Lipocalin 2 deficiency impairs thermogenesis and potentiates diet-induced insulin resistance in mice

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**Objective:** Lipocalin 2 (LCN2) belongs to the lipocalin subfamily of low molecular mass secreted proteins that bind small hydrophobic molecules. LCN2 has been recently characterized as an adipose-derived cytokine; and its expression is up-regulated in adipose tissue in genetically obese rodents. The objective of this study was to investigate the role of LCN2 in diet-induced insulin resistance and metabolic homeostasis in vivo.

**Research design and methods:** Systemic insulin sensitivity, adaptive thermogenesis, and serum metabolic and lipid profile were assessed in LCN2 deficient mice fed a high fat diet or regular chow diet.

**Results:** The molecular disruption of LCN2 in mice resulted in significantly potentiated diet-induced obesity, dyslipidemia, fatty liver disease, and insulin resistance. LCN2 -/- mice exhibit impaired adaptive thermogenesis and cold intolerance. Gene expression patterns in white and brown adipose tissue, liver, and muscle indicate that LCN2-/- mice have increased hepatic gluconeogenesis, decreased mitochondrial oxidative capacity, impaired lipid metabolism, and increased inflammatory state under the high fat diet condition.

**Conclusions:** LCN2 has a novel role in adaptive thermoregulation and diet-induced insulin resistance.
Obesity is a major risk for developing insulin resistance, a hallmark of type 2 diabetes and other metabolic complications such as fatty liver, dyslipidemia and atherosclerosis. Adipose tissue plays a central role in body weight homeostasis, inflammation, and insulin resistance via regulating lipid metabolism/storage and releasing a range of adipokines/cytokines (1-4). Adipose tissue in a variety of insulin resistant states has been characterized by dysregulated lipid metabolism and altered production of adipokines/cytokines that in sum are important contributors to systemic inflammation and related metabolic disorders.

Lipocalin 2 (LCN2; also known as neutrophil gelatinase-associated lipocalin (NGAL)), a lipocalin subfamily member, has been recently identified by our group and others (5, 6) as an adipose-derived cytokine. LCN2 is a 25-kDa secreted protein initially identified from human neutrophils (7, 8) and other immune cells and tissues that are exposed to microorganisms in the respiratory and gastrointestinal tract and is present abundantly in the circulation (9). Interestingly, lipocalins have structural similarity with fatty acid binding proteins (FABPs) and both are members of the multigene family of up and down β-barrel proteins (10). Both the intracellular FABPs and the extracellular lipocalins have a clearly defined β-barrel motif that forms either an interior cavity (FABP) or a deep pit (lipocalins) that constitutes the lipid binding domain (10). The extracellular lipocalins such as LCN2, retinol binding protein (RBP) 4 and α2-microglobulin use a series of β-strands to form a globular domain with a deep depression resembling the calyx of a flower. Because of the unique structure, the lipocalins function as efficient transporters for a number of different hydrophobic ligands in extracellular milieu including a variety of retinoids, fatty acids, biliverdin, pheromones, porphyrins, odorants, steroids, and iron. RBP4, one of the extracellular lipocalins, affects glucose metabolism and insulin sensitivity (11).

Previous studies have demonstrated that LCN2 gene expression is up-regulated in adipose tissue and liver of genetically obese animals (6). Rosiglitazone administration significantly reduces LCN2 expression in adipose tissue in obese animals (6) suggesting that the protein may function as a pro-inflammatory factor. Unexpectedly, the addition of LCN2 protein to the culture media of adipocytes and macrophages leads to the suppression of TNFα- and LPS-induced cytokine/chemokine production indicating an anti-inflammatory function (6). Most strikingly, LCN2 appears to protect against TNFα-induced insulin resistance in adipocytes. Unlike RBP4, increased production of LCN2 in obesity may be a protective mechanism against inflammation and insulin resistance.

To evaluate this hypothesis, we assessed the metabolic and regulatory consequences of LCN2 deficiency. Herein we show that the ablation of LCN2 profoundly impairs adaptive thermogenesis and exacerbates high fat diet (HFD) - or age-induced insulin resistance and glucose homeostasis. LCN2 deficient mice have increased hepatic gluconeogenesis and inflammatory state, and exhibit a cold sensitive phenotype.

**RESEARCH DESIGN AND METHODS**

**Animals.** LCN2/-/- mice were kindly provided by Dr. Alan Aderem, Institute for Systems Biology, Seattle, Washington, USA, where they were originally generated by Dr. Shizuo Akira, Research Institute for Microbial Diseases, Osaka University, Japan. LCN2 -/- mice were generated by gene targeting in ES cells from mouse strain 129 and Targeted ES cells were injected into...
C57BL/6 blastocysts as described previously (12). C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). LCN2 null mice were backcrossed onto the B6 background for 10 generations before mice were used for the experiments. Heterozygous mating scheme was used to generate WT and LCN2-/- mice for the experiments.

Animals were housed in Specific Pathogen Free facility at the University of Minnesota. Animal handling were follow National Institutes of Health guidelines, and experimental procedures were approved by the University of Minnesota animal care and use committee. Age-matched male WT and LCN2-/- mice were allocated into groups (3-4 mice per cage) and fed a high fat (fat calories: 60%) diet obtained from Bio-Serv, Inc. (F3282, New Brunswick, NJ) or a regular chow diet, with free access to water for all studies. In the adaptive thermogenesis study, age-matched WT and LCN2-/- mice on RCD were exposed to 4°C, with free access to diet and water. Rectal temperature of the mice was measured at the indicated time points using MicroTherma Thermometer with rectal probe for mice (Braintree Scientific, Inc. Braintree, MA).

Metabolic Studies. During the experimental period of high fat diet feeding, glucose and insulin tolerance tests were conducted by the intraperitoneal (i.p.) injection of glucose and insulin. Mice were fasted overnight (12 hr) for glucose tolerance test (GTT) and for 6 hr for insulin tolerance tests (ITT). GTT and ITT were conducted by intraperitoneal (i.p.) injection of glucose (1 mg/g body weight) or insulin (0.75u/kg body weight) with blood collection via the tail vein at 0, 15, 30, 60, 90, and 120 min. Blood glucose was measured using an Ascensia glucometer.

Triglyceride content measurement. Lipid extraction was performed using the Bligh-Dyer method (13). Briefly, frozen liver tissue (100 mg) was homogenized in 1 ml water. Lipid was extracted using chloroform:methanol (2:1). An aliquot of the organic phase was collected and dried with nitrogen, and then dissolved in isopropanol alcohol containing 1% Triton. Triglyceride content was determined using commercially available kits (Stanbio lab, reference No.: 2150-101).

Primary mouse adipose cell isolation. Preparation of isolated adipose cells from WT and LCN2-/- mice was performed as described previously (14, 15). After mincing, epididymal fat pads were digested with collagenase (2 mg/mL solution) in digestion vials containing Krebs-Ringer bicarbonate Hepes (KRBH) buffer, pH 7.4, 200 nM adenosine, and 3.5% BSA. After a 2-hour digestion, adipose cells were separated by centrifugation at 1200 rpm for 10 min and washed twice with KRBH buffer. After the final wash, adipose cells were collected for RNA extraction.

Adipose cell size analysis. Adipose tissue was obtained from epididymal and inguinal fat pad of WT and LCN2-/- mice fed on RCD. Tissue samples (20–30 mg) were immediately fixed in osmium tetroxide and incubated in a water bath at 37°C for 48 h as described previously (16), and then adipose cell size was determined by a Beckman Coulter Multisizer III with a 400 μm aperture. The range of cell sizes that can effectively be measured using this aperture is 20–240 μm. After collection of pulse sizes, the data were expressed as particle diameters and displayed as histograms of counts against diameter using linear bins and a linear scale for the x-axis. The figures were generated using GraphPad Prism 5.01 for Windows (GraphPad Software Inc., LaJolla, CA).

Statistical Analysis. Results were expressed as mean ± standard error of mean (SEM). Differences in parameters between wild-type and LCN2-/- mice were evaluated using a two group t-test with a 0.05 two-sided significance.
level. A p-value < 0.05 was considered significant.

RESULTS

LCN2-/- mice are more susceptible to diet-induced obesity. At 3 weeks of age, male wild-type and LCN2-/- mice were fed either HFD for 12 weeks or regular chow diet (RCD). Throughout the experimental period, the body weight was significantly different between wild-type and LCN2-/- mice fed HFD (Fig. 1A) and RCD (Fig. 1B). LCN2-/- mice were significantly heavier than wild-type mice on either HFD or RCD. The weight of white and brown fat pads was significantly higher in HFD-fed (Fig. 1C) or aged LCN2-/- mice (Fig. 1D) than that in wild-type mice. Both HFD-fed and aged LCN2-/- mice showed a significantly higher weight of liver, heart, kidney, and spleen (Fig. 1E and 1F). Similar metabolic phenotypes were also observed in female LCN2-/- mice (data not shown). However, the overall growth did not appear to be significantly affected, as the body length and bone length (trabecular) were not significantly different between the genotypes (data not shown). Figure 1G and 1H demonstrated the fat cell size distributions of WT and LCN2-/- mice on RCD at 15 weeks of age, as measured by Multisizer analysis using cells isolated from osmium-fixed epididymal and inguinal adipose tissue. The average peak diameter of the large cell population was significantly larger for LCN2-/- mice compared to WT mice (Fig. 1H), indicating that LCN2-/- adipose tissue contains larger adipose cells.

LCN2-/- mice display impaired adaptive thermogenesis. Because LCN2 deficient mice gained more body weight and increased adiposity, particularly when mice were fed high fat diet or aged, we examined food intake and energy expenditure in LCN2-/- mice. An analysis of indirect calorimetry measurements showed that food intake, ambulatory activity, VO2, and RQ were not significantly different between RCD-fed WT and LCN2-/- mice at 18 weeks of age (Fig. S1 in the online appendix available at http://diabetes.diabetesjournals.org). We then assessed thermogenic activity of BAT under thermoneutral and thermal stress conditions. Body temperature was measured in an ambient temperature of 28°C and 22°C as well as during acute exposure to 4°C. WT and LCN2-/- mice at 12 weeks of age exhibited a similar body temperature in an ambient temperature of 28°C (WT, 38.34 ± 0.34; LCN2-/-, 38.2 ± 0.30). However, female LCN2-/- mice had a significantly lower rectal temperature as compared with WT mice (Fig. 2A), while male LCN2-/- mice had a trend towards decrease in rectal temperature when kept in an ambient temperature of 22°C. More strikingly, LCN2-/- mice displayed cold sensitive and could not survive when exposed to 4°C for longer than 10 hours. LCN2-/- mice (7 out of 7) died after exposed to 4°C for 12 hours, while all of WT mice (n=7) in the experiment survived. When acutely exposed to 4°C, rectal temperature of LCN2-/- mice dropped significantly within 3h as compared to WT mice (Fig. 2B). Five hour cold exposure caused a 10-fold increase in LCN2 gene expression in adipose tissue in WT mice (Fig. 2C), suggesting that LCN2 is a critical regulator of energy metabolism.

To explore the possible mechanisms for cold intolerance in LCN2-/- mice, UCP-1 mediated thermogenic activity and capacity of BAT was first evaluated under the conditions of high fat diet feeding and cold exposure. We observed that upon high fat diet feeding, LCN2-/- mice expressed significantly lower levels of UCP-1 and PGC-1α genes in BAT than WT mice (Fig. 2D). However, UCP-1 and PGC-1α expression in BAT were markedly stimulated at an even higher level in LCN2-/- mice as WT mice after exposed to 4°C for 5h (Fig. 2E). This suggests that
LCN2/-/- BAT remains normal UCP-1 dependent thermogenic activity. We next examined whether the regulation of substrate provision is altered in LCN2/-/- mice. Figure 2F showed that the protein expression levels of ATGL (adipose triglyceride lipase) in WAT were slightly reduced in LCN2/-/- mice under the HFD and cold condition as compared with WT mice. However, cold-induced HSL gene expression in BAT and WAT was completely diminished in LCN2 -/- mice (Fig. 2G). Lastly, we investigated the mitochondrial oxidative capacity of BAT as this function of BAT is critical for providing the energy for thermogenesis. Interestingly, we observed that there was a significant reduction in the expression levels of mitochondrial genes involved in mitochondrial biogenesis and oxidative function such as nuclear respiratory factor 1 (Nrf1), transcription factor A, mitochondrial (Tfam), ATP synthase 5β (ATP5β), cytochrome c oxidase 4 (COXiv), and carnitine palmitoyltransferase 1β (CPT1β) in BAT of LCN2/-/- mice fed a HFD (Fig. 2H). In addition, the expression of transcription factors PPARδ, Nrf1 and Tfam and CPT-1 gene in skeletal muscle was also significantly down-regulated in LCN2/-/- mice fed a HFD (Fig. 2D).

LCN2 -/- mice develop more severe diet-induced insulin resistance, and increased inflammatory state. To assess the effects of LCN2 deficiency on systemic insulin sensitivity, glucose and insulin tolerance tests were performed. Glucose clearance curves in response to glucose administration were significantly increased in HFD-fed (Fig. 3A), but not RCD-fed LCN2/-/- mice (Fig. 3B), indicating that LCN2 -/- mice are more glucose intolerant upon HFD feeding. In the insulin tolerance test, increased insulin-stimulated glucose disposal curve was observed in LCN2/-/- mice indicating that LCN2 -/- mice are more insulin resistant than WT controls when fed HFD (Fig. 3C) or aged (Fig. 3D). Analysis of serum metabolic parameters as shown in Table 1 demonstrated that levels of fasting serum glucose and insulin were significantly elevated, while adiponectin levels were reduced in HFD-fed LCN2 -/- mice when compared to WT controls. Serum levels of leptin and PAI-1 were not significantly changed in LCN2/-/- mice on RCD although serum leptin levels had a trend towards decrease in HFD-fed LCN2/-/- mice. After adjusted to body weight, the serum levels of glucose, insulin, and adiponectin were consistently different at the statistically significant level, indicating that the changes in glucose tolerance and insulin sensitivity after HFD in LCN2 -/- mice is not simply due to body weight effects. To further confirm that LCN2/-/- mice have decreased insulin sensitivity, in vivo insulin-stimulated Akt phosphorylation was evaluated. WT and LCN2/-/- mice on RCD were injected intraperitoneally with insulin, sacrificed after 10 min, and tissues were collected for evaluation of Akt phosphorylation. Consistently, insulin-stimulated Akt phosphorylation in liver, muscle and adipose cells was significantly reduced in LCN2/-/- mice as compared to WT mice (Fig. 4). The examination of LCN2 effect on upstream signaling components of insulin signaling pathway demonstrated that the expression levels of IRS-1 protein were markedly reduced in WAT of LCN2/-/- mice on HFD compared to WT mice (Fig. S2). This data further support that LCN2-/- mice developed more insulin resistance and LCN2 regulates insulin signaling activity likely at the upstream level.

To test the role of LCN2 in inflammatory response, expression of LCN2 and inflammatory molecules was examined in adipose tissue and liver of HFD-induced WT and LCN2-/- mice. The protein expression levels of LCN2 were decreased in adipose tissue (Fig. 5A) and bone marrow-derived macrophages (BM-MAC) of normal mice upon a high fat diet feeding (Fig. 5B).
Furthermore, the gene expression of proinflammatory cytokine TNFα and MCP-1 were up-regulated, while expression of arginase 1, an anti-inflammatory marker, was down-regulated in adipose tissue of HFD-fed LCN2-/- mice as compared to WT control mice, (Fig. 5C). Downregulation of arginase 1 and IL-10 was also observed in the liver of HFD-fed LCN2-/- mice (Fig. 5C). 

**LCN2-/- mice develop dyslipidemia and fatty liver.** To investigate whether increased liver weight in LCN2 -/- mice is associated with the development of fatty liver and dyslipidemia, liver triglyceride content and blood lipid profiles were measured. In comparison with WT controls, HFD-fed and aged LCN2 -/- mice demonstrated a significant increase in lipid accumulation as detected by Oil-Red O staining of liver section (Fig. 6A) and the increased levels of liver triglyceride (Fig. 6B). The development of more severe fatty liver disease, together with elevated fasting blood glucose levels suggest hepatic insulin resistance in LCN2 -/- mice. To elucidate hepatic glucose production in WT and LCN2 deficient mice fed HFD, mice were fasted for 18 hours and gene expression of two key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were detected by qPCR. As illustrated in Fig. 6C, expression levels of PEPCK1, PEPCK2 and G6Pase genes were significantly higher in the liver of LCN2 -/- mice than that in WT controls, suggesting that increased hepatic glucose production is attributable to hyperglycemia in LCN2 -/- mice. We next examined the hepatic capabilities for fatty acid synthesis and oxidation to explore the possible mechanism for the development of fatty liver in LCN2 -/- mice. As illustrated in Fig. 6D, the lipogenic genes sterol regulatory element binding protein-1c (SRECP-1c), Acetyl-CoA carboxylase 1 (ACC1), Spot 14 (S14), and stearoyl-CoA desaturase 1 (SCD-1) were expressed at a significantly higher level in HFD-fed LCN2 -/- mice compared to WT controls, while genes involved in fatty acid oxidation such as PPARα, CPT-1, and ACC2 were similarly expressed between genotypes (data not shown). Therefore, the development of fatty liver results primarily from the increased capability for fatty acid synthesis in the liver in LCN2 -/- mice.

**DISCUSSION**

We show here that LCN2 plays a critical role in lipid metabolism, adaptive thermoregulation, and diet-induced obesity and insulin resistance in vivo. Lack of LCN2 in mice potentiates high fat diet-induced obesity, dyslipidemia, fatty liver, glucose intolerance, and insulin resistance. We also discovered that LCN2 is a critical regulator of adaptive thermogenesis and mitochondrial oxidative function in BAT. LCN2 deficiency impairs adaptive thermogenesis and increases
the susceptibility of LCN2-/- mice to HFD-induced obesity.

Our data demonstrated that LCN2-/- mice gained more body weight when they were fed HFD or aged. Increased body fat mass together with enlarged multiple organs may contribute to the development of higher body weight in LCN2-/- mice. In particular, increased organ weights may largely account for increased body weight in LCN2-/- mice under the RCD condition. In general, WT and LCN2-/- mice at a young age did not demonstrate significantly different metabolic phenotypes when they were fed RCD. However, LCN2-/- mice developed more server systemic insulin resistance, hyperglycemia, hyperinsulinemia and hypoadiponectinemia under the HFD condition. One of the most profound phenotypic features of LCN2-/- mice is cold sensitive and lower body temperature under thermal stress conditions. It has been know for a long time that nonshivering thermogenesis is used to maintain constant body temperature and energy balance, controlling body weight, especially in small mammals (17 - 19). Both neural and hormonal signals including norepinephrine, epinephrine, thyroid hormones, glucagon, insulin, glucocorticoid, and leptin are known to regulate thermogenesis (17, 20) through different thermogenic mechanisms involving stimulating ATP utilization, increasing UCP-1 mediated proton leak and heat production, and affecting substrate availability and utilization for energy production. In rodents, BAT is the main tissue that is responsible for sympathetic nervous system regulated non-shivering thermogenesis with the involvement of norepinephrine and epinephrine (17, 20, 21). UCP-1 and PGC-1α are the key genes that mediate sympathetic thermogenic responses in BAT. Low sympathetic activity is known to be associated with obesity (22). For example, UCP-1 gene expression in BAT of ob/ob mice was reduced (23). However, BAT UCP-1 expression could be normally stimulated in response to cold environment in young ob/ob mice albeit ob/ob mice show decreased capability of utilizing fat and cold intolerance (23).

In this study, we found that five hour cold exposure leads to a significant increase in LCN2 expression in WAT, which is in agreement with the result in a recent study (24). In the absence of LCN2, mice were cold intolerant and failed to maintain the body temperature when acutely exposed to cold temperature. Interestingly, cold exposure could still significantly stimulate gene expression of UCP1 and PGC-1α in brown adipose tissue in LCN2-/- mice, suggesting that cold-induced sympathetic thermogenesis remained intact in the absence of LCN2. The role of leptin in thermogenesis has been well documented and its thermogenic effect is considered to be primarily central (25). The peripheral role of leptin in thermogenesis is controversial. For example, leptin administration has been reported to rescue cold-induced hypothermia in the absence of UCP-1 in leptin and UCP-1 double knockout mice (26, 27), while the opposite results were perceived in the other two independent studies (28, 29). In LCN2-/- mice on RCD, serum leptin levels were not different from that in WT mice albeit serum leptin levels had a trend towards decreased in LCN2-/- mice in response to HFD feeding. Additionally, WT and LCN2-/- mice on RCD did not exhibit a difference in body temperature in thermoneutral conditions. Our results suggest that the central control of body temperature may function normally. Therefore, it seems rather unlikely that leptin could be the major contributor to cold intolerance in LCN2-/- mice; leptin may be only partially involved in LCN2-regulated thermogenesis.

In addition to hormonal signals, defects in key metabolic regulators of substrate availability for energy production, ATP synthesis and mitochondrial biogenesis
Lipocalin 2 deficient mice develop insulin resistance and cold intolerance

... and oxidative function could all possibly affect thermogenic function. Indeed, studies in mice with the genetic disruption of key metabolic regulators have proved this assumption; mice without expressing ATGL could similarly result in cold intolerance (30). In the present study, the disruption of LCN2 completely diminished cold-induced HSL gene expression in BAT and WAT, whereas ATGL expression in WAT was only slightly affected in LCN2-/- mice under both the HFD and cold conditions. Since HSL-/- mice are not cold sensitive (31), HSL is not an important regulator for adaptive thermogenesis. Therefore, the effect of changes in ATGL levels on the supply of substrates for energy production for thermogenesis is minimal in LCN2-/- mice. On the other hand, the production of the energy necessary for thermogenesis requires normal mitochondrial oxidative function; and mitochondrial dysfunction could have a significant impact on adaptive thermogenesis. This hypothesis has been supported by a previous study in estrogen related receptor α (EERα) knockout mice. In EERα deficient mice, mitochondrial biogenesis and oxidative function in BAT was disturbed; and mice failed to maintain body temperature when exposed to cold (32). To this end, we further investigated the mitochondrial oxidative capacity of BAT and skeletal muscle. Interestingly, we found that the expression levels of mitochondrial genes involved in mitochondrial biogenesis and oxidative function such as Nrf1, Tfam, ATP5b, COXiv, and CPT1b were significantly reduced in BAT of LCN2-/- mice fed a HFD. In the skeletal muscle, the expression of β oxidation genes PPARδ and CPT-1 as well as mitochondrial genes Nrf1 and Tfam was also down-regulated in HFD-fed LCN2-/- mice.

All the above data together suggest that mitochondrial metabolism is dysfunctional in BAT and muscle of LCN2-/- mice, which mainly contributes to decreased thermogenic capacity, decreased energy expenditure, and the high susceptibility to diet-induced obesity and insulin resistance during the thermal stress conditions (mice have been living at 22°C). In acute cold, muscle shivering thermogenesis is considered to be the primary mechanism for a body temperature defense (21, 33, 34). BAT non-shivering thermogenesis participates in combating the cold temperature only when the extra capacity is already recruited for using during the acute cold exposure (35). Therefore, cold intolerance (acute exposure to 4°C for 5 h) in LCN2-/- mice may largely result from decreased oxidative capacity in skeletal muscle and BAT as well as decreased ATGL expression in WAT.

Our results clearly demonstrated that insulin responsiveness in muscle, adipose tissue and liver was reduced, indicating systemic insulin resistance in LCN2-/- mice. Furthermore, fasting for 18 hours induces significantly higher levels of expression for PEPCK and G6Pase in the liver of HFD-fed LCN2-/- mice, supporting that the increased hepatic glucose production is an important contributor to fasting hyperglycemia in LCN2-/- mice. Dyslipidemia such as hypertriacylglycerolemia and hypercholesterolemia is another prominent feature perceived in LCN2-/- mice. This phenomenon is likely due to the impaired regulation of lipid metabolism in adipose tissue and liver in LCN2-/- mice. We showed that LCN2-/- mice on RCD had higher serum levels of triglyceride, but lower serum triglyceride levels when fed high fat diet as compared with WT mice. Lower serum TG in HFD-fed LCN2-/- mice could be due to the increased TG synthesis and decreased VLDL (or TG) secretion into the circulation resulting from the development of fatty liver in LCN2-/- mice. This explanation is strengthened by a previous study in LIRKO (insulin receptor knockout) mice with hepatic insulin resistance and 50% decrease in serum TG levels (36).
As reported in our previous in vitro study, LCN2 is capable of suppressing inflammatory response of macrophages to LPS stimulation (6). We speculated that LCN2 possesses anti-inflammatory role in metabolic regulation. In this in vivo study, we show that LCN2 expression is down-regulated in adipose tissue and BM-MAC in HFD-induced obese mice. In the absence of LCN2, the expression of proinflammatory cytokines such as MCP-1 and TNFα are up-regulated in adipose tissue, while expression levels of anti-inflammatory markers arginase 1 and IL-10 are decreased in adipose tissue and liver, suggesting an increased inflammatory state in LCN2-/- mice. These results further suggest that LCN2-/- mice are more susceptible to high fat diet-induced proinflammatory response than WT mice.

In summary, herein we discovered a novel role for LCN2 in energy metabolism, adaptive thermogenesis and insulin resistance. Mice lacking LCN2 display impaired adaptive thermogenesis and deteriorated diet-induced obesity and insulin resistance. Decreased mitochondrial oxidative capacity in BAT and skeletal muscle may be the primary mechanisms for the development of diet-induced insulin resistance and cold sensitive in LCN2-/- mice. However, the mechanisms for how LCN2 regulates mitochondrial function and lipid metabolism remain to be further identified. It is likely that the binding of LCN2 to small hydrophobic molecules such as retinoic acids, fatty acids and other unknown ligands or to the cell-surface receptor such as megalin or low density lipoprotein-related protein 2 (LRP2) (37) could potentially involve or mediate LCN2 effects in metabolism. Our findings provide new insight into the role of LCN2 in lipid metabolism and metabolic homeostasis. In this manner, LCN2 may be a potential therapeutic target for controlling obesity-associated type 2 diabetes and metabolic complications.

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Lipocalin 2 deficient mice develop insulin resistance and cold intolerance

REFERENCES
Lipocalin 2 deficient mice develop insulin resistance and cold intolerance

Lipocalin 2 deficient mice develop insulin resistance and cold intolerance

Table 1. Serum metabolic parameters in wild-type and LCN2-/- mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Glucose (mg/ml)</th>
<th>Insulin (ng/ml)</th>
<th>Leptin (ng/ml)</th>
<th>Adiponectin (µg/ml)</th>
<th>PAI-1 (ng/ml)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>RCD</td>
<td>82.33±3.27</td>
<td>0.56±0.15</td>
<td>3.33±1.56</td>
<td>14.78±1.85</td>
<td>3.59±0.51</td>
</tr>
<tr>
<td>LCN2-/-</td>
<td>RCD</td>
<td>100.38±4.99*</td>
<td>0.79±0.11</td>
<td>3.06±0.95</td>
<td>15.46±1.02</td>
<td>3.22±0.62</td>
</tr>
<tr>
<td>WT</td>
<td>HFD</td>
<td>157.10±19.55</td>
<td>1.85±0.36</td>
<td>24.23±3.86</td>
<td>16.50±0.34</td>
<td>2.83±0.42</td>
</tr>
<tr>
<td>LCN2-/-</td>
<td>HFD</td>
<td>238.13±6.45#</td>
<td>3.02±0.57#</td>
<td>16.62±2.07</td>
<td>13.50±0.88#</td>
<td>3.43±0.79</td>
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</table>

Measurements were performed on 6h fasted mice fed RCD and HFD (n = 9-11). Values are expressed as Mean ± SEM. * P < 0.01, comparison between WT and LCN2-/- mice on RCD; # P < 0.01, comparison between WT and LCN2-/- mice on HFD.

Table 2. Plasma lipid profile in wild-type and LCN2 null mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Triglycerides (mg/dl)</th>
<th>Cholesterol (mg/ml)</th>
<th>HDL Direct (mg/dl)</th>
<th>LDL Direct (mg/dl)</th>
<th>NEFA (mEq/l)</th>
<th>Beta-hydroxybutyrate (mmol/L)</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>RCD</td>
<td>89.76±3.39</td>
<td>168.14±5.56</td>
<td>85.26±3.59</td>
<td>7.07±0.29</td>
<td>1.31±0.03</td>
<td>0.36±0.03</td>
</tr>
<tr>
<td>LCN2-/-</td>
<td>RCD</td>
<td>100.07±4.05*</td>
<td>191.82±6.17*</td>
<td>94.99±4.75</td>
<td>6.71±0.38</td>
<td>1.58±0.07</td>
<td>0.27±0.05</td>
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<tr>
<td>WT</td>
<td>HFD</td>
<td>110.23±3.34</td>
<td>194.72±4.59</td>
<td>90.5±2.23</td>
<td>6.74±0.33</td>
<td>1.23±0.07</td>
<td>0.39±0.04</td>
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<tr>
<td>LCN2-/-</td>
<td>HFD</td>
<td>99.34±1.78</td>
<td>197.26±14.82</td>
<td>86.63±2.79</td>
<td>17.38±3.19*</td>
<td>1.11±0.04</td>
<td>0.48±0.02</td>
</tr>
</tbody>
</table>

Measurements were performed on 6h fasted mice fed RCD and HFD (n = 9-11). Values are expressed as Mean ± SEM. * P < 0.01, comparison between WT and LCN2-/- mice on RCD; # P < 0.01, comparison between WT and LCN2-/- mice on HFD.

Figure Legends

Figure 1. Growth curve, weight of white and brown fat pads and organs in LCN2-/- mice. Growth curve of LCN2-/- mice on HFD (n = 10-14) (A) and RCD (n = 13) (B). Fat pad weight of LCN2-/- mice on HFD at 16 weeks of age (C) and on RCD at 30 weeks of age (D). Organ weight of LCN2-/- mice on HFD at 16 weeks of age (E) and on RCD at 30 weeks of age (F). The data are represented as mean ± SEM. * P < 0.05; ** P < 0.01. Fat cell size distribution of mice on RCD diet at 15 weeks of age (G and H).

Figure 2. Adaptive thermogenesis in LCN2-/- mice. Basal rectal body temperature of 8 week old male and female WT and LCN2 mice on RCD (n = 7-10) measured during the daytime in an ambient temperature of 22°C (A). Body temperature curve of LCN2-/- mice (n = 8) and WT mice (n = 7) exposed to 4°C (B). Gene expression of LCN2 in WAT of WT mice exposed to 4°C for 5h (C). Gene expression of UCP-1 and PGC-1α in BAT and PPARδ, CPT-1, Tfam, and Nrf1 in skeletal muscle of HFD-fed mice (n = 6) (D). Gene expression of UCP-1 and PGC-1α in BAT (E). ATGL protein expression in WAT under the HFD and cold conditions (F). Gene expression of HSL in BAT and WAT of mice exposed to 4°C for 5h (G). Gene expression of mitochondrial genes in BAT of LCN2-/- mice under the HFD condition (H). The data are represented as mean ± SEM. * P < 0.05; ** P < 0.01.

Figure 3. Assessment of insulin sensitivity. Glucose (A) and insulin (C) tolerance tests conducted in LCN2-/- mice on HFD (n = 10-12, age = 14-15 weeks). Glucose (B) and insulin (D) tolerance tests conducted in LCN2-/- mice on RCD (n = 10-12, age = 28-29). The data are represented as mean ± SEM. Experiments were repeated on two independent sets of mice, yielding similar results. * P < 0.05; ** P < 0.01.
Lipocalin 2 deficient mice develop insulin resistance and cold intolerance

Figure 4. In vivo insulin-stimulated Akt phosphorylation in LCN2-/- mice. Representative Western blot analysis of Serine-phosphorylated Akt in muscle, liver, and isolated primary adipose cells from LCN2-/- (n = 4 per group) and WT mice (n = 4 per group) under basal and in vivo insulin-stimulated conditions.

Figure 5. Protein expression levels of LCN2 and gene expression of proinflammatory molecules in LCN2-/- mice. Protein expression levels of LCN2 in adipose tissues of WT mice fed on RCD and HFD (A). Protein expression levels of LCN2 in bone marrow macrophages isolated from WT mice fed on RCD and HFD (B). Gene expression of inflammatory molecules in adipose tissue and liver of WT and LCN2-/- mice fed on HFD (C). The data of gene expression are represented as mean ± SEM. * P < 0.05.

Figure 6. Liver triacylglyceride content in LCN2-/- mice. (A) Oil-red O staining of liver section of LCN2-/- mice. (B) Liver triacylglyceride levels in LCN2-/- mice on regular chow (n = 6-8, age = 30 weeks) and HFD (n = 11, age = 15-16 weeks). (C) The mRNA expression of gluocneogenic genes in liver (n = 6). (D) The mRNA expression of lipogenic genes in liver (n = 6). The data are represented as mean ± SEM. * P < 0.05; ** P < 0.01.
Lipocalin 2 deficient mice develop insulin resistance and cold intolerance

Figure 1
Lipocalin 2 deficient mice develop insulin resistance and cold intolerance

Figure 2

- Panel A: Bar graph showing rectal temperature (°C) in male and female mice with LCN2+/+ and LCN2-/-. 
- Panel B: Line graph showing rectal temperature (°C) over time (min) for LCN2+/+ and LCN2-/- mice. 
- Panel C: Bar graph showing LCN2 expression in WAT at 22°C and 4°C. 
- Panel D: Bar graph showing target gene/actin (AU) for muscle and BAT with PPARγ, CPT-1, Nrfl, Tfr1, UCP-1, PGC-1α. 
- Panel E: Bar graph showing target gene/actin (AU) for BAT at 22°C and 4°C with LCN2+/+, LCN2-/-, UCP-1, PGC-1α. 
- Panel F: Western blot showing ATGL and β-actin expression under HFD and Cold conditions. 
- Panel G: Bar graph showing target gene/actin (AU) for HSL at 22°C and 4°C with LCN2+/+, LCN2-/-, BAT, WAT. 
- Panel H: Bar graph showing target gene/actin (AU) for ATP5β, COXiv, CPT1β, Tfr1, Nrfl with LCN2+/+, LCN2-/-.
Figure 4

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Muscle

Liver

Adipocyte
Lipocalin 2 deficient mice develop insulin resistance and cold intolerance

Figure 5
Lipocalin 2 deficient mice develop insulin resistance and cold intolerance

Figure 6

[Image of Figure 6 showing tissue staining and bar graphs comparing LCN2+/+ and LCN2-/- mice under RCD and HFD conditions, along with gene expression analysis in liver tissue.]