Supporting Online Material for

A Stress Signaling Pathway in Adipose Tissue Regulates Hepatic Insulin Resistance

Guadalupe Sabio, Madhumita Das, Alfonso Mora, Zhiyou Zhang, John Y. Jun, Hwi Jin Ko, Tamera Barrett, Jason K. Kim, Roger J. Davis*

*To whom correspondence should be addressed. E-mail: roger.davis@umassmed.edu

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Materials and Methods

Mice.
We have previously described Jnk1−/− mice (S1) and Jnk1fl/fl mice (S2). Fabp4-Cre mice (S3) and Lys-Cre mice (S4) were obtained from the Jackson Labs. The mice were backcrossed to the C57BL/6J strain (Jackson Laboratories) and were housed in facilities accredited by the American Association for Laboratory Animal Care (AALAC). The mice were genotyped by PCR analysis of genomic DNA (S2). All studies were performed using male mice. Radiation chimeras were generated by exposure of recipient mice to two doses of ionizing radiation (525 Gy) and reconstitution of the mice with 10⁷ donor bone marrow cells by injection into the tail vein. The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School and Pennsylvania State University College of Medicine.

Tissue culture.
Primary bone marrow-derived macrophages were prepared and cultured using methods described previously (S5)

RNA analysis.
The expression of mRNA was examined by quantitative PCR analysis using a 7500 Fast Real Time PCR machine (Applied Biosystems). Taqman® assays were used to quantitate Adiponectin (Mm00456425_m1), Cd68 (Mm00839636_g1), Fabp4 (Mm00445880_m1), Glucose 6 kinase Mm00439129_m1), Glucose 6 phosphatase (Mm00839363_m1), Il6 (Mm00446190_m1), Il13 (Mm00434204_m1), Jnk1 (Mm0048915_m1), Lysozyme (Mm00727183_m1), Rbp4 (Mm00803266_m1), Steap4 (Mm004754022_m1), Phef1 (Mm00451938_m1), Pepck (Mm00440636_m1), Tnfa (Mm00443258_m1) (Applied Biosystems). Amplimers for Pparγ (TGTGGGGATAAAGCAGGCT and CCGGCAGTTAAGATCACCTA), Mcp-1 (S6), and Mij (S6) were employed using Sybr Green detection. The relative mRNA expression was normalized by measurement of the amount of Gapdh or β-Actin mRNA in each sample using Taqman® assays (Applied Biosystems).

Immunoblot analysis.
Tissue extracts were prepared using Triton lysis buffer [20 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethyalsulfonyl fluoride, and 10 µg/mL of aprotinin and leupeptin]. Extracts (20-50 µg of protein) and immunoprecipitates were examined by protein immunoblot analysis by probing with antibodies to AKT, phosphoSer-308 AKT, phosphoSer-473 AKT (Cell Signaling), insulin receptor (Santa Cruz), phosphotyrosine (4G10, Upstate), JNK1 (Pharmingen), α-Tubulin and β-Actin (Sigma). The IRS1 antibody was prepared by immunization of a rabbit
with a peptide that corresponds to the carboxy-terminal 14 amino acids of rat IRS1 ([C]YASINFQKQPEDRQ). Immunocomplexes were detected by enhanced chemiluminescence (NEN).

**Cytokine analysis.**
Cytokines in plasma and culture medium were measured by multiplexed ELISA using a Luminex 200 machine (Millipore) and serum mouse adipokine, adiponectin and cytokine kits (Millipore).

**Lipid analysis.**
Total Cholesterol, HDL and triglycerides were measured, and the amount of LDL was calculated, using a Cardiocheck PA (PTS, Inc.). The concentration of free fatty acids (Roche) and glycerol (Sigma) was measured using kits purchased from the indicated suppliers.

**Protein kinase assays.**
JNK activity was measured using an in vitro protein kinase assay with the substrates cJun and [γ-32P]ATP as substrates (S7).

**Glucose tolerance, insulin tolerance, and pyruvate challenge tests.**
The mice were fed a standard chow diet or a high fat diet (Iso Pro 3000, Purina and F3282, Bioserve Inc.) for 16 wks. Glucose tolerance tests, insulin tolerance tests, and pyruvate challenge tests were performed using methods described previously (S8).

**Hyperinsulinemic-euglycemic clamp studies.**
These assays were performed at the Penn State Diabetes & Obesity Mouse Phenotyping Center. Briefly, F\textsuperscript{WT} and F\textsuperscript{KO} mice were fed a HFD diet (55% fat by calories; Harlan Teklad) or chow diet for 3 weeks, and whole body fat and lean mass were non-invasively measured using \textsuperscript{1}H-MRS (Echo Medical Systems). Following an overnight fast, a 2-hr hyperinsulinemic-euglycemic clamp was conducted in awake mice with a primed and continuous infusion of human insulin (2.5 mU/kg/min; Humulin; Eli Lilly), and 20% glucose was infused at variable rates to maintain euglycemia (S9). Whole body glucose turnover was assessed with a continuous infusion of [3-\textsuperscript{3}H]glucose and 2-deoxy-D-[1-\textsuperscript{14}C]glucose (PerkinElmer) was administered as a bolus (10 µCi) at 75 min after the start of clamps to measure insulin-stimulated glucose uptake in individual organs. At the end of the clamps, mice were anesthetized, and tissues were taken for biochemical analysis (S9).

**Analysis of tissue sections.**
Sections (7 µm) prepared from tissue frozen in O.C.T. compound (Tissue-Tek) were stained with Oil-red-O (Sigma). Tissue fixed in 4% paraformaldehyde (24 h) was processed and embedded in paraffin. Sections (5 µm) were prepared and mounted on coverslips for staining with haematoxylin & eosin. De-paraffinized sections (4 µm) were also stained, following microwave antigen retrieval and incubation (1h, 25°C) in Tris-buffered saline supplemented with 0.4% Triton and 10% goat serum, with an ALEXA FLUOR 647-conjugated antibody (clone CI:A3-1; Serotech) to the macrophage antigen F4/80 (16h, 4°C). The sections were washed, the coverslips were mounted on slides in medium with DAPI (Vector Labs), and examined by confocal fluorescence microscopy (Leica).
Statistical analysis.
Differences between groups were examined for statistical significance using the Student's test or analysis of variance (ANOVA) with the Bonferroni post-test.
Fig. S1. Genotype analysis of mice with adipose tissue- or myeloid cell-specific deficiency of JNK1.

(A) The strategy to create a conditional Jnk1 allele (Jnk1H) using loxP sites that flank exon 7 is illustrated schematically. PCR primers 5'-CTTCAGGAAGAAGGGCTTATTTC-3' and 5'-GAACCACTGTTCCATTCCATCC-3' that can be used to distinguish between the Jnk1+, Jnk1H, and ΔJnk1 alleles are indicated. (B) Cre-mediated deletion of the Jnk1 gene in adipose tissue. Genomic DNA isolated from adipose tissue was examined by PCR analysis using amplimers that target introns 6 and 7. The analysis of adipose tissue from Fabp4-Cre+ Jnk1H/- (FWT) and Fabp4-Cre+ Jnk1H/- (FKO) mice is presented. (C) Cre-mediated deletion of the Jnk1 gene in macrophages. The analysis of macrophage genomic DNA isolated from FWT, FKO, Jnk1H-, Lyzs-Cre+ Jnk1+/H (MWT), and Lyzs-Cre+ Jnk1H/- (MKO) mice is presented.
Fig. S2. Effect of JNK1-deficiency in myeloid cells.

(A) Lyzs-Cre\textsuperscript{+} Jnk1\textsuperscript{+/+} (M\textsuperscript{WT}) and Lyzs-Cre\textsuperscript{+} Jnk1\textsuperscript{f/f} (M\textsuperscript{KO}) mice were fed a normal chow diet (ND) or a high fat diet (HF). The lean and fat mass of the mice was measured (mean ± SD; n = 8). The blood glucose concentration in mice fed ad libitum or fasted overnight was measured...
(mean ± SD; n = 14). The IL6 concentration in the blood of mice fasted overnight was measured (mean ± SD; n = 14). No statistically significant differences between \(M^{KO}\) mice and \(M^{WT}\) mice were detected (\(P > 0.05\)). (B) Glucose tolerance test (GTT). Mice fasted overnight were injected intraperitoneally with glucose (1 mg/g). Blood glucose concentration was measured at the indicated times (mean ± SD; n = 14). No statistically significant differences between \(M^{KO}\) mice and \(M^{WT}\) mice were detected (\(P > 0.05\)). (C) Insulin tolerance test (ITT). Mice fed \textit{ad libitum} were injected intraperitoneally with insulin (0.75 mU/g). Blood glucose concentration was measured at the indicated times (mean ± SD; n = 14). No statistically significant differences between \(M^{KO}\) mice and \(M^{WT}\) mice were detected (\(P > 0.05\)).
Fig. S3. Bone marrow chimeric mice demonstrate that JNK1 in hematopoietic cells is not required for diet-induced glucose and insulin intolerance.

Lethally irradiated C57BL/6J mice were transplanted with bone marrow derived from wild-type (hWT) or Jnk1−/− (hJnk1−/−) mice. These mice were maintained for 5 wks to enable the reconstitution of the hematopoietic compartment in the recipient mice with cells derived from the
donor mice. The mice were then fed a standard chow (ND) diet or a high fat (HF) diet for 16 wk. (A) The expression of Jnk1 mRNA in blood was examined by quantitative RT-PCR analysis (Taqman®) and is presented as relative mRNA expression (mean ± SD; n = 6). Control studies were performed to detect Gapdh mRNA. The amount of Jnk1 mRNA was significantly reduced in the blood of mice transplanted with Jnk1−/− bone marrow compared with Jnk1+/+ bone marrow (asterisk, P < 0.01). (B) The mice were fasted overnight and the concentration of blood glucose was measured (mean ± SD; n = 10). No statistically significant differences between mice transplanted with Jnk1−/− or Jnk1+/+ bone marrow were detected (P > 0.05). (C) The body mass of the mice during the 16 wk diet using a standard chow (ND) or a high fat (HF) diet was measured (mean ± SD; n = 10). No statistically significant differences between mice transplanted with Jnk1−/− or Jnk1+/+ bone marrow were detected (P > 0.05). (D) Glucose tolerance test (GTT). Mice fasted overnight were injected intraperitoneally with glucose (1 mg/g). Blood glucose concentration was measured at the indicated times (mean ± SD; n = 10). No statistically significant differences between mice transplanted with Jnk1−/− or Jnk1+/+ bone marrow were detected (P > 0.05). (E) Pyruvate challenge. Mice fasted overnight were injected intraperitoneally with pyruvate (1 mg/g). Blood glucose concentration was measured at the indicated times (mean ± SD; n = 10). No statistically significant differences between mice transplanted with Jnk1−/− or Jnk1+/+ bone marrow were detected (P > 0.05). (F) Insulin tolerance test (ITT). Mice fed ad libitum were injected intraperitoneally with insulin (0.75 mU/g). Blood glucose concentration was measured (30 mins) and the area under the curve (AUC) was calculated (mean ± SD; n = 10). No statistically significant differences between mice transplanted with Jnk1−/− or Jnk1+/+ bone marrow were detected (P > 0.05).
Fig. S4. JNK1-deficiency in adipose tissue does not protect against obesity.

(A) The body mass of Fabp4-Cre⁺ Jnk1⁺⁻ (F⁷WT) and Fabp4-Cre⁺ Jnk1⁻⁻ (FKO) mice fed a normal chow diet (ND) or a high fat diet (HF) was measured at the indicated times (mean ± SD; n = 14). No statistically significant differences between FKO mice and FWT mice were
detected (P > 0.05). (B) Sections of epidymal fat pads stained with H&E (left panel) and the mass of epididymal fat pads (right panel) of mice fed a chow or a high fat diet for 16 wk are presented (mean ± SD; n=14). No statistically significant differences between F^KO mice and F^WT mice were detected (P > 0.05). (C) The mass of brown fat, heart, liver and muscle (quadriceps) of mice fed a chow or a high fat diet for 16 wk is presented (mean ± SD; n=14). No statistically significant differences between F^KO mice and F^WT mice were detected (P > 0.05). (D) The lean mass and whole body fat of mice fed a chow or high fat diet (3 wks) were non-invasively measured by ^1H-MRS (mean ± SD; n=8). No statistically significant differences between F^KO mice and F^WT mice were detected (P > 0.05).
Fig. S5. Effect of adipose tissue-specific JNK1-deficiency on blood lipids.

(A-F) *Fabp4-Cre*<sup>+</sup> *Jnk1<sup>+/−</sup>* (F<sup>WT</sup>) and *Fabp4-Cre*<sup>+</sup> *Jnk1<sup>−/−</sup>* (F<sup>KO</sup>) mice were fed a normal chow diet (ND) or a high fat diet (16 wk). The mice were fasted overnight and blood was taken for the measurement of cholesterol, triglycerides, HDL, LDL, glycerol, and free fatty acids (FFA) (mean ± SD; n = 10). No statistically significant differences between F<sup>KO</sup> mice and F<sup>WT</sup> mice were detected (P > 0.05).
Fig. S6. Effect of adipose tissue-specific JNK1-deficiency on the response to a pyruvate challenge and hepatic gene expression.

(A) Fabp4-Cre+ Jnk1+/− (FWT) and Fabp4-Cre+ Jnk1−/− (FKO) mice were fed a normal chow diet (ND) or a high fat diet (HF) for 16 wk. Mice fasted overnight were injected intraperitoneally with pyruvate (1 mg/g). Blood glucose concentration was measured at the indicated times (mean ± SD; n = 14). Statistically significant differences between FKO mice and FWT mice are indicated (*, P < 0.01). The data demonstrate that feeding a HF diet suppresses pyruvate-induced hepatic gluconeogenesis in FWT mice, but not in FKO mice. (B-D) Total RNA was isolated from the liver and the expression of mRNA from the Pepck, glucose 6 phosphatase, and glucose 6 kinase genes was examined by quantitative RT-PCR (mean ± SD; n = 8). The data are normalized for the amount of Actin mRNA in each sample. Significant differences in the expression of Pepck, glucose 6 phosphatase, and glucose 6 kinase mRNA detected between FWT
and FKO mice are indicated (*, P < 0.05). The data demonstrate that altered expression of *Pepck*, *glucose 6 phosphatase*, or *glucose 6 kinase* does not account for the failure of a HF diet to suppress hepatic gluconeogenesis in FKO mice. It is possible that the reduced steatosis observed in HF diet-fed in FKO mice, compared with FWT mice, contributes to the effects on adipose tissue-specific JNK1-deficiency on pyruvate-induced gluconeogenesis (Fig. 4A). (E) Total RNA was isolated from the liver and the expression of the myeloid-specific gene *Cd68* was examined by quantitative Taqman® RT-PCR analysis (mean ± SD; n = 8). The data are normalized for the amount of *Actin* mRNA in each sample. No significant differences (P < 0.05) in the expression of *Cd68* mRNA were detected between livers isolated from FWT and FKO mice. These data indicate that adipose tissue-specific JNK1-deficiency does not affect myeloid cell infiltration of the liver.
**Fig. S7. Effect of adipose-specific JNK1-deficiency on muscle insulin resistance.**

*Fabp4-Cre*<sup>+</sup> *Jnk1<sup>+/−</sup>* (*F<sup>WT</sup>*) and *Fabp4-Cre*<sup>+</sup> *Jnk1<sup>−/−</sup>* (*F<sup>KO</sup>*) mice were fed a chow diet or a high fat diet (16 wk). The mice were fasted overnight and then treated with insulin (1.5 U/kg body mass) by intraperitoneal injection. Muscle (quadriiceps) was isolated at 30 min post-treatment with insulin and examined by immunoblot analysis to detect phospho-AKT, AKT, and GAPDH.

Feeding a high fat diet (HFD) suppressed the effects of insulin to cause increased AKT activation in the muscle of control (*F<sup>WT</sup>*) mice. The adipose tissue-specific JNK1-deficient (*F<sup>KO</sup>*) mice were not protected against the effects of the HFD to suppress insulin-stimulated AKT activation. The HFD therefore causes similar insulin resistance in the muscle of *F<sup>WT</sup>* and *F<sup>KO</sup>* mice. This conclusion is consistent with the results of hyperinsulinemic-euglycemic clamp studies that demonstrate no significant differences between muscle glucose uptake by HFD-fed *F<sup>WT</sup>* and *F<sup>KO</sup>* mice (*P > 0.05; n = 7). Together, these data demonstrate that *F<sup>KO</sup>* mice do not exhibit defects in diet-induced muscle insulin resistance.

While it is established that IL6 can cause hepatic insulin resistance, the effects of IL6 on muscle insulin resistance are unclear. Thus, administration of IL6 to mice causes insulin resistance in liver, but not in muscle (S10). Nevertheless, IL6 treatment of L6 myotubes in culture increases both basal and insulin-stimulated glucose uptake (S11). Moreover, transgenic mice that express IL6 in muscle (and have very high circulating concentrations on IL6) exhibit decreased muscle glucose uptake in the absence of changes in AKT activation (S12). However, neutralization of IL6 does not affect muscle glucose uptake in hyperphagic *ob/ob* mice (S13). These studies indicate that the effects of IL6 on muscle insulin resistance are complex. One possibility is that the acute effects of IL6 may be different from the chronic effects of IL6. Indeed, it has been reported that acute IL 6 treatment increases insulin-stimulated muscle glucose uptake and systemic insulin sensitivity, but chronic exposure caused insulin resistance (S14). In contrast, another study demonstrated that acute IL-6 treatment reduced insulin-stimulated glucose uptake in skeletal muscle (S9).
Further studies are required to define the physiological role of IL6 in muscle insulin resistance. In the context of this study, it appears that the decreased blood IL6 concentration of HFD-fed FKO mice, compared with FWT mice, does not lead to altered muscle insulin resistance. The absence of a muscle insulin resistance phenotype in the FKO mice may result from dominant effects of other regulators of insulin resistance, including blood lipids.
Fig. S8. Effect of adipose-specific JNK1-deficiency on blood cytokines.

*Fabp4-Cre*^+^ *Jnk1^+/−* (F^WT^) and *Fabp4-Cre*^+^ *Jnk1^−/−* (F^KO^) mice were fed a chow diet or a high fat diet for 16 wk and then fasted overnight. The plasma levels of different cytokines were examined (mean ± SD; n=10 in each group). No statistically significant difference in the serum concentration of IL1, IL2, IL4, IL5, IL10, and IL12 between F^WT^ and F^KO^ mice was detected (P > 0.05).
Fig. S9. Effect of adipose tissue-specific JNK1-deficiency on adipokine expression.

(A) *Fabp4-Cre* $^+\text{Jnk}^{	ext{WT}}$ and *Fabp4-Cre* $^+\text{Jnk}^{	ext{KO}}$ mice were fed a normal chow diet or a high fat diet for 16 wk. High molecular weight adiponectin in the blood was examined by immunoblot analysis of plasma examined by native gel electrophoresis. Each lane represents the
analysis of serum from one mouse. Feeding a HFD caused a small decrease in serum adiponectin in F\textsuperscript{WT} mice, but not in F\textsuperscript{KO} mice. (B-I) The expression of mRNA from the Adiponectin, Lysozyme, Rbp4, Steap4, Pbef1, Ppar\textgamma, Mcp-1, and Mif genes was examined by quantitative RT-PCR (mean ± SD; n = 6). No significant differences (P > 0.05) in the expression of Rbp4, Pbef1, or Mcp-1 mRNA were detected between F\textsuperscript{WT} and F\textsuperscript{KO} mice. In contrast, the HFD-induced increase in Mif and Steap4 mRNA was reduced in F\textsuperscript{KO} compared with F\textsuperscript{WT} mice (*, P < 0.01). This reduction in Mif and Steap4 mRNA expression is consistent with previous reports indicating that Mif expression is JNK1-dependent (S6) and that Steap4 expression is IL6-dependent (S15). Marker gene expression analysis did not indicate differences in adipose tissue differentiation (Ppar\textgamma) or macrophage infiltration (Lysozyme) between HFD-fed F\textsuperscript{WT} and F\textsuperscript{KO} mice.
Fig. S10. Effect of adipose-specific JNK1-deficiency on the expression of IL13.

(A) Fabp4-Cre$^+$ Jnk1$^{+/+}$ (F$^\text{WT}$) and Fabp4-Cre$^+$ Jnk1$^{f/-}$ (F$^\text{KO}$) mice were fed a normal chow diet or a high fat diet for 16 wk. Total RNA isolated from epididymal fat pads was employed to measure the expression of mRNA from the Il13 gene by quantitative Taqman® RT-PCR analysis (mean ± SD; n = 6). The data are normalized for the amount of Gapdh mRNA in each sample. (B) The concentration of IL13 in the blood was measured (mean ± SD; n = 10).

No significant differences between F$^\text{WT}$ and F$^\text{KO}$ mice were detected (P > 0.05) in the expression of the Il13 mRNA in adipose tissue or IL13 protein in blood.

Polarization of tissue macrophages to the inflammatory M1 or anti-inflammatory M2 phenotypes can contribute to metabolic regulation (S16). It is established that IL4 and IL13 can induce alternative activation of macrophages to the M2 phenotype (S17) and these cytokines are implicated in metabolic regulation (S18, S19) because M2 cells express the anti-inflammatory cytokine IL10 that can protect against insulin resistance (S20). We found no significant
differences in IL4 and IL10 expression in F\textsuperscript{WT} and F\textsuperscript{KO} mice (fig. S8), suggesting that M1/2 polarization of tissue macrophages was not altered by adipose tissue-specific deficiency of JNK1. Adipocytes can also express IL13 (S19). Altered expression of IL13 could therefore contribute to the metabolic phenotype of F\textsuperscript{KO} mice. However, no significant difference in adipose tissue \textit{Il13} mRNA, or the serum concentration of IL13, was detected in studies of F\textsuperscript{WT} and F\textsuperscript{KO} mice (see above). The observation that JNK1-deficiency in adipose tissue does not affect IL4, IL10, or IL13 expression suggests that altered M1/2 polarization of tissue macrophages does not contribute to the metabolic phenotype of F\textsuperscript{KO} mice.
Fig. S11. Effect of adipose-specific JNK1-deficiency on infiltration by myeloid cells.

(A) Fabp4-Cre\(^+\) Jnk1\(^{+/+}\) (F\(^{WT}\)) and Fabp4-Cre\(^+\) Jnk1\(^{/-}\) (F\(^{KO}\)) mice were fed a normal chow diet or a high fat diet for 16 wk. Total RNA isolated from epididymal fat pads was employed to measure the expression of mRNA from the Cd68 gene by quantitative Taqman\(^\circledR\) RT-PCR analysis (mean ± SD; n = 6). The data are normalized for the amount of Gapdh mRNA in each sample. No significant differences in the expression of Cd68 gene between F\(^{WT}\) and F\(^{KO}\) mice were detected (P > 0.05). CD68 is expressed selectively in myeloid cells. These data indicate that there is no difference in the macrophage infiltration of adipose tissue between F\(^{WT}\) and F\(^{KO}\) mice. (B) Macrophage infiltration of the adipose tissue of F\(^{WT}\) and F\(^{KO}\) mice fed a normal chow diet or a high fat diet for 16 wk. was examined by staining sections of epididymal fat with a monoclonal antibody to F4/80 (green). Cell nuclei were identified by staining DNA (red). The sections were examined by confocal fluorescence microscopy. Representative images are presented; the white box indicates a sub-region of each image that is also presented at higher magnification. These data indicate that no differences in the infiltration of adipose tissue by macrophages between F\(^{WT}\) and F\(^{KO}\) mice were detected.
Fig. S12. Effect of JNK1-deficiency in macrophages on TNFα and IL6 expression.

(A-B) Bone marrow-derived macrophages from (A) WT and Jnk1<sup>−/−</sup> mice or (B) Fabp4-Cre<sup>+</sup> Jnk1<sup>−/−</sup> (F<sub>WT</sub>) and Fabp4-Cre<sup>+</sup> Jnk1<sup>−/−</sup> (F<sub>KO</sub>) mice were stimulated with 0.5mM palmitate. The amount of TNFα and IL6 in the culture medium was measured at the indicated time points (mean ± SD; n = 5). Statistically significant differences between F<sub>WT</sub> and F<sub>KO</sub> mice are indicated (*, P < 0.05; **, P < 0.01).
Supplementary References