is unclear (Fig. 4C).

Two observations complicate the interpretation of the effect of the Thr103Ala JIP1 mutation on the response of the liver to feeding of an HFD. First, liver-specific JNK1 knockout mice are not protected against HFD-induced hepatic insulin resistance (29). The effects of JIP1 on the liver may therefore not be mediated by hepatic JNK1. Second, JIP1 is expressed by white adipose tissue and brown adipose tissue, but JIP1 is expressed only at very low levels in the liver (Fig. 1E). It is therefore unclear that the effects of JIP1 on the liver are mediated by a cell autonomous mechanism.

We have previously reported that adipose JNK1 can regulate hepatic insulin sensitivity by a mechanism that involves JNK1-dependent expression of interleukin 6 (IL-6) by adipose tissue and subsequent IL-6-induced SOCS3-mediated inhibition of hepatic insulin receptor signaling (30). This mechanism could account for JIP1-mediated regulation of hepatic insulin resistance. Indeed, the HFD-induced activation of JNK1 in adipose tissue (Fig. 2C) and the increase in the blood IL-6 concentration (Fig. 8A) observed in JIP1\(^{WT}\) mice were markedly reduced in JIP1\(^{TA}\) mice. Control studies demonstrated that the Thr103Ala JIP1 mutation caused no change in the blood concentration of tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)) (Fig. 8B). However, the blood concentrations of leptin and resistin were decreased in HFD-fed JIP1\(^{TA}\) mice (Fig. 9A and B) and the expression of adiponectin was increased (Fig. 9C) compared with those in HFD-fed JIP1\(^{WT}\) mice. These changes in blood adipokines may also contribute to the hepatic insulin sensitivity of HFD-fed JIP1\(^{TA}\) mice.

To test whether suppression of HFD-induced expression of IL-6 in the blood of JIP1\(^{TA}\) mice contributes to the metabolic phenotype of JIP1\(^{TA}\) mice, we examined the expression of IL-6 target genes in the liver, including suppressor of cytokine signaling 3 (Socs3) (36) and very low density lipoprotein receptor (Vldlr) (9). Feeding an HFD to JIP1\(^{WT}\) mice but not JIP1\(^{TA}\) mice caused increased expression of Socs3 (Fig. 8C and E) and Vldlr (Fig. 8D). These data demonstrate that JIP1\(^{TA}\) mice exhibit a marked defect in HFD-induced activation of the hepatic IL-6 signal transduction pathway in vivo. Since IL-6-induced Socs3 expression can cause hepatic insulin resistance (5, 6, 26, 30, 32, 33), reduced expression of Socs3 in liver may contribute to the increased hepatic insulin sensitivity of HFD-fed JIP1\(^{TA}\) mice.

**DISCUSSION**

The JIP1 protein is implicated in HFD-induced JNK activation (23, 39). Indeed, Jip1\(^{-/-}\) mice exhibit a major defect in HFD-induced JNK activation and insulin resistance (13), and it is established that the JIP1 protein can assemble a functional JNK signaling module (40, 42). However, JIP1 appears to be a multifunctional protein that may have additional biological activities (23). Thus, JIP1 may act as an adapter protein for kinesin-mediated (11, 12, 16, 38, 42) and dynein-mediated (35) trafficking on microtubules. It is not known whether this proposed transport function of JIP1 might contribute to HFD-induced insulin resistance. However, other proposed functions of JIP1, including interactions with AKT and insulin receptor substrate proteins (8, 17, 18, 34, 35), may contribute to HFD-induced insulin resistance. The mechanism that accounts for the effect of JIP1 deficiency to protect mice against HFD-induced insulin resistance is therefore unclear (13).

To test whether JIP1-mediated JNK activation is required for HFD-induced insulin resistance, we examined the effect of a point mutation in JIP1 that selectively prevents JIP1-induced JNK activation. It is established that phosphorylation of JIP1 on Thr\(^{103}\) is required for JIP1-mediated JNK activation by the MLK pathway (25). This requirement of Thr\(^{103}\) phosphorylation reflects the dynamics of JIP1 function in the activation of MLK isoforms. JNK phosphorylation of JIP1 on Thr\(^{103}\) causes the dissociation of bound MLK isoforms that subsequently become activated by leucine zipper-mediated dimerization and...
activation. Recent studies indicate that hepatic JNK1 (29) and insulin resistance regulation caused by JIP1-mediated JNK1 may functionally compensate defects in the brains of JIP1TA mice (23). The presence of these JIP family proteins (JIP3, and JIP4) of the JIP protein family that can form heterocomplexes (1, 27), a tissue that expresses four members (JIP1, JIP2, JIP3, JIP4) of the JIP family, may not be fully compensated by other JIP isoforms in the brains of JIP1/TA mice but not JIP1TA mice. A JNK1-independent action of JIP1 may contribute to obesity regulation. Alternatively, the effects of JIP1 on obesity may be mediated by a tissue-specific role of JNK1. It is established that JNK1-deficient mice exhibit a defect in obesity regulation (13). In contrast, JIP1 WT mice and JIP1TA mice become similar obese when fed an HFD (Fig. 2A). These data demonstrate that a loss of JIP1-mediated JNK1 activation (in JIP1TA mice) can phenocopy the effects of JIP1 deficiency (in JIP1/TA mice) on insulin resistance but not obesity. This conclusion is consistent with the finding that JNK1 deficiency in peripheral tissues can protect against diet-induced obesity (28–30). The mechanism of diet-induced obesity suppression in Jip1/TA mice but not JIP1 TA mice is unclear. A JNK1-independent action of JIP1 may contribute to obesity regulation. Alternatively, the effects of JIP1 on obesity may be mediated by a tissue-specific role of JNK1. It is established that JNK1-regulated obesity is mediated by a central action of JNK1 in the brain (1, 27), a tissue that expresses four members (JIP1, JIP2, JIP3, and JIP4) of the JIP protein family that can form heterocomplexes (23). The presence of these JIP family proteins may functionally compensate defects in the brains of JIP1TA mice, but the more severe defects caused by JIP1 deficiency may not be fully compensated by other JIP isoforms in the brains of JIP1/TA mice.

A major unresolved question relates to the mechanism of insulin resistance regulation caused by JIP1-mediated JNK1 activation. Recent studies indicate that hepatic JNK1 (29) and JNK1-mediated phosphorylation of the adapter protein IRS1 (3) may not cause insulin resistance. In contrast, JNK1 regulation of muscle lipoprotein lipase expression (28), the hypothalamic-pituitary-thyroid axis (1, 27), and adipokines (30) appear to play important roles in the development of obesity-induced insulin resistance. A goal for future studies will be to fully define the mechanism of insulin resistance caused by JNK activation mediated by the JIP1 scaffold protein pathway.

Conclusions. The JIP1TA mice described in this report exhibit a selective defect in JIP1-mediated JNK activation. These mice can be used to rigorously test whether JIP1 contributes to JNK activation in response to a specific stimulus. The results of the present study demonstrate that the JIP1 scaffold complex plays a key role in JNK activation caused by feeding of an HFD. We propose that JIP1TA mice will be useful for future studies to identify pathways of JNK activation that are mediated by the JIP1 scaffold protein.


