A minor role for lipocalin 2 in high-fat diet-induced glucose intolerance

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Jun LS, Siddall CP, Rosen ED. A minor role for lipocalin 2 in high-fat diet-induced glucose intolerance. Am J Physiol Endocrinol Metab 301: E825–E835, 2011. First published July 19, 2011; doi:10.1152/ajpendo.00147.2011.—Adipose tissue controls energy homeostasis and systemic insulin sensitivity through the elaboration of a series of cytokines and hormones, collectively termed “adipokines.” We and others have identified Lcn2 as a novel adipokine, but its exact role in obesity-induced insulin resistance remains controversial. The aim of this study was to examine the metabolic phenotype of Lcn2−/− mice to clarify the role of Lcn2 in metabolism. Male and female Lcn2−/− and wild-type (WT) littermates were placed on either chow or high-fat diet (HFD) to characterize their metabolic phenotype. Studies included body weight and body composition, glucose and insulin tolerance tests, and adipokine expression studies in serum and in white adipose tissue (WAT). Neither chow nor HFD cohorts showed any differences in body weight or body composition. Chow-fed Lcn2−/− mice did not exhibit any difference in glucose homeostasis compared with WT mice. Fasting serum glucose levels were lower in the chow-fed Lcn2−/− mice, but this finding was not seen in the HFD cohort. Serum adiponectin, leptin, resistin, and RBP4 levels were not different between WT and Lcn2−/− on chow diet. HFD-fed male Lcn2−/− mice did display a small improvement in glucose tolerance, but no difference in insulin sensitivity was seen in either male or female Lcn2−/− mice on HFD. We conclude that the global ablation of Lcn2 has a minimal effect on obesity-associated glucose intolerance but does not appear to affect either age- or obesity-mediated insulin resistance in vivo.

insulin resistance; adipokine

OBESITY, INSULIN RESISTANCE, AND TYPE 2 DIABETES have become public health problems of epidemic proportions. In the US, more than one-half of all adults and ~25% of all children are overweight or obese (15). Type 2 diabetes can be characterized by peripheral insulin resistance in the skeletal muscle and the liver, which are important sites of glucose disposal (3, 8, 13, 15, 36, 38). Numerous studies have shown the causal relationship between excess adiposity and impaired insulin action in these tissues, often causing deleterious consequences such as hyperglycemia, hyperinsulinemia, and dyslipidemia (3, 8, 13, 15, 36, 38). Therefore, understanding how insulin sensitivity is regulated is paramount if we are to find effective targets for treatment of type 2 diabetes.

Adipose tissue was long regarded as a passive site of energy storage, but we now know that it acts as perhaps one of the largest endocrine organs in the body (5, 15, 16, 22, 42). Fat produces numerous hormones and cytokines, collectively referred to as adipokines, that play crucial roles in physiology and are involved in obesity-related complications (5, 15, 16, 34, 38, 42). These adipokines include leptin, adiponectin, tumor necrosis factor-α (TNFα), retinol-binding protein 4 (RBP4), and others (5, 15, 16, 34, 38, 42). Recently, lipocalin 2 (Lcn2) was proposed as a novel adipokine with potentially important actions on systemic glucose homeostasis (21, 26, 33, 45, 46).

Adipokines can act positively or negatively on whole body glucose metabolism. Leptin is known for its role in appetite control by anorexigenic peptide stimulation in the hypothalamus as well as the reduction of lipids in skeletal muscle, enhancing insulin sensitivity and glucose uptake (40). Adiponectin activates adenosine monophosphate (AMP)-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-α, both of which promote fatty acid oxidation and glucose uptake in skeletal muscle while decreasing inflammation (5, 38). RBP4 has been shown in both human and rodent models of obesity to be elevated in the serum and is negatively correlated with insulin sensitivity (16, 27, 30, 37, 47). Studies show that transgenic expression of RBP4 increases phosphoenolpyruvate carboxykinase (PEPCK) expression in the liver and decreases insulin receptor substrate-1 phosphorylation in skeletal muscle (16, 37).

Lcn2 is produced by adipose tissue and, in particular, white adipose tissue (WAT) (21, 31, 46). Lcn2 (24p3 in mouse, neutrophil gelatinase-associated lipocalin in human) is a 25-kDa secretory glycoprotein identified originally in mouse kidney cells and human neutrophil granules (17–20). The Lcn2 protein fold is a highly symmetrical β-barrel, which in its cross-section encloses an internal ligand-binding site (19, 20). Lipocalins bind a wide array of small hydrophobic ligands, such as bacterial siderophores (4, 19, 20). The lipocalin family is thought to regulate growth and development by binding to cell surface receptors and activating downstream targets (4, 19, 20). Lcn2 in particular has been implicated in innate immunity and apoptosis and is expressed in brain, liver, lung, kidney, adipocytes, and macrophages (4, 19). Lipopolysaccharides (LPS) and interleukin-1β can induce Lcn2 in these cells, at least in part through nuclear factor-κB binding a consensus motif in the promoter region of the Lcn2 gene (6, 28, 32, 43).

Our previous work demonstrated that Lcn2 protein expression is elevated in multiple animal models of obesity in both serum and WAT (46). Furthermore, Lcn2 acts to reduce insulin sensitivity in 3T3-L1 adipocyte and H4IIE hepatocyte cell lines (46). Studies have also shown that obese humans also have increased serum Lcn2 levels (45). Lcn2 synthesis by adipocytes is increased by several inducers of insulin resistance, including TNFα, glucocorticoids, and hyperglycemia (16, 33, 46), and is reduced by thiazolidinedione, an insulin-sensitizing agent (46). On the basis of these data, we postulated that Lcn2 might act as an adipocyte-derived mediator of insulin resistance in obesity and inflammation.

The goal of this study was the characterization of the global Lcn2−/− mouse to determine whether Lcn2 has an impact on...
whole body glucose metabolism in both lean and obese animals. On the basis of our previous findings in vitro, we hypothesized that *Lcn2*+/− mice would be protected from insulin resistance due to the absence of Lcn2 and its deleterious effects on hepatic glucose metabolism (46). While these studies were in progress, two groups published data that also addressed the role of Lcn2 in modulation of insulin sensitivity (21, 31). Interestingly, their results were strikingly discordant, with one group showing that *Lcn2*−/− mice are leaner and more insulin sensitive and the other showing that *Lcn2*−/− mice are more obese and insulin resistant (21, 31). Contrary to both prior studies, we find that only male *Lcn2*+/− mice on a high-fat diet (HFD) display a small improvement in glucose tolerance, but that ablation of Lcn2 had no effect on whole body glucose metabolism in the chow-fed mice of either sex or in female HFD-fed mice.

**MATERIALS AND METHODS**

*Animals.* All animal-related procedures described in this study were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. The lipocalin 2-knockout mouse was a gift from Dr. Alan Aderem (17). All mice used in our studies were backcrossed onto C57BL/6 for over 13 generations.

All animals were housed one to five per cage under 12:12-h light-dark conditions at an ambient temperature of 73°F (22.8°C). Chow-fed animals were given Purina Diet (no. 5008), and the HFD cohort was given a diet with 60% kcal from fat (Research Diets no. 124921i). All cohorts were generated by mating heterozygous males and females. Offspring were born in Mendelian ratios and *Lcn2*+/− offspring, and wild-type (WT) littermates were used for all studies.

**Metabolic studies.** Body weights were monitored weekly. All animals were subjected to glucose (GTTs) and insulin tolerance tests (ITTs) at the indicated ages. For GTTs mice were fasted overnight, after which a solution of 20% glucose in normal saline was injected intraperitoneally (ip) at a dose of 1 g/kg body wt. Tail bleeds were taken for whole blood glucose readings using OneTouch hand-held glucometers (Roche) at 0, 15, 30, 45, 60, and 120 min after ip injection. Ad libitum-fed glucose measurements were taken in the same manner.

ITTs were performed on *Lcn2*−/− and WT littermates after a 4-h fast in the morning on the day of the test. The mice were then given an ip injection of human insulin (Humulin; Eli Lilly) at a dose of 0.6 U/kg body wt for males on chow or HFD and 0.3 or 0.4 U/kg body wt for females on chow or HFD, respectively. Females are more prone to fatal hypoglycemia, and therefore, their insulin doses were adjusted accordingly. Again, blood glucose readings were taken at 0, 15, 30, 45, 60, and 120 min after insulin injection. For pyruvate tolerance tests (PTTs), all animals were given ip injections of pyruvate at 1 g/kg body wt dose. Sodium pyruvate (Sigma) was dissolved in PBS at a concentration of 0.43 g/ml and sterile-filtered prior to injection. Blood glucose readings were taken at 0, 15, 30, 60, and 90 min after injection.

*Echo MRI.* Echo MRI was performed on all animals to quantify body fat and lean mass (3-in-1 Composition Analyzer; Echo Medical Systems, Houston, TX). Briefly, awake animals were placed into glass MRI tubes prior to scanning. Anesthesia was not used for the Echo MRI procedure.

**Adipokine and insulin measurements.** Fed and fasted serum insulin values were obtained by tail-vein bleeds from ad libitum-fed or overnight-fasted mice using heparinized capillary tubes (Fisher Scientific). Blood samples were also collected during GTT in males on HFD to assess insulin secretion. After clotting on ice for at 1–2 h, the whole blood was centrifuged in 1.5-ml eppendorf tubes at 8,000 rpm for 10 min at 4°C. After centrifugation, the serum was collected into fresh eppendorf tubes and stored at −20°C until the insulin levels could be quantified using ELISA (Chrysal Chem, cat. no. 90080). Terminal serum levels of leptin were also quantified using ELISA (Chrysal Chem, cat. no. 90030). Terminal serum resistin (cat. no. MRSNN0) and adiponectin (cat. no. EZMADP-60K) levels were quantified using ELISA kits from Millipore. All ELISAs were performed according to the manufacturers’ protocols.

Serum RBP4 levels were quantified by Western blot with a rabbit polyclonal anti-human RBP4 antibody, which has been demonstrated by other studies to have the best sensitivity and specificity for murine RBP4 (Dako, cat. no. A0040) (37). Serum was diluted 30-fold in lysis buffer and run on a 5–20% polyacrylamide gel (Bio-Rad) for 2 h at 120 V. The gel was transferred onto a PVDF membrane (Millipore) using a Criterion transfer apparatus at 100 V for 1 h at 4°C. The membrane was blocked using 5% milk in PBS-T for 1 h before incubating overnight in primary antibody (1:500 in 1% milk–PBS-T). After three to four washes in PBS-T, the membrane was incubated in rabbit secondary antibody (GE Healthcare) at a concentration of 1:2,000 in 5% milk–PBS-T. After several washes in PBS-T, the membrane was exposed to film after reacting with SuperSignal West Pico Maximum Sensitivity Substrate (Pierce). All blots were quantified using ImageQuantTL software.

**Serum endotoxin measurement.** Serum endotoxin, or LPS, was measured using the terminal blood that was collected at the end of the study after an overnight fast. The blood was collected from all of the animals in the morning, and plasma was collected after centrifugation. The limulus amebocyte lysate QCL-1000 detection kit (Cambrex, cat. no. 50-647U) was used to measure the plasma endotoxin levels. First, all samples were diluted fivefold in endotoxin-free water and heat-inactivated at 70°C for 10 min. Then, 50 µl of the samples and standards were measured in a prewarmed, 37°C, 96-well, endotoxin-free microplate per the manufacturer’s instructions. First, 50 µl of the samples and standards were measured in a prewarmed, 37°C well was added to the wells and incubated for 16 min. Finally, 100 µl of the stop reagent, acetic acid, 25% vol/vol glacial acetic acid in water, filtered through a 0.22-µm filter, was added to all wells to halt the reaction. All reactions were carried out inside a heating block at 37°C to ensure the proper reaction temperature, and absorbance was measured at 405 nm.

**Insulin signaling in vivo.** Animals were fasted overnight and injected ip with 10 µg/kg of insulin prior to termination. Animals were euthanized 7 min after insulin injections, and their tissues were harvested immediately upon euthanization and snap-frozen in liquid nitrogen. Tissues were then homogenized in RIP-A buffer containing both MiniComplete Protease Inhibitor (Roche) and phosphatase inhibitor cocktails (Boston BioProducts). Total protein was quantified using the DC method (Bio-Rad), and all lysates were treated with Laemmli buffer and boiled for 5 min at 95°C. A total of 50 µg protein/lane was loaded into 4–20% gradient polyacrylamide gels (Bio-Rad). All gels were run at 100 V for 2 h before being transferred onto a PVDF membrane (Millipore) in Criterion transfer systems at 100 V for 1 h. All blots were blocked in 5% milk in TBS-T before being incubated overnight in primary rabbit anti-phospho-(p)-Akt (Cell Signaling Technology). The blots were then washed with TBS-T and incubated in secondary anti-rabbit antibody for 1 h. The blots were rinsed in TBS-T and incubated in SuperSignal West Pico Chemiluminescent substrate (Pierce) before being exposed to film. All blots were quantified using ImageQuantTL software.

**Gene expression study.** All RNA was harvested using Trizol (Invitrogen), and cDNA was made using a reverse-transcription kit from Applied Biosystems using 1–2 µg total RNA. Real-time quantitative PCR (qPCR) was performed using the Applied Biosystems 7900-HT qPCR apparatus, and relative expression was calculated using the ΔΔCt method. qPCR primers were as follows: CCL5, CCCCCACCATCATCACCTC (forward) and CTTCTGAGTGA-CAACAC (reverse); IL-6, TTCCATCAGTGGCCTTCTT (forward) and CAGAATTGCCATTCGCAAC (reverse); IFNβ, GCACATGCTGAGTCATCTGAG; IL-10, GTAGGTCTTTGCTGAGTGGT (forward) and CAGAATTGCCATTCGCAAC (reverse); PPARγ, CAATTCAGTGGGAGCTCTCC (forward) and CAGAATTGCCATTCGCAAC (reverse).
GGTGGAATGAGACT (forward) and AGTGGAGAGCAGTTGAG-GACA (reverse); TNFα, TGAAGGGAATGGGTGTTCAT (forward) and GGTCACGTCCAGCAGATCT (reverse); and 36B4, GAGGAATCAGATGAGGATATGGGA (forward) and AAGCAGGCTGACTTGTTGC (reverse).

Statistical analyses. Unpaired t-tests or ANOVA were applied to the data with either Bonferroni or Fisher post hoc tests. All statistical analyses were performed using the Prism Software (GraphPad, La Jolla, CA).

RESULTS

Chow-fed Lcn2−/− mice have no body weight or body composition phenotype. Weekly body weight measurements were performed on both male and female Lcn2−/− and their WT littermates on chow diet. Body weights were indistinguishable between Lcn2−/− and WT mice at 40 wk of age in both sexes (Fig. 1A). Echo MRI for body composition revealed no differences between Lcn2−/− and WT mice at 40 wk of age (Fig. 1B).

Fig. 1. Body weight and composition on chow diet. A: body weights of male and female lipocalin 2 (Lcn2−/−) and their wild-type (WT; Lcn2+/+) littermates on chow diet were measured throughout the study (n = 7). B: Echo MRI analysis of body composition on chow diet revealed no differences between Lcn2−− and WT. Data are presented as means ± SE.

Fig. 2. Serum glucose and insulin levels on chow diet. A: fed and fasted blood glucose levels of all chow animals were measured at 12 mo of age. Fasting insulin levels were significantly lower in Lcn2−/− mice compared with WT (n = 7). B: fasting and fed serum insulin levels were measured at 11 mo of age (n = 7). Data are presented as means ± SE. *P < 0.002.
differences between the two genotypes in lean or fat mass at 10 mo of age (Fig. 1B). Despite the advanced age of the cohort, there was no divergence in either body weight or lean/fat mass distribution in either Lcn2−/− or WT mice at any point in the study.

Chow-fed Lcn2−/− mice have lower fasting glucose levels. Fasting serum glucose levels showed a slight but significant reduction in male Lcn2−/− mice (Fig. 2A), whereas fed glucose levels remained the same between the two groups. Females on chow diet showed no significant difference in fed or fasted

![Glucose levels in male and female mice at 12 weeks and 9 months](image1.png)

![Glucose levels in male and female mice at 14 weeks and 10 months](image2.png)

Fig. 3. Glucose (GTTs) and insulin tolerance tests (ITTs) of chow-fed mice A: glucose tolerance tests and calculated areas under the curve (AUC) at 12 wk of age and again at 9 mo of age show no difference between Lcn2−/− and WT on chow diet (n = 7). B: ITTs and calculated AUC at 14 wk of age and again at 10 mo of age. Data are presented as means ± SE.
Neither males nor females on the chow diet exhibited differences in serum insulin concentrations between Lcn2−/− mice and WT controls in the fed or fasted state (Fig. 2B).

Chow-fed Lcn2−/− mice do not display improved glucose tolerance. Lcn2−/− mice were found to be equally glucose tolerant to their WT littermates at 12 wk and 9 mo of age on a chow diet (Fig. 3A). GTTs did not show any difference in glucose metabolism in Lcn2−/− mice compared with WT controls (Fig. 3A). We did note that there was no difference in fasting glucose levels at the start of these GTTs at 9 mo of age (Fig. 3A), although Lcn2−/− mice exhibited lower fasting glucose levels later, at 12 mo (Fig. 2A). The ITT showed that Lcn2−/− mice display insulin sensitivity comparable with their WT littermates at 14 wk and 10 mo of age (Fig. 3B).

Chow-fed Lcn2−/− mice show no differences in serum adipokine levels. We saw no differences in fasted serum resistin, leptin, or adiponectin levels (Fig. 4, A and B) in chow-fed Lcn2−/− mice compared with WT. We also examined whether any compensatory rise in RBP4 could be taking place in Lcn2−/− mice, possibly masking an insulin-sensitive phenotype. We did see a slight increase in serum RBP4 levels in the chow-fed male Lcn2−/− mice, but this was not statistically significant after Western blot quantification (Fig. 4C). Serum RBP4 levels were not different in chow-fed females (Fig. 4C).

Lcn2−/− mice on HFD show no difference in body weight or composition. We placed a second cohort of mice on a HFD (60% kcal from fat) immediately after weaning at 21 days of age. As with chow-fed mice, Lcn2−/−/− mice maintained the same body weights and body composition as WT controls throughout the study (Figs. 5, A and B). Fasting and fed serum glucose levels were the same in both Lcn2−/−/− and WT mice on an HFD at 12 wk of age (Fig. 6A). Fed and fasted insulin levels also did not differ between the two groups (Fig. 6B).

Male Lcn2−/− mice on HFD show small improvement in glucose metabolism. Glucose tolerance testing at 13 wk of age showed that high-fat-fed male Lcn2−/− mice displayed a small enhancement in their glucose clearance rates compared with WT littermates (Fig. 7A). However, male Lcn2−/− mice showed no difference in insulin sensitivity or glucoseogenesis when subjected to ITT and PTT, respectively (Fig. 7, B and C). Furthermore, the males showed no statistically significant difference in glucose-stimulated insulin secretion during the GTT (Fig. 7D). Females on HFD did not show any difference between Lcn2−/− and WT controls in their glucose tolerance, insulin sensitivity, or glucoseogenesis when tested (Fig. 7, A and C).

Lcn2−/− mice and WT littermates show no significant changes in insulin signaling on HFD. At 6 mo of age, HFD-fed Lcn2−/−/− and WT littermates were injected with insulin 7 min prior to euthanization and tissue harvest to determine insulin signaling parameters. Overall, there were no significant differences in Akt phosphorylation between male Lcn2−/− and WT mice on HFD in liver, WAT (Fig. 8A), or skeletal muscle (data not shown). WT HFD males showed modest induction of insulin-induced p-Akt in liver, whereas Lcn2−/− mice showed little induction over basal p-Akt levels (Fig. 8A). WT and Lcn2−/−/− males showed little induction of p-Akt in WAT (Fig. 8A). Overall, female Lcn2−/− mice on HFD showed slightly higher levels of Akt phosphorylation in liver, WAT (Fig. 8B), and muscle (data not shown). Female WT mice on HFD had slightly depressed Akt phosphorylation in WAT, whereas the Lcn2−/− littermates did display slightly improved Akt phosphorylation in response to insulin (Fig. 8B).

Lcn2−/− mice on HFD show differential inflammatory gene expression. Next, several proinflammatory parameters were examined in the HFD cohort. Males and females on the HFD showed no differences in serum LPS levels at 6 mo of age (Fig. 9A). Male WT did not show any differential expression in chemokine (C-C motif) ligand 5 (CCL5), interleukin-6 (IL-6), interferon-β (INFβ), or TNFα expression in the Lcn2−/− compared with WT mice (Fig. 9B). The females showed significantly lower expression of IL-6 in WAT, but CCL5, IFN-β, and TNFα expression remained similar between Lcn2−/− and WT mice (Fig. 9B).
DISCUSSION

Emerging research now implicates several members of the lipocalin family as potential players in whole body metabolism. One well-known example is serum RBP4, which has been shown to be elevated in obese human subjects and rodent models of obesity (37). When RBP4 levels are lowered in mice using fenretinide, both insulin sensitivity and glucose tolerance improve (37). RBP4 is also more highly expressed in visceral than in subcutaneous fat, implicating a role as a marker of intra-abdominal fat mass, the depot believed to exacerbate the underlying pathophysiology of the metabolic syndrome (25, 30, 47). On the other side of the ledger, Lcn13 is a newly

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**Fig. 5.** Body weight and composition of high-fat diet (HFD) cohorts. A: body weights of Lcn2<sup>−/−</sup> and their WT littermates on HFD were measured throughout the study (n = 7). B: Echo MRI analysis of body composition. Data are presented as means ± SE.

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**Fig. 6.** Metabolic phenotyping of female Lcn2<sup>−/−</sup> mice on HFD. A: fed and fasting blood glucose levels in high-fat-fed Lcn2<sup>−/−</sup> vs. WT mice at 12 mo of age; n = 7. B: fed and fasting serum insulin levels were measured at 11 mo of age (n = 7). Data are presented as means ± SE.
Fig. 7. GTT and ITT of HFD cohort. A: GTTs and calculated AUC at 13 wk of age (n = 6–11). B: ITTs and calculated AUC on males and females on HFD. C: pyruvate tolerance tests and calculated AUC on HFD males and females at 16 wk of age. Data are presented as means ± SE. D: insulin secretion was measured during GTT in HFD males. Data are presented as means ± SE. Two-way ANOVA was performed on all data with P values <0.05. *P < 0.05.
characterized family member, shown in a recent study to improve glucose tolerance and enhance insulin sensitivity when overexpressed (11). Furthermore, Lcn13 expression was demonstrated to be downregulated in rodent models of metabolic syndrome (11). Last, major urinary protein-1 is another lipocalin family member that appears to ameliorate hyperglycemia and glucose intolerance in \( db/db \) mice and rodents with diet-induced obesity and type 2 diabetes (48, 49).

We hypothesized that global Lcn2 ablation would be beneficial for diet-induced metabolic syndrome based on work we published previously that showed that Lcn2 was induced by factors that promote insulin resistance, such as TNFα and dexamethasone (24), and that knocking down Lcn2 in 3T3-L1 cells enhanced insulin-stimulated glucose uptake (46). We also demonstrated that the addition of recombinant Lcn2 protein to H4IIe cells increased the expression of both PEPCK and glucose-6-phosphatase mRNA, which led to increased glucose production (46). Studies have shown that Lcn2 circulates at higher levels in multiple rodent models of obesity, including \( ob/ob \), \( db/db \), and diet-induced obesity mouse models, as well as in obese and diabetic human subjects (27, 45, 46). Therefore, we sought to use a knockout mouse model to determine the extent to which Lcn2 contributes to insulin resistance.

Fig. 8. In vivo insulin signaling in HFD cohort. Males (A) and females (B) were injected with insulin prior to termination to assess Akt phosphorylation (p-Akt) in liver and white adipose tissue (WAT). p-Akt bands were quantified using densitometry and normalized to total Akt. Lcn2\(^{-/-}\) p-Akt levels were compared with WT levels. Data are presented as means ± SE. Two-way ANOVA was performed on all data with \( P \) values <0.05.
We determined that male Lcn2\(^{-/-}\) mice fed a HFD are somewhat protected from obesity-associated glucose intolerance, although the effect was small and not seen in females. Despite a minor improvement in glucose tolerance in the males on HFD, we saw no statistically significant differences in insulin secretion during the GTT in Lcn2\(^{-/-}\) mice compared with WT controls (Fig. 7C). This argues against the possibility of differential \(\beta\)-cell function in Lcn2\(^{-/-}\) mice, because they appear to respond with equal levels of insulin secretion during GTT. Furthermore, we were not able to find a clear effect on insulin sensitivity because neither serum insulin levels nor ITTs differed between WT and Lcn2\(^{-/-}\) mice on a HFD. Consistent with these findings, we saw no significant differences in insulin signaling in liver, skeletal muscle (data not shown), or WAT, as evidenced by similar levels of Akt phosphorylation in male Lcn2\(^{-/-}\) and WT controls on a HFD (Fig. 8). Males on a HFD in general showed impaired response to insulin irrespective of genotype, which is most likely due to the age of these animals at the time of euthanization exceeding the age or diet. Consistent with this, we see almost no corresponding effect on glucose tolerance or insulin sensitivity.

Our results differ from two other studies characterizing the metabolic phenotype of the Lcn2\(^{-/-}\) mouse that were published recently. One group (31) reported a profound difference in body weight between Lcn2\(^{-/-}\) compared with WT, with Lcn2\(^{-/-}\) mice significantly leaner than WT mice under HFD conditions. Lcn2\(^{-/-}\) mice on both chow and HFD in the Law et al. (31) study also displayed a striking improvement in whole body glucose metabolism when subjected to both GTTs and ITTs. Their study showed that Lcn2\(^{-/-}\) mice on chow diet had a minimal body weight phenotype but displayed significantly lower fasting glucose as well as fasting insulin levels beginning at 11 wk of age, an age in which mice already display high insulin sensitivity (31).

A subsequent study by Guo et al. (21) reported very different results. They found that Lcn2\(^{-/-}\) mice were significantly heavier than WT controls on both chow and HFD, with body weight differences that were apparent starting at 4 wk of age. Consistent with their greater degree of adiposity, Lcn2\(^{-/-}\) mice showed markedly impaired glucose tolerance and insulin sensitivity on both chow and HFD. They also reported that both HFD- and chow-fed Lcn2\(^{-/-}\) mice had higher fasting glucose levels starting 4 and 8 wk of age, respectively. Our study contrasts with both prior reports in that we see no effect of Lcn2 ablation on body mass or body composition regardless of age or diet. Consistent with this, we see almost no corresponding effect on glucose tolerance or insulin sensitivity.

There are a number of possible factors to consider that could explain such widely discordant phenotypes. First, we used the same Lcn2\(^{-/-}\) mice as Guo et al. (21), in which exons 2 through 5 were deleted (17). Law et al. (31) used a different Lcn2\(^{-/-}\) mouse, in which exons 1 through 6 were deleted (2). It is formally possible that this difference in targeting strategy could explain the observed phenotypic differences, although serum Lcn2 levels are clearly absent in both models (2, 17). The different approaches to generating the Lcn2\(^{-/-}\) mouse may have affected the gene deletion in unexpected ways, perhaps giving rise to truncation products or differentially spliced proteins that may have residual function, and may not be detectable with the commercial anti-Lcn2 antibodies. Guo et al. (21) used the same Lcn2\(^{-/-}\) mice as we did in this study, but the key difference between our studies appears to be body weights of the mice (17). The Guo et al. (21) study showed that Lcn2\(^{-/-}\) mice were heavier than their WT littermates at all time points in the study on both chow and HFD. This may indicate that the litters themselves were born to heavier parents or that the survival of the individuals in a litter somehow
favored larger Lcn2−/− pups, since the body weight difference appeared so early in the lives of these cohorts. As it stands, the differences in phenotype between our study and that of Guo et al. (21) seem to depend on the presence or absence of a body weight difference on chow or HFD.

Another possible confounder is diet; different HFD formulations were used in all three of these studies, which may have elicited varying phenotypes (7, 44). Housing conditions unique to each study may also affect weight gain, such as ambient temperature or the cleanliness of the mouse facilities. Given that Lcn2−/− mice are moderately immune deficient (2, 17), it is plausible that the difference between a barrier facility and a nonbarrier facility could yield markedly different phenotypes since the animals experience various levels of stress on their immune systems. Finally, genetic strain appears to be an unlikely source of variation, because all studies used mice that were extensively back-crossed onto C57Bl/6, although small effects from residual non-C57 genomic loci that were not bred off cannot be formally ruled out.

One interesting aspect of Lcn2 action that has not been addressed in any of these studies is its role in gastrointestinal mucosal regeneration. Lcn2 has been demonstrated to aid in the regeneration of the gastrointestinal mucosa in mice after chemical or physical injury by promoting cellular migration (39). The integrity of the gut and the gut microbiome have been shown by several groups to play a key role in energy and glucose homeostasis (12). Studies have shown that mice fed a HFD have an increased gram-negative/gram-positive bacterial ratio in their gut, which is associated with elevated plasma LPS levels (12). When we measured circulating LPS (endotoxin) levels in the HFD cohorts, we did not see any differences between Lcn2−/− and WT mice (Fig. 9A). It is interesting to note that these levels were considerably lower than previously reported, but given that these mice are reared in a full barrier facility throughout their lives, the low levels of LPS may reflect the overall cleanliness of the facility in which they were reared (9, 10). It would be interesting to assess serum LPS levels in the Law et al. (31) and Guo et al. (21) cohorts. LPS can trigger the release of proinflammatory cytokines when bound to CD14/TLR4 receptors present on immune cells as can trigger the release of proinflammatory cytokines when bound to CD14/TLR4 receptors present on immune cells as

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acknowledged as one causal factor in obesity-induced insulin resistance as a classic proinflammatory cytokine in many studies, but its exact contribution to glucose metabolism is still incompletely understood (14). For example, IL-6 has been shown to induce insulin resistance in adipose tissue, but acute exposure in skeletal muscle actually enhances insulin signaling and glucose metabolism in rodents (29). A 2010 study by Matthews et al. (35) reported that global IL-6 deletion mice developed systemic insulin resistance and hepatic insulin resistance. Our study showed that despite lower IL-6 expression in WAT, the female Lcn2−/− on HFD did not exhibit any improvement in glucose tolerance or insulin sensitivity.

Many members of the lipocalin family have been shown to play important roles in glucose metabolism. However, our results suggest that Lcn2 plays a minimal role, if any, on glucose homeostasis or insulin sensitivity in mice.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


Lcn2 IS A MINOR CONTRIBUTOR TO GLUCOSE METABOLISM IN VIVO

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