

Adiponectin resistance exacerbates insulin resistance in *insulin receptor* transgenic knockout mice

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Abstract

Objective: Adiponectin increases insulin sensitivity and contributes to insulin's indirect effects on hepatic glucose production.

Research Design and Methods: To examine adiponectin's contribution to insulin action, we analyzed adiponectin levels and activation of AMP-dependent kinase (AMPK) in insulin receptor transgenic/knockout mice (L1), a genetic model of resistance to insulin's indirect effects on hepatic glucose production.

Results & Conclusions: In euglycemic, insulin-resistant L1 mice we detected hyperadiponectinemia with normal levels of adiponectin receptor-1 and -2. Moreover, adiponectin administration is unable to lower glucose levels or induce activation of AMP activated kinase (AMPK), consistent with a state of adiponectin resistance. In a subset of hyperglycemic L1 mice, we observed decreased mRNA expression of AdipoR2 in liver and muscle, as well as decreased PPAR α target gene expression in liver, raising the possibility that deterioration of adiponectin/AdipoR2 signaling via PPAR α activation contributes to the progression from compensated insulin resistance to diabetes. In contrast, we failed to detect changes in other markers of the systemic or local inflammatory response. These data provide evidence for a mechanism of adiponectin resistance and corroborate the notion that adiponectin potentiates hepatic insulin sensitivity.

Type 2 diabetes¹ is characterized by insulin resistance and impaired pancreatic β cell function (1). Fasting hyperglycemia in diabetics results primarily from the inability of insulin to inhibit endogenous glucose production (2). Insulin regulates hepatic glucose production (HGP) through both direct (hepatic) and indirect (extrahepatic) mechanisms (3). Chronic effects of insulin are primarily mediated by direct mechanisms via hepatic insulin receptor (*Insr*)/PI3K/forkhead box O1 (*FoxO1*) signaling to suppress the expression of gluconeogenic enzymes (4). Acute effects of insulin on HGP are mediated by both direct and indirect mechanisms (5-9). Multiple mechanisms have been proposed to account for insulin's indirect effects on HGP, including glucagon (10; 11), gluconeogenic substrates released from muscle (12) and fat (13), and hypothalamic signals (9; 14). In addition, adipocytokines have been shown to either increase (e.g., resistin) or decrease glucose production (e.g., adiponectin) (15; 16).

In previous studies, we have carried out genetic reconstitution experiments with an allelic series of tissue-specific transgenes to disentangle the complex interactions underlying the integrated physiology of insulin action (17-20). These studies have emphasized the central role of the liver in insulin action (19; 20), and the role of indirect mechanisms in insulin control of hepatic glucose production (18). Thus, mice in which insulin signaling is restricted to liver, selected regions of the brain and pancreatic β cells (referred to as L1) are, surprisingly, resistant to insulin's direct effect on HGP (18). In this study, we

asked whether the impairment of insulin's indirect actions in the liver of L1 mice could be explained by altered adipocytokine action. We report that L1 mice display hyperadiponectinemia, associated with inability to lower plasma glucose levels and blunted hepatic AMP-activated kinase (AMPK) response in response to adiponectin. These findings delineate a condition of "adiponectin resistance", previously recognized in IGF-1 receptor dominant-negative transgenic mice (21) that may contribute to the impairment of insulin's direct control of HGP, as previously reported (18).

Research Design and Methods

Mice

Transgenic mice expressing human *INSR* cDNA from the transthyretin (*Ttr*) promoter were intercrossed with *Insr*^{+/-} mice. The resulting progeny were further intercrossed to generate *Insr*^{-/-}, *Ttr-INSR* (L1) mice and *Insr*^{+/+} littermates. Animals were maintained on a mixed background derived from 129/Sv, C57BL/6, and FVB. Genotyping was performed as previously described (19) with the following modification. WT and null *Insr* alleles were detected using primers 5'-AGCTGTGCACTTCCCTGCTCAC-3', 5'-TTAAGGGCCAGCTCATTCCTCC-3', and 5'-TCTTTGCCTGTGCTCCACTCTCA-3'. The product of the WT allele is 232bp in length and that of the null allele is 355bp. All animal procedures have been approved by the Columbia University Institutional Animal Care and Utilization Committee.

Metabolic analyses

Plasma glucose was measured by the One-Touch Ultra meter (LifeScan, Milpitas, CA). Free fatty acids,

¹ Abbreviations: *Insr*: insulin receptor; AMPK: AMP-activated protein kinase

triglycerides, and β -hydroxybutyrate were measured by NEFA C test kit (Wako Chemicals, Richmond, VA), free glycerol reagent (Sigma, St. Louis, MO), and β -hydroxybutyrate reagent set (Pointe Scientific, Canton, MI), respectively. Insulin was measured by ELISA, and adiponectin and resistin were measured by RIA (Linco Research Inc., St. Charles, MO). Cytokines were measured by the mouse cytokine 10-plex Luminex kit (Biosource, Camarillo, CA). Body composition was determined using GE Lunar PIXImus scan.

Adiponectin treatment

Production and purification of recombinant full-length murine adiponectin were performed as described (22). In the morning, food was withdrawn 2 hours before the experiment. Mice were then injected with either saline or adiponectin via the tail vein (2 μ g/g body weight). Blood glucose was measured at 0, 1, 2, 3, 4, 6, 8, and 48 hours after injection from tail sampling. Mice were re-fed 8 hours after injection. For Western analysis, mice were fasted for 6 hours, injected with saline or adiponectin via the tail vein (2 μ g/g body weight), and sacrificed 20 minutes after injection.

RNA isolation and RT-PCR analyses

We extracted total RNA using RNeasy Mini Kit, RNeasy Mini Fibrous Tissue Kit, and RNase-Free DNase Set (Qiagen, Valencia, CA). RNA was reverse transcribed using oligo-dT and SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative PCR reactions were performed in triplicate using a DNA Engine Opticon 2 System (MJ Research, Bio-Rad, Hercules, CA) and DyNAmo HS SYBR green Q-PCR kit (New England Biolabs, Ipswich, MA). The sequences of

the primers used were: 5'-CCCTGAACCCTAAGGCCAACCGTGAA AA-3' and 5'-TCTCCGGAGTCCATCACA-ATGCCTGTG-3' for β -actin, 5'-AAGGGCAAGCGGGCAGCCAGCA-3' and 5'-ATCT-TCTCCATGGCATGGTGGGCTTG-3' for AdipoR1, 5'-CAACTACCAAGGAGATTTG-GAGCCCAGC-3' and 5'-GCGGGGACATGCCATAAACCCCTTCA-3' for AdipoR2, 5'-TTGGCCAAGCTATTGCGACA-3' and 5'-GCAAAGGCATTGGCTGGAAG-3' for CD36, 5'-GTGCAGCTCAGAGTCTGTCCAA-3' and 5'-TACTGCTGCGTCTGAAAATCCA-3' for Acox1, 5'-CCATCATGGGTCCTTCTGGAG-3' and 5'-GAACAGTGAGGTGAGGCA-GCA-3' for ABCg1, 5'-TGCACTACGGAGTCCTGCAA-3' and 5'-GGACAACCTCCAT-GGCTCAG-3' for Cpt1 α , 5'-GGGTACCACTACGGAGTTCACG-3' and 5'-CAGACAG-GCACTTGTGAAAACG-3' for PPAR α , 5'-AGTCCTTCCCCTGACCAAAG-3' and 5'-TCGAAACTGGCACCCTTGAAA-3' for PPAR γ . Relative mRNA levels were calculated using standard curves, with the PCR product for each primer set normalized to β -actin RNA.

Western blotting

We prepared detergent extract from liver in buffer containing 20mM Tris (pH7.6), 150mM NaCl, 1mM DTT, 10mM EGTA, 1% NP40, 2.5mM Na₄P₂O₇, 1mM NaVO₃, 1mM β -glycerophosphate, and protease inhibitor cocktail (Roche, Indianapolis, IN). Protein concentration of extracts was determined by BCA assay (Pierce,

Rockford, IL). We resolved equal amounts of protein (100 μ g) on SDS-PAGE, transferred them onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), and probed the membranes with antibodies against phospho-AMPK α (Thr172), total AMPK α , and actin (Cell Signaling, Danvers, MA).

Immunohistochemistry

Adipose and liver samples were fixed for 12-16 hours at room temperature in Z-Fix (Anatech, Battle Creek, MI) and embedded in paraffin. 5 μ m-thick sections were mounted on slides and stained to visualize F4/80 as described (23), using a rat monoclonal antibody against mouse F4/80 and rat IgG2a isotype as control (Caltag, Burlingame, CA). For each liver sample, 5 different high power fields (40X objective) were analyzed, and F4/80-positive cells were counted. For each adipose sample, 10 different high power fields were analyzed.

Results

General characteristics of the experimental animals

L1 mice lack endogenous *Insr*, but carry an *INSR* transgene driven by the transthyretin promoter that reactivates *INSR* expression in liver, pancreatic β cells, and various brain regions (19). At 3 months of age, approximately 65% of the male L1 mice showed normal glucose levels, while 35% were frankly diabetic. All L1 mice showed increased circulating insulin levels compared to WT under both fasting and fed conditions (19)(Fig. 1A), indicative of systemic insulin resistance. In addition, we have shown that, despite restored insulin signaling in liver by transgenic expression of *INSR*, L1 mice remain resistant to insulin suppression of

HGP during hyperinsulinemic/euglycemic clamps (18).

Hyperadiponectinemia and normoresistinemia in L1 mice

We hypothesized that hepatic insulin resistance in L1 mice is due to impaired adipocytokine signaling. We measured plasma adiponectin levels in 3-month-old L1 mice and WT littermates under fed conditions, and detected a 140% increase in L1 mice (Table 1 and Fig. 1C). Notably, every L1 mouse examined has higher adiponectin values than the mean WT value, and only two WT mice had adiponectin values in the low range of the L1 mice. Furthermore, we observed an inverse correlation between adiponectin and glucose values in L1 mice (Fig. 1D), indicating that falling adiponectinemia correlates with hyperglycemia. Resistin levels in 3-month-old L1 and WT mice were similar; but by 5 months of age, L1 mice had a 42% increase of resistin levels compared WT littermates (Fig. 1B). This difference appears to result from diverging age-dependent trends, such that resistinemia decreased with age in WT (3.31ng/ml at 3 months vs. 2.47ng/ml at 5 months, $P=0.06$), but not in L1 mice (3.76 ng/ml at 3 months vs. 3.51ng/ml at 5 months, $P=0.53$). Since L1 mice exhibited hepatic insulin resistance at 3 months of age, when their resistinemia was similar to WT mice, it is unlikely that altered resistin levels contribute to insulin resistance in L1 mice. In addition, we previously showed that leptin levels are not significantly different between L1 and WT mice (18). Finally, there were no differences in mean body weight, fat mass and fat-free mass between 3-month-old WT and normoglycemic L1 mice. Diabetic L1 mice displayed increased body weight that was largely accounted for by increased fat-free mass

(Table 1). Histological analyses of skeletal muscle of diabetic L1 mice did not reveal significant changes in intramuscular lipid accumulation or in slow twitch/fast twitch fiber composition (data not shown). Postprandial FFA and TG levels were increased in both groups of L1 mice (Table 1), while fasting levels were similar to WT (18). Fasting β -hydroxybutyrate levels were increased by 75% in normoglycemic L1 mice compared to WT (Table 1).

L1 mice are resistant to the rapid glucose-lowering action of adiponectin

The correlation between hyperadiponectinemia and hyperinsulinemia in L1 mice led us to hypothesize that hyperadiponectinemia is a marker of adiponectin resistance, and that impaired adiponectin action contributes to hepatic insulin resistance in L1 mice. To test this hypothesis, we administered a single dose of purified recombinant full-length murine adiponectin by i.v. injection in 3 month-old normoglycemic L1 mice and WT littermates. The recombinant protein was produced in mammalian cells as previously described (22) and had normal distribution of trimer, hexamer, and HMW complexes [(21) and data not shown]. Consistent with previous reports (21; 22), administration of an adiponectin bolus in WT mice resulted in a 38% decrease in glucose levels 2 hr after injection (Fig. 2A), followed by a return to normal values by 6 hr post-injection (Fig. 2A). In contrast, we detected no difference in glucose levels following adiponectin injection L1 mice over an 8 hr-period, compared to saline control (Fig. 2B). Adiponectin-treated L1 mice displayed a trend toward hyperglycemia at 2 days after injection (134% of basal glycemia, $P=0.08$), while control (saline-injected) animals did not.

These data indicate that L1 mice are resistant to the acute action of adiponectin to lower plasma glucose levels.

Reduced AdipoR2 expression correlates with diabetes susceptibility in L1 mice

The metabolic effects of adiponectin are mediated by two putative receptors, AdipoR1 and AdipoR2. The former is primarily expressed in muscle, the latter in liver (24). In addition, T-cadherin has been proposed to serve as an adiponectin receptor (25), although the metabolic effects of this interaction have not been studied. We investigated whether the decrease in adiponectin's ability to lower plasma glucose levels in L1 mice was due to decreased expression of AdipoR1 and/or R2. We measured levels of mRNAs encoding AdipoR1 and R2 in liver and skeletal muscle samples from 3-month-old normoglycemic and diabetic L1 mice as well as WT littermates by quantitative RT-PCR. Hepatic AdipoR1 mRNA levels were similar in L1 and WT mice. Hepatic AdipoR2 mRNA levels in normoglycemic L1 mice were also similar to WT, whereas diabetic L1 mice showed a $33 \pm 9\%$ reduction in hepatic AdipoR2 levels compared to WT (Fig. 3A). We observed a similar expression profile in skeletal muscle. AdipoR1 mRNA expression in muscle exhibited no significant difference between WT and L1 mice. AdipoR2 expression in muscle was unaffected in normoglycemic L1 mice, but was reduced by $33 \pm 11\%$ in diabetic L1 mice compared to WT (Fig. 3B). These data suggest that the systemic "adiponectin resistance" observed in normoglycemic L1 mice (Fig. 2) is independent of mRNA expression levels of AdipoR1 and R2 in liver or muscle. However, reduction in AdipoR2 mRNA in both liver and muscle

is associated with hyperglycemia in L1 mice.

Adiponectin-induced AMPK phosphorylation and PPAR α activation in L1 mice

Adiponectin has been shown to inhibit glucose production via activation of AMPK (26-28) and fatty acid oxidation via PPAR α (24; 29). We used western blotting with a phospho-Thr172-specific antibody to examine AMPK activation in response to i.v. adiponectin injection in normoglycemic L1 and WT littermates. As expected, adiponectin treatment in WT mice significantly increased phospho-AMPK levels in liver (Fig. 4), without affecting AMPK expression. Interestingly, basal phospho-AMPK levels were elevated in liver of saline-injected L1 mice compared to WT, possibly resulting from hyperadiponectinemia. However, acute adiponectin treatment in L1 mice failed to enhance AMPK phosphorylation (Fig. 4). These data indicate that adiponectin resistance in L1 mice correlates with impaired hepatic AMPK activation by adiponectin.

The insulin-sensitizing effects of adiponectin can be partly explained by activation of PPAR α (26). We examined PPAR α mRNA expression in liver and skeletal muscle of L1 mice. Hepatic PPAR α mRNA levels in both normoglycemic and diabetic L1 mice were similar to WT mice (Fig. 5A), as were those of PPAR α target genes, acyl-coA oxidase (*Acox1*) and carnitine palmitoyl-transferase 1 α (*Cpt1 α*). In contrast, mRNA expression of both *Acox1* and *Cpt1 α* in liver of diabetic L1 mice was reduced compared to WT (Fig. 5A). Expression of CD36, another PPAR α target gene, was not significantly altered in L1 mice (Fig. 5A). This suggests that PPAR α expression/activity is not affected

in normoglycemic L1 mice, while diabetic L1 mice may exhibit reduced PPAR α activity, hence reduced fatty acid oxidation, independent of PPAR α mRNA levels. This is consistent with the trend toward increased hepatic triglycerides in diabetic L1 mice (15.4 ± 7.6 mg/g liver) compared to WT (4.6 ± 0.5 mg/g liver, $P=0.19$), but not in normoglycemic L1 mice (4.4 ± 2.0 mg/g liver, $n=4$ for all groups). Additionally, mRNA levels of PPAR α , *Acox1*, *Cpt1 α* , and CD36 in muscle of L1 mice and WT littermates showed no significant differences (Fig. 5B). Therefore changes in PPAR α expression and activity cannot account for adiponectin resistance in L1 mice.

Systemic inflammatory markers and reduced resident hepatic macrophages

A chronic, low-grade inflammatory stage, characterized by macrophage accumulation in adipose tissue, and changes in circulating pro-inflammatory cytokines has been demonstrated in insulin resistance, obesity and diabetes (15). L1 mice macrophages lack *Insr* (30), and may therefore contribute to systemic inflammation and predisposes to hepatic insulin resistance. To address this possibility, we measured circulating levels of interleukin-1 β , IL-2, IL-6, IL-12, granulocyte macrophage-colony stimulating factor (GM-CSF), and tumor necrosis factor (TNF)- α in plasma, and found no significant difference between L1 mice and WT littermates (Table 2). In addition, histological surveys of macrophages in perigonadal adipose tissue by immunostaining for F4/80, a marker specific for mature macrophages (23; 31), showed no morphometric differences between WT, normoglycemic L1 and diabetic L1 mice (data not shown). Quantitative analyses revealed that the percentage of F4/80-positive cells was

similar between WT and normoglycemic L1 mice ($6.8 \pm 0.5\%$ vs. $6.1 \pm 0.6\%$, $P=0.35$), but was moderately increased in the diabetic L1 group ($8.1 \pm 0.8\%$, $P=0.16$ and 0.05 vs. WT and normoglycemic L1, respectively). Since no difference was detected between WT and normoglycemic L1 mice, these data indicate that systemic inflammation does not contribute to insulin resistance in L1 mice.

We next asked whether there are alterations of Kupffer cells number or distribution in L1 mice, potentially leading to intra-hepatic inflammation and cytokine production, and thus causing hepatic insulin resistance. F4/80 immunostaining failed to reveal differences in Kupffer cell morphology between WT, normoglycemic L1 and diabetic L1 mice (Fig. 6A-C). Indeed, quantitative analyses revealed that both normoglycemic and diabetic L1 mice had fewer Kupffer cells than WT (Fig. 6D, 80% of WT and 61% of WT, respectively, $P=NS$). We also examined expression of macrophage-specific transcripts in liver samples of L1 and WT mice. Hepatic CD36 expression failed to show significant differences between WT and normoglycemic or diabetic L1 mice (Fig. 5A). In addition, mRNA expression of ATP-binding cassette transporter G1, a macrophage-specific cholesterol efflux transporter, in liver of normoglycemic L1 mice was not significantly different from that of WT, but was reduced by 60% in diabetic L1 mice (Fig. 6E). The transcriptional activity of PPAR γ is required for the activation of both CD36 and ABCg1 in macrophages (32; 33). We did not detect differences in PPAR γ mRNA levels in liver lysates (Fig. 6F), although it's possible that changes in macrophage PPAR γ expression were masked by PPAR γ transcripts in hepatocytes. In summary, our data

indicate that liver of L1 mice has a reduced Kupffer cell population, which correlates with decreases in macrophage-specific gene expression. Therefore, we found no evidence for local activation of inflammation that may account for hepatic insulin resistance in L1 mice.

Discussion

Tissue-specific insulin resistance is associated with hyperadiponectinemia and adiponectin resistance

The antidiabetic properties of adiponectin have attracted considerable attention. A number of studies in humans, non-human primates, and rodents have shown that hypoadiponectinemia is a common feature of obesity and insulin resistance (34). In contrast, L1 mice have elevated adiponectin levels. The association between hyperadiponectinemia and insulin resistance was previously reported in another model of selective *Insr* ablation, the adipocyte-specific *Insr* knockout (FIRKO) (35), suggesting that hyperadiponectinemia in L1 mice may be explained by the lack of *Insr* signaling in adipose cells. This hypothesis is also consistent with a recent study demonstrating that FoxO1, a transcription factor inhibited by insulin signaling, activates adiponectin gene transcription in adipocytes (36). Moreover, Kim et al. have recently shown that mice with muscle-specific insulin resistance due to trans-dominant inhibition of *Insr* and IGF-1 receptor are also hyperadiponectinemic and adiponectin-resistant (21). When considered with the present data, this burgeoning literature begins to outline a mechanism by which adiponectin production is controlled in response to changes in systemic insulin sensitivity. This mechanism is likely to be conserved in humans, as a recent study of patients

with severe insulin resistance found that subjects with *INSR* mutations have elevated plasma adiponectin levels, whereas moderate insulin resistance correlates in hypoadiponectinemia (37). Since three major forms of adiponectin are present in circulation, potentially with different signaling activities (34), it will be interesting to determine whether all three are elevated in *Insr*-deficient models.

In this study, we show that acute adiponectin treatment failed to lower glycemia in L1 mice (Fig. 2), consistent with systemic adiponectin resistance. The mechanism for this failure appears to correlate with a blunted response of AMPK activation, but not with changes in AdipoR1 and R2 mRNA expression. The reduced AMPK response is associated with increased basal AMPK phosphorylation. Therefore, although our data are consistent with adiponectin resistance in L1 mice, we cannot rule out the possibility that adiponectin/AMPK signaling is maximally activated in L1 mice under basal conditions, and is no longer sensitive to acute increases in circulating adiponectin. Interestingly, mice with muscle-specific expression of dominant-negative IGF-1 receptor (MKR) also display systemic adiponectin resistance, although adiponectin-induced AMPK activation was normal (21). Therefore, adiponectin resistance may occur via multiple mechanisms, and MKR mice may represent a state of “AMPK resistance”.

Progressive reduction in adiponectin signaling and diabetes

The metabolic phenotype of L1 mice is inherently heterogeneous. While all L1 mice are markedly hyperinsulinemic and insulin-resistant, ~35% of male L1 mice are frankly diabetic, possibly as a result of differences in their genetic make-up (38).

In this subset of mice, we identified two differences in the adiponectin pathway: a trend toward lower adiponectin levels compared to normoglycemic L1 mice; and a reduction of AdipoR2 mRNA levels in liver and muscle compared to WT. These data are consistent with the possibility that hyperglycemia acts as an independent variable in the control of adiponectin sensitivity. In this regard, we have previously shown that AdipoR1 and R2 are negatively regulated by insulin via FoxO1 (39). Since FoxO1 is also regulated by glucose via oxidative stress (40), it is possible that the changes in AdipoR2 levels reflect decreased FoxO1-dependent transcription. In contrast, AdipoR1 mRNA expression was not changed in liver and tended to be reduced in skeletal muscle of both normoglycemic and diabetic L1 mice. These data may reflect differential regulation of AdipoR1 and R2 under insulin resistant/diabetic conditions. In addition, the tendency for reduced AdipoR1 expression in muscle of L1 mice may also contribute to adiponectin resistance and insulin resistance.

Inflammation in a non-obese model of insulin resistance

A mechanistic link between innate immunity and metabolism has emerged over the past few years (15). Obesity is associated with chronic low-grade inflammation, macrophage recruitment to WAT, as well as increased pro-inflammatory cytokines (41). However, whether inflammation also plays a role in insulin sensitivity in the lean state is unknown. Moreover, the relative contribution of insulin resistance in adipocytes vs. macrophages to activation of the inflammatory state is unclear. L1 mice maintain normal body weight and composition, despite lack of *Insr* in

macrophages and adipocytes. Thus, they represent an excellent non-obese model of insulin resistance/diabetes to address these questions. Our findings indicate that the serum cytokine profile of L1 and WT mice was similar, as was number and morphology of adipose tissue macrophages. Although we observed a 33% increase in macrophage number in the diabetic L1 group, it was far less pronounced than the 4~6 fold increase seen in diet-induced obesity or *Lep^{ob/ob}* mice (23). Therefore, these data indicate that, in the absence of obesity, insulin resistance in macrophages and adipocytes is insufficient to trigger macrophage accumulation and stimulate cytokine production.

Previous studies have also suggested that activation of liver-resident macrophages, or Kupffer cells, plays a role in obesity/insulin resistance (42; 43). Since L1 mice retained hepatic insulin resistance despite restored *Insr* signaling in hepatocytes, we asked whether the neighboring *Insr*-deficient Kupffer cells contribute to local activation of inflammation and act in a paracrine fashion to cause insulin resistance. Interestingly, L1 mice showed a moderate decrease in Kupffer cell population compared to WT and a corresponding decrease in macrophage-specific gene expression in liver (Figs. 5 and 6). It's unclear whether this represents defects in Kupffer cell development and/or infiltration in the absence of *Insr*. In summary, our data suggest that insulin resistance in Kupffer cells does not play a major role in exacerbating hepatic insulin resistance in L1 mice.

Adiponectin signaling pathways and hepatic insulin sensitivity

Both intracellular effectors of adiponectin, AMPK and PPAR α , have been shown to

regulate glucose homeostasis and lipid metabolism (44; 45). In liver, AMPK activation suppresses gluconeogenesis (46). The blunting of adiponectin-dependent AMPK activation in liver of L1 mice can thus explain our prior observations of increased HGP during hyperinsulinemic clamps in this model (18). In contrast, PPAR α expression/activity was not affected in normoglycemic L1 mice, while diabetic L1 mice exhibited decreases in PPAR α target gene expression in liver (Fig. 5). These data raise the possibility that decreases in PPAR α activation by adiponectin/AdipoR2 signaling, and consequently reduction of PPAR α -dependent fatty acid oxidation, exacerbates hepatic insulin resistance and result in diabetes. This finding is consistent with our recent demonstration that gain-of-function in the transcription factor Foxo1, as seen in insulin resistance, results in decreased levels of Ppar α targets (47).

The interactions between insulin and adiponectin are increasingly complex. In this study, we used L1 mice (18) to probe the contribution of adiponectin to insulin sensitivity in a model of tissue-specific insulin resistance. Our data indicate that insulin resistance can result in hyperadiponectinemia and adiponectin resistance. Moreover, stepwise decreases in adiponectin and AdipoR1 and R2 levels are associated with the progression from insulin resistance to overt diabetes, raising the question of whether "adiponectin failure" is an integral component of the natural history of type 2 diabetes. Further work will be required to test this hypothesis in a mechanistic fashion.

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Table 1 Metabolic characteristics of the animals

Groups	WT	normoglycemic L1	diabetic L1
Body weight (g)	25.6 ± 0.9	24.9 ± 0.9	29.3 ± 1.2 * ††
Fat mass (g)	4.8 ± 0.5	5.1 ± 0.4	5.6 ± 0.6
Fat-free mass (g)	20.7 ± 1.1	19.8 ± 0.7	23.7 ± 1.2 †
Glucose (mg/dL)	160 ± 9	154 ± 8	240 ± 23 * †
FFA (mEq/L)	0.20 ± 0.04	0.37 ± 0.06 *	0.33 ± 0.06
Triglycerides (mg/ml)	0.10 ± 0.04	0.24 ± 0.04 *	0.24 ± 0.06 *
β-OH butyrate (mM)	1.31 ± 0.15	2.29 ± 0.38 *	ND
Insulin (ng/ml)	1.0 ± 0.3	12.3 ± 2.1 **	19.3 ± 7.8 ** ††
Adiponectin (μg/ml)	8.5 ± 1.6	21.2 ± 3.1 **	15.4 ± 2.1 *
Resistin (ng/ml)	3.3 ± 0.4	4.1 ± 0.5	3.1 ± 0.4

β-OH butyrate was measured after a 16-hour fast. All other measurements were made in 3-month-old fed male mice. *= $P<0.05$ versus WT, **= $P<0.01$ versus WT. †= $P<0.05$ vs. normoglycemic L1, ††= $P<0.01$ vs. normoglycemic L1. ND: not determined.

Table 2 Plasma cytokine levels

Cytokines (pg/ml)	WT	normoglycemic L1	diabetic L1
IL-1 β	37.6 \pm 4.4	46.9 \pm 9.7	46.2 \pm 5.2
IL-2	33.6 \pm 1.4	34.0 \pm 1.5	ND
IL-6	24.5 \pm 2.1	28.3 \pm 4.7	27.8 \pm 2.2
IL-12	119.9 \pm 27.4	121.4 \pm 13.6	ND
GM-CSF	24.4 \pm 3.8	31.7 \pm 7.1	31.2 \pm 4.2
TNF α	36.4 \pm 0.9	37.7 \pm 2.2	38.6 \pm 1.6

Measurements were made in 3-month-old male mice under fed conditions. Data are mean \pm SEM. ND: not determined.

Figures

Figure 1 Metabolic profile. (A) Scatter plot of insulin vs. glucose values in 3 month-old L1 and WT male littermates. (B) Resistin values in 3- and 5-month-old mice. (C) Adiponectin values in 3-month-old mice. (D) Scatter plot of adiponectin vs. glucose values in 3-month-old mice. Blue circles=WT; red diamonds=L1; light blue line=mean of each group. **= $P < 0.001$ by 2-tailed Student's t Test.

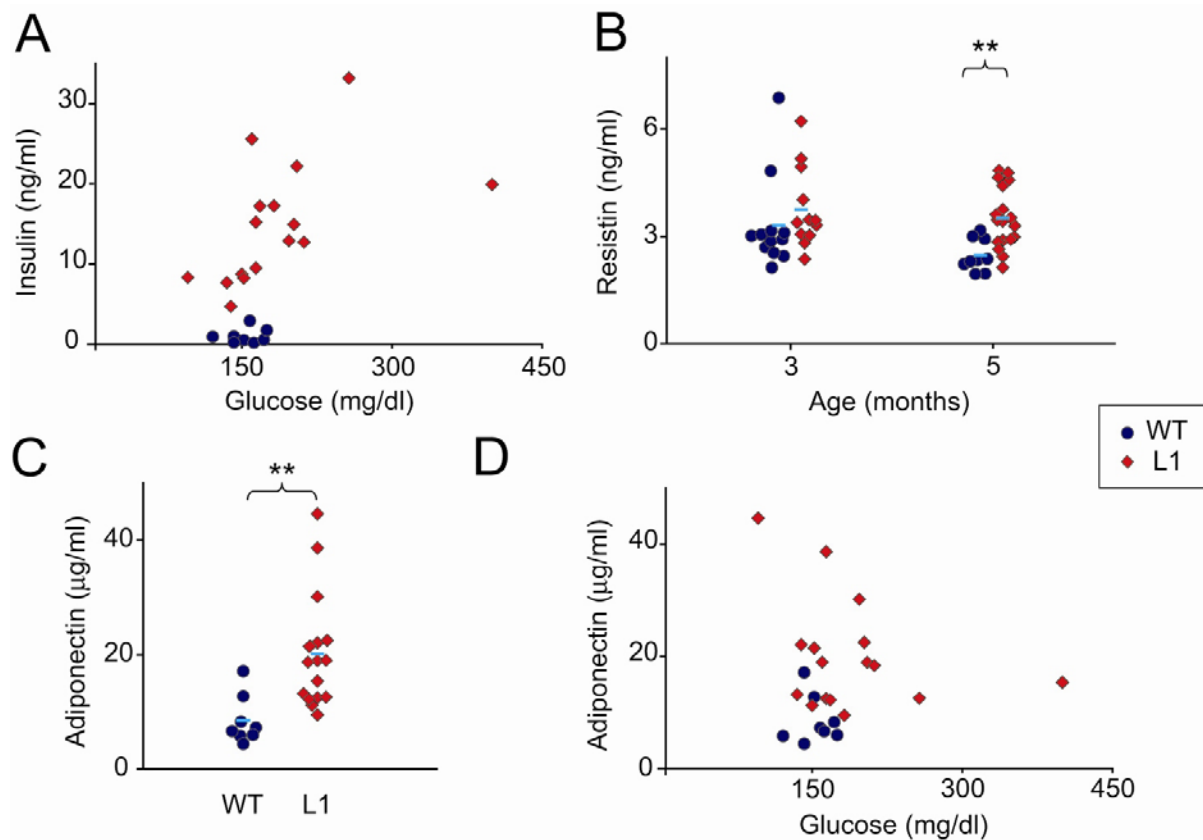


Figure 2 Effects of i.v. adiponectin administration (2 μ g/g body weight) on blood glucose levels in WT (A) and L1 (B) mice. Mice were fasted for 2 hours before injection and re-fed 8 hours after injection. n=4 for WT adiponectin injection, n=6-10 for all other groups. Data are mean \pm SEM. White circles = saline injection; black circles = adiponectin injection. *= P <0.05 vs. saline group by 2-tailed Student's t Test, **= P <0.01 vs. saline group, †= P <0.05 vs. WT adiponectin group.

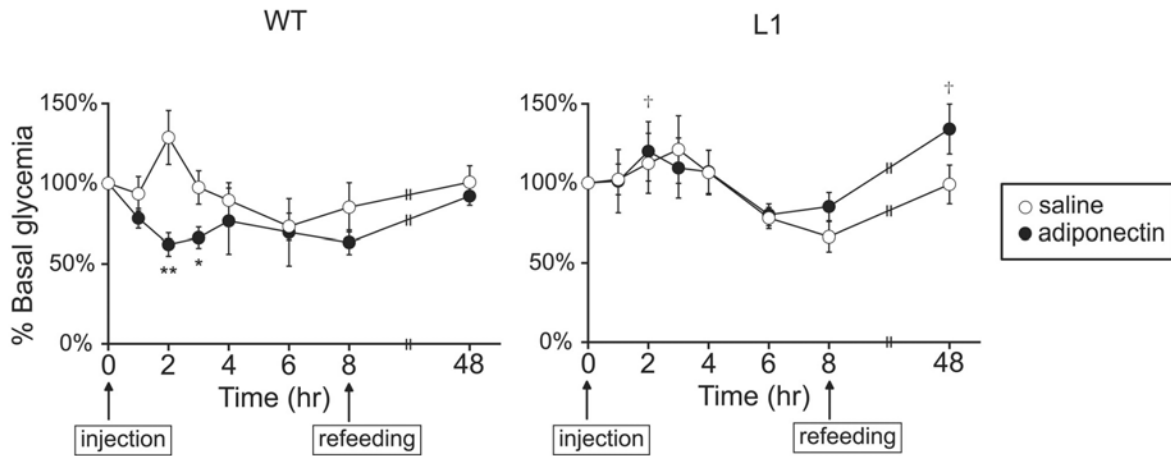
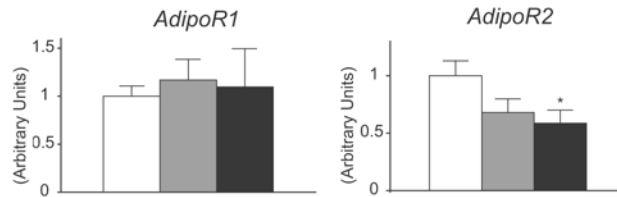


Figure 3 mRNA expression of *adipoR1* and *adipoR2* in liver (A) and hind limb skeletal muscle (B) of WT, normoglycemic and diabetic L1 mice. Mice were sacrificed and liver and hind limb skeletal muscles were dissected and snap frozen in liquid nitrogen. Total RNA was isolated. *adipoR1* and *adipoR2* mRNA expression was measured by quantitative RT-PCR and normalized against β -actin mRNA. Data are mean \pm SEM. n=8-14. White bar = WT; gray bar = normoglycemic L1; black bar = diabetic L1. *= P <0.05 vs. WT.

A Liver



B Skeletal Muscle

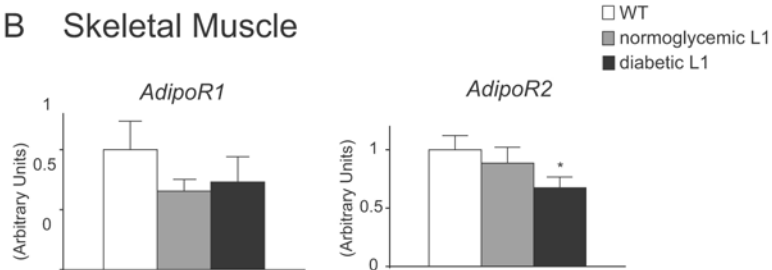


Figure 4 Adiponectin stimulated AMPK α phosphorylation in liver of 8 week-old WT and normoglycemic L1 mice. Mice were fasted for 6 hours, injected with saline or adiponectin i.v. (2 μ g/g body weight) and sacrificed 20 minutes after injection. Representative phospho-Thr12 and total AMPK α immunoblots of liver lysates are shown. Ratio of phosphorylated vs. total AMPK α was calculated and shown as mean \pm SEM. n=4-6. *= P <0.05 vs. WT saline group, **= P <0.01 vs. WT saline group.

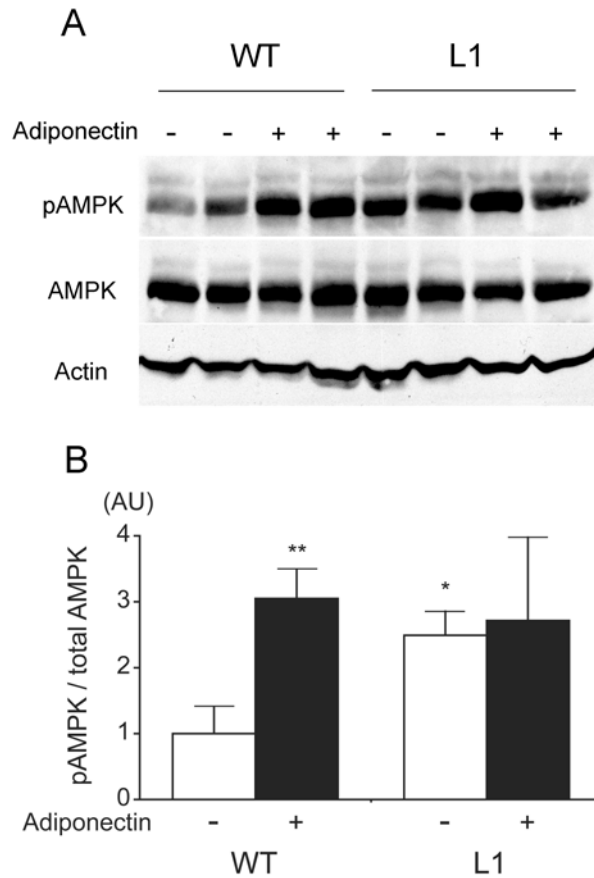
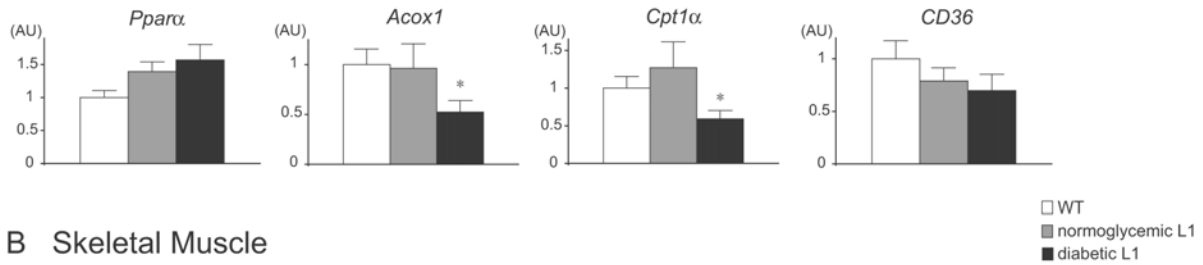


Figure 5 mRNA expression of *pparα*, *acox1*, *cpt1α* and *cd36* in liver (A) and hind limb skeletal muscle (B) of WT, normoglycemic and diabetic L1 mice. mRNA expression was measured by quantitative RT-PCR and normalized against β -actin mRNA. Data are mean \pm SEM. n=6-8. AU = arbitrary units. White bar = WT; gray bar = normoglycemic L1; black bar = diabetic L1. *= P <0.05 vs. WT.

A Liver



B Skeletal Muscle

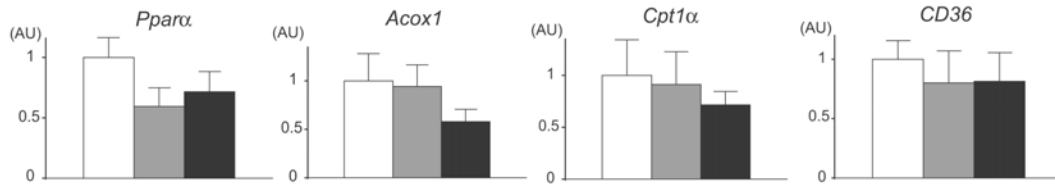


Figure 6 Immunohistochemical and gene expression analyses of liver macrophages of WT and L1 mice. Kupffer cells were immunostained with F4/80 using DAB (brown). Representative images of WT (A), normoglycemic L1 (B), and diabetic L1 mice (C) are shown. (D) Kupffer cells were quantified as number of F4/80 positive cells per 5 optical fields (40X objective) in each animal (n=8 for each group). mRNA expression of *AbcG1* (E) and *Pparg* (F) was determined by quantitative RT-PCR in liver of WT, normoglycemic and diabetic L1 mice (n=6~8). Data were normalized against β -actin mRNA. Data are mean \pm SEM. AU = arbitrary units. White bar = WT; gray bar = normoglycemic L1; black bar = diabetic L1. * P <0.05 vs. WT, ** P <0.01 vs. WT.

