Lipocalin 2 Regulates Brown Fat Activation via a Nonadrenergic Activation Mechanism*

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Background: Lipocalin 2 is a recently characterized adipokine/cytokine known to be a critical regulator of metabolic homeostasis.
Results: Lcn2 deficiency impairs BAT activation by reducing peroxisomal and mitochondrial oxidation of lipids and the recruitment of functional brown adipocytes.
Conclusion: We demonstrate a novel role of Lcn2 in oxidative metabolism and BAT activation via a nonadrenergic pathway.
Significance: Our findings advance understanding of the adrenergic independent mechanism for BAT activation.

In this study, we report that lipocalin 2 (Lcn2), a recently characterized adipokine/cytokine, is a novel regulator of brown adipose tissue (BAT) activation by modulating the adrenergic independent p38 MAPK-PGC-1α-UCP1 pathway. Global Lcn2 knock-out (Lcn2−/−) mice have defective BAT thermogenic activation caused by cold stimulation and decreased BAT activity under high fat diet-induced obesity. Nevertheless, Lcn2−/− mice maintain normal sympathetic nervous system activation as evidenced by normal catecholamine release and lipolytic activity in response to cold stimulation. Further studies showed that Lcn2 deficiency impairs peroxisomal and mitochondrial oxidation of lipids and attenuates cold-induced Pgc1α and Ucp1 expression and p38 MAPK phosphorylation in BAT. Moreover, in vitro studies showed that Lcn2 deficiency reduces the thermogenic activity of brown adipocytes. Lcn2−/− differentiated brown adipocytes have significantly decreased expression levels of brown fat markers, decreased p38 MAPK phosphorylation, and decreased mitochondrial oxidation capacity. However, Lcn2−/− brown adipocytes have normal norepinephrine-stimulated p38 MAPK and hormone-sensitive lipase phosphorylation and Pgc1α and Ucp1 expression, suggesting an intact β-adrenergic signaling activation. More intriguingly, recombinant Lcn2 was able to significantly stimulate p38 MAPK phosphorylation in brown adipocytes. Activating peroxisome proliferator-activated receptor γ, a downstream effector of PGC-1α, by thiazolidinedione administration fully reverses the BAT function of Lcn2−/− mice. Our findings provide evidence for the novel role Lcn2 plays in oxidative metabolism and BAT activation via an adrenergic independent mechanism.

Adipose tissue plays a central role in metabolic homeostasis, inflammation, and insulin resistance. The main functions of white adipose tissue (WAT) are to regulate lipid storage and mobilization, glucose homeostasis, and inflammation; many WAT functions in metabolism and insulin sensitivity are exerted by adipose-derived adipokines and cytokines (1, 2). Adipose tissue dysfunction is the major contributor to the development of obesity and its associated metabolic disorders. In contrast to WAT, brown adipose tissue (BAT) has recently been known to exist in adult humans and exhibit high metabolic activity (3–12). More importantly, BAT plays a potential role in metabolic regulation; activation of BAT has a significant influence on glucose and lipid homeostasis (13). A decline in BAT activation has been associated with metabolic dysregulation in obesity and diabetes. Thus, activating BAT function is an attractive therapeutic approach for obesity and obesity-related metabolic complications in humans. Increasing sympathetic nervous system (SNS) tone has been known as one of the therapeutic approaches to activate BAT thermogenesis, thereby promoting weight loss. However, this approach causes nonspecific activation of SNS leading to cardiovascular side effects that prevent the therapeutic use of adrenergic activators. Therefore, recent attempts have been aimed at identifying nonadrenergic activators of BAT thermogenesis as potential novel therapeutic targets for obesity and diabetes (14). To this end, it is of great importance to fully understand the nonadrenergic regulation of BAT activation.

Multiple metabolic processes determine the efficiency of energy production and BAT thermogenesis, i.e. fuel mobilization from storage tissues to BAT, lipolysis, and lipogenesis in BAT, intracellular delivery of substrates (fatty acids) from lipid droplets to peroxisome and mitochondria for oxidation, and

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3 The abbreviations used are: WAT, white adipose tissue; Lcn2, lipocalin 2; BAT, brown adipose tissue; PPAR, peroxisome proliferator-activated receptor; SNS, sympathetic nervous system; TAG, triacylglycerol; β-AR, β-adrenergic receptor; NE, norepinephrine; Iso, isoproterenol; VLCFA, very long chain fatty acid; HFD, high fat diet; Rosi, rosiglitazone; 5V, stromal vascular; AMPK, AMP kinase; HSL, hormone-sensitive lipase.
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UCP1-mediated proton leak and heat production. These metabolic processes become highly active during cold adaptation. Both fatty acid synthesis and oxidation are simultaneously increased in BAT, which constitutes a feed-forward cycle allowing us to continuously provide substrates for thermogenesis during cold adaptation (15). Among these processes, mitochondrial function and UCP1-mediated uncoupling directly contribute to the efficiency of fuel utilization and energy production, thereby playing an indispensable role in energy metabolism. Cold stimulation significantly increases the number of mitochondria in brown adipocytes of animals (16, 17).

It is well established that the β-adrenergic cAMP-PKA pathway is the major mediator of adaptive thermogenesis in BAT by activating PGC-1α and UCP1, which control mitochondrial biogenesis and thermogenesis. Additionally, studies have demonstrated that p38 MAPK is the key downstream target of β-AR-PKA pathway activation and is activated in response to cold stimulation (18). The activation of p38 MAPK is essential for PGC-1α and UCP1 expression by adrenergic stimulation (19). In addition to β-adrenergic stimulation, natriuretic peptides (20), insulin (21–23), and cytokines such as TNFα and interleukin 1 have been known as activators of p38 MAPK (24). This nonadrenergic activation of the p38 MAPK-PGC-1α-UCP1 pathway may have great significance in anti-obesity and diabetes therapeutics. However, the mechanisms and regulators involved in this oxidative and thermogenic pathway in BAT have not been fully understood.

Lipocalin2 (Lcn2), also named neutrophil gelatinase-associated lipocalin (NGAL), is a newly identified adipokine with abundant expression in adipose tissue (25, 26). As a member of the multigene family of up and down β-barrel proteins, Lcn2 has structural similarity to other member proteins, such as fatty acid-binding proteins and retinol-binding proteins (27). We have recently characterized Lcn2 as a critical regulator of obesity, glucose and lipid homeostasis, and insulin resistance (28–31). The primary focus of this study is on the role and mechanism of Lcn2 in BAT activation and function. We report that Lcn2 has a novel role in BAT activation; Lcn2 deficiency impairs mitochondrial oxidative metabolism, thermogenic function of brown adipocytes, as well as adrenergic independent activation of the p38 MAPK-PGC-1α-UCP1 pathway.

EXPERIMENTAL PROCEDURES

Animal Studies—Mice were housed in a temperature-controlled room at ~22 °C on a 12:12-h light/dark cycle in the animal facility at the University of Minnesota. Animal handling followed National Institutes of Health guidelines, and experimental procedures were approved by the University of Minnesota Animal Care and Use Committee. Control (wild type) and global Lcn2 knock-out (Lcn2−/−) mice were maintained on a C57BL/6 background by a heterozygous breeding scheme, as described previously (28). In the cold adaptive study,agematched wild type (WT) and Lcn2−/− mice on a regular chow diet were exposed to 4 °C for 4 h, with free access to water. Mice were individually housed and had no access to food during the whole period of cold exposure. In the rosiglitazone (Rosi) rescue studies, WT and Lcn2−/− mice at 10–12 weeks of age were subjected to oral gavage of Rosi (10 mg/kg body weight/day) for 2 weeks, followed by the exposure to 4 °C for 4 h. Rectal temperature of mice was measured every 30 min using the Micro Therma Thermometer (Braintree Scientific, Braintree, MA). After cold exposure, mice were sacrificed immediately; blood and tissue samples were collected for various assessments.

Isolation and Differentiation of Primary BAT Stromal-Vascular Cells—Isolation of primary stromal vascular (SV) cells was performed as described previously (32). BAT was removed from wild type and Lcn2−/− mice, minced, and digested with Krebs-Ringer bicarbonate HEPES buffer containing 2 mg/ml collagenase. After 1.5-h digestion, SV cells were separated from floating adipocytes by centrifuging at 1200 rpm for 5 min and washed with KRBH buffer twice. After the final wash, SV cells were plated on 6-well plates and cultured in DMEM containing 20% fetal bovine serum and 100 IU/ml penicillin/streptomycin (Invitrogen) until confluence. Cells were then treated with the differentiation mixture consisting of DMEM, 10% fetal bovine serum (Sigma), 100 IU/ml penicillin/streptomycin (Invitrogen), 115 μg/ml methylisobutylxanthine (Sigma), 1 μg/ml insulin (Sigma), 100 ng/ml dexamethasone (Sigma), 125 μM indomethacin (Sigma), and 1 nM 3,3′,5-triiodo-l-thyronine sodium (Sigma). Three days later, the differentiation mixture was replaced with DMEM containing 10% fetal bovine serum, 100 IU/ml penicillin/streptomycin, 1 μg/ml insulin, and 1 nM 3,3′,5-triiodo-l-thyronine sodium. The cultures were continued for another 6 days. On day 9 of differentiation, differentiated brown adipocytes were used for the experiments.

BAT TAG Analysis—TAG extraction and composition analysis were performed as described previously (28). Snap-frozen BAT tissues were homogenized in sterile water and extracted twice with chloroform/methanol. The chloroform layer was isolated and dried under nitrogen gas, and lipids were dissolved in Triton X-100. For analysis of TAG composition, lipid extracts were separated with TLC, and bands corresponding to TAG were scraped and methylated with HCl/methanol. Fatty acid methyl esters were extracted with multiple hexane washes followed by GC analyses with a fused silica capillary column (Supelco Omegawax, model 122-7032), 30 m × 0.25-mm inner diameter × 0.25-μm film thickness and a Hewlett-Packard Agilent 5890 GC system with flame ionization detector. The temperature program was as follows: 50 °C with a 2-min hold; ramp, 10 °C/min to 250 °C with a 15-min hold. Constant pressure of 20 p.s.i. was applied throughout the run of 37 min per sample. Analyses were initiated by injection of 1 μl of sample at a split ratio of 20:1 and injector temperature of 250 °C. The FID temperature was set at 300 °C with air and hydrogen flow rates of 433 and 37 ml/min. Specific fatty acid methyl esters were identified based on retention time using a reference standard purchased from Nu-Chek Prep, Inc.

Triglyceride Content Measurement—Lipid extraction was performed using the Bligh-Dyer method as described previously (28). BAT (100 mg) was homogenized in water. The homogenates were transferred to a glass tube and mixed with chloroform and methanol (chloroform/methanol 2:1), followed by the centrifugation at 1700 rpm for 5 min at 4 °C. The lower
chloroform phase was transferred to a new glass tube, dried down with nitrogen, and resuspended in 1 ml of chloroform. An aliquot of a 100-μl sample was dried down with nitrogen one more time and resuspended in 100 μl of isopropyl alcohol with 1% Triton X-100. The sample was kept at room temperature for at least 1 h and vortexed occasionally to fully resuspend lipids. Triglyceride content was finally measured with a commercially available kit (Stanbo Laboratory, Boerne, TX) and normalized to protein content.

**Fatty Acid Oxidation in Adipocytes**—SV cells were isolated from BAT of WT and Lcn2−/− mice and induced to differentiate into brown adipocytes as described above. Differentiated brown adipocytes were cultured in 0.5% FBS/DMEM, 5 mM glucose, and 50 μM L-carnitine overnight. The next morning, media were changed to serum-free DMEM, 5 mM glucose, 0.1% fatty acid-free BSA, and 1 mM carnitine. After 2 h, cells were exposed to 250 μM [1-14C]oleate for 90 min. One subset of wells was harvested to measure label in the acid-soluble metabolite fraction (pulse), and the media were changed to label free media in another subset of wells, and acid-soluble metabolites were quantified 3 h later (chase).

**Electron Microscopy**—One to two cubic millimeters of brown adipose tissue from WT and Lcn2−/− mice after 4 h of exposure to 4 °C was collected and fixed immediately in 1–2 ml of 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1M sodium cacodylate buffer and post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer overnight at 4 °C. Samples were washed three times with 0.1 M sodium cacodylate buffer and post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer. After three washes in distilled water, samples were dehydrated using a 25–100% acetone gradient and then infiltrated with 1:2 acetone/Embed 812 resin mixture for 2 h. Finally, tissue sections were infiltrated with 100% resin and embedded in 100% resin, followed by incubation at 58 °C for 24 h to polymerize the resin.

**Sectioning**—Embedded samples were trimmed and sectioned on a Leica UC6 Ultramicrotome (Leica Microsystems, Vienna, Austria). Thin sections (60–70 nm) were obtained and collected on a 200 mesh copper grid (Electron Microscopy Sciences) using a perfect loop (Electron Microscopy Sciences).

**Staining**—Grids were stained with 5% uranyl acetate for 20 min and Sato’s lead citrate for 6 min. These sections were observed under a JEOL 1200 EX II transmission electron microscope (JEOL Ltd., Tokyo, Japan). Images were obtained using a Veleta 2Kx2K camera with iTEM software (Olympus SIS, Munich, Germany).

**Analysis**—A qualitative and quantitative assessment was performed. All tissue sections were blindly examined; criteria include mitochondrial size, shape, cryptation, and matrix densities as well as lipid droplets. For the quantitative analysis, 10 cells were randomly selected from each WT and Lcn2−/− sample (four mice per genotype were included). The number of mitochondria and lipid droplets in each cell was tallied. Average areas of the mitochondria and lipid droplets were calculated from a total of 100 mitochondria. All the measurements were acquired using iTEM software (Olympus SIS). Dimensions were compared between both the wild type and knock-outs.

**Measurement of Serum Levels of Glucose, Lactate, Fatty Acid, Glycerol, and Catecholamines**—Mice serum glucose levels were detected with Autokit Glucose (Wako Diagnostics, Richmond, VA). Lactate and fatty acid levels were determined with the lactate assay kit (Biovision, Mountain View, CA) and free fatty acid quantification kit (Biovision). Serum and media glycerol levels were detected with free glycerol reagent (Sigma). Serum and BAT catecholamine levels were detected with 2-CAT ELISA Fast Track (Rocky Mountain Diagnostics, Colorado Springs, CO) following the instructions provided by the manufacturer.

**Adipose Lipolysis Assay**—WAT (100 mg) or BAT (50 mg) were collected from overnight-fasted mice and minced into small pieces. Minced tissues were washed twice with PBS in a 12-ml tube and cultured in 1 ml of KRB for WAT or 0.5 ml of KRB for BAT for 2 h. After removal of KRB buffer, tissue explants were cultured in 1 ml of fresh KRB containing 2% fatty acid-free BSA and 0.1% glucose with or without 10 μM isoproterenol (Iso) or NE. Culture medium was collected after 3 h of incubation with or without Iso or NE. Free fatty acids and glycerol release were measured in aliquots from incubation buffer using the NEFA C Kit (Wako) and free glycerol reagent (Sigma), respectively.

**Glucose Uptake Assay**—Uptake of 2-deoxy-D-[3H]glucose (Amersham Biosciences) was measured in differentiated brown adipocytes as described previously (25). Briefly, brown adipocytes at day 8 of differentiation were serum-starved in KRB buffer supplemented with 0.5% BSA and 2 mM/liter sodium pyruvate, pH 7.4, for 3 h, followed by incubation with or without 173 nmol/liter insulin for 30 min at 37 °C. Glucose uptake was initiated by the addition of 2-[^3H]deoxy-d-glucose to a final assay concentration of 100 μmol/liter for 5 min. The incorporated radioactivity was determined by scintillation counting. Nonspecific 2-deoxyglucose uptake was measured in the presence of 20 μmol/liter cytochalasin B and subtracted from the total glucose uptake assay to obtain specific uptake.

**NAD/NADH Assay**—The levels of cellular NAD+ and NADH in differentiated brown adipocytes were determined using the NAD+/NADH assay kit (Sigma) according to the manufacturer’s instructions. Briefly, after washing twice in PBS, brown adipocytes at day 8 of differentiation were harvested with 500 μl of extraction buffer. After centrifugation, half of the supernatant was heated at 60 °C for 30 min to decompose NAD. Both native and heated samples were then added to a 96-well plate for the measurement. Total NAD and NADH levels were calculated according to a standard curve, and the ratio of NAD/NADH was calculated using the formula (NAD − NADH)/NADH.

**Quantitative Real Time RT-PCR**—Total RNAs were extracted from tissues and cells using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was performed using SYBR GreenER qPCR SuperMix universal kit (Invitrogen) with an ABI 7500 real time PCR system (Applied Biosystems). Results were analyzed using the software supplied with the 7500 system and presented as levels of expression relative to that of controls after normalizing to β-actin.
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using $\Delta \Delta C_t$ method. Statistical significance was determined by two-tailed Student’s $t$ test.

**Immunoblotting**—Tissue samples were homogenized and solubilized in RIPA buffer (Sigma). Cell lysates were prepared in lysis buffer containing 25 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 25 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1% Nonidet P-40 and protease inhibitor mixtures (Roche Diagnostics). Protein concentrations of homogenized samples and cell lysates were measured using the bichinonic acid method (Pierce). Equal amounts of proteins were separated on SDS-PAGE and immunoblotted with anti-Lcn2 (R&D Systems, Minneapolis, MN), anti-UCP1, anti-HSL, anti-phospho-HSL (Ser-563), anti-phospho-p38 MAPK (Thr-180/Tyr-182), anti-p38 MAPK (Cell Signaling Technology), and anti-actin antibodies according to the recommendations of the manufacturers. After incubation with primary antibodies, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase. ECL Western blotting detection systems (GE Healthcare) were used to detect antibody reactivity.

**Histology**—Brown adipose tissues were fixed in 10% neutral buffered formalin (Thermo Scientific, Rockford, IL) for 24 h and embedded in paraffin. After deparaffinization and rehydration, tissues were sectioned into 4 μm and stained with hematoxylin and eosin (H&E) using standard techniques.

**RESULTS**

**Lcn2 Deficiency Impairs Adaptable Thermogenesis by Cold Stimulation**—Global Lcn2 knock-out (Lcn2$^{-/-}$) mice were cold-sensitive and failed to maintain their body temperature when acutely exposed to 4 °C (Fig. 1A). Four-hour cold exposure caused a significant up-regulation of Lcn2 protein expression in BAT in WT mice (Fig. 1B). After a 4-h cold exposure, the levels of serum glucose were unaltered in WT mice but were markedly reduced in Lcn2$^{-/-}$ mice (Fig. 1C). In contrast, serum lactate levels were significantly increased in Lcn2$^{-/-}$ mice compared with WT controls (Fig. 1D). Serum fatty acid levels did not differ between the two genotypes (data not shown). The assessment of glycolysis and gluconeogenesis demonstrated that Lcn2$^{-/-}$ mice had significantly lower liver glycocontent than WT mice after a 4-h cold exposure (Fig. 1E). The mRNA expression levels of gluconeogenic genes glucose-6-phosphatase (G6pase) but not phosphoenolpyruvate carboxykinase 1 (Pepck1) in liver were increased in WT and Lcn2$^{-/-}$ mice to a similar extent in response to cold stimulation (data not shown).

Lcn2 deficiency does not affect sympathetic nervous system activation by cold stimulation. The SNS plays a key role in mediating central control of thermogenesis, primarily promoting glucose uptake and UCP1 expression in BAT as well as lipolysis in BATs and WATs to meet the increased energy demand during cold adaptation (33). We therefore determined the response of SNS activation to cold stimulation in Lcn2$^{-/-}$ mice. After 4 h of exposure to 4 °C, Lcn2$^{-/-}$ mice released similar or even higher levels of catecholamine compared with WT mice as determined by serum levels of noradrenaline and adrenaline (Fig. 1F). We then further evaluated β-adrenergic signaling activation by cold stimulation. As shown in Fig. 1G, HSL phosphorylation at Ser-563 in BAT and WAT was more significantly stimulated by cold in Lcn2$^{-/-}$ mice compared with WT mice. The basal levels of glycerol release from BAT and WAT explants isolated from Lcn2$^{-/-}$ mice were slightly lower than those from WT mice (Fig. 1, H and I), whereas NE- or Iso-stimulated glycerol release was not different between WT and Lcn2$^{-/-}$ mice (Fig. 1, H and I). Furthermore, TAG hydrolyase activity (data not shown) and Adg1 protein expression (data not shown) in BAT were not changed in Lcn2$^{-/-}$ mice under either normal or cold exposure conditions. In the analysis of lipogenic gene expression, there was no significant difference in the mRNA levels of fatty-acid synthase (Fasn), stearoyl-CoA desaturase 1 (Scd1), and fatty acid elongase 3 (Elov3) in BAT between WT and Lcn2$^{-/-}$ mice in either the normal or cold condition (data not shown). The above results indicate that Lcn2$^{-/-}$ mice have normal adrenergic response in mobilizing energy substrates.

**Lcn2 Deficiency Reduces UCP1 Protein and PGC-1α Gene Expression in BAT during Cold Exposure and HFD Feeding**—We examined the expression of Ucp1 and thermogenic genes, the markers of metabolically active BAT in Lcn2$^{-/-}$ mice in response to cold stimulation. Compared with WT mice, the UCP1 protein levels were not significantly altered in Lcn2$^{-/-}$ BAT under the normal physiological condition. However, the cold-induced UCP1 protein increase was significantly blunted in Lcn2$^{-/-}$ BAT (Fig. 2A). Interestingly, UCP1 protein levels were decreased in Lcn2$^{-/-}$ BAT after cold exposure compared with Lcn2$^{-/-}$ controls at 22 °C (Fig. 2A). Unlike UCP1 protein, Ucp1 mRNA levels were increased after cold exposure in Lcn2$^{-/-}$ BAT, although this increase was attenuated (Fig. 2B). This result suggests that UCP1 protein degradation is increased in response to cold in Lcn2$^{-/-}$ BAT via an unknown mechanism. Additionally, we found that the mRNA levels of Pgc1a (Fig. 2C) and Prdm16 (Fig. 2D) were markedly lower in BAT of Lcn2$^{-/-}$ mice than that of WT mice after 4 h of cold exposure, suggesting that cold-induced thermogenic programming, especially UCP1 activation and mitochondrial biogenesis, is impaired in Lcn2$^{-/-}$ mice.

To further provide evidence for the role of Lcn2 in BAT function, we examined the BAT activity in Lcn2$^{-/-}$ mice under the conditions of HFD-induced obesity. We showed that Lcn2$^{-/-}$ mice on HFD had a significantly lower rectal temperature as compared with WT mice (Fig. 2E) when kept in an ambient temperature of 22 °C. Upon HFD feeding, Lcn2$^{-/-}$ mice expressed significantly lower levels of UCP1 protein (Fig. 2F) and Pgc1a gene (Fig. 2G) in BAT than WT mice. Moreover, H&E staining showed that Lcn2$^{-/-}$ mice had significantly enlarged and lipid-filled brown adipocytes in BAT compared with WT mice upon HFD feeding (Fig. 2H).

Previous studies suggest that M2 macrophage activation may play a role in adaptive thermogenesis (34). Because Lcn2 has been implicated in the regulation of immune response (25, 35), we sought to address whether inflammatory signaling is changed in BAT of Lcn2$^{-/-}$ mice serving as a potential mechanism. In the first experiment, we examined the expression of M2 and M1 macrophage markers in BAT of mice in response to cold exposure. As shown in Fig. 2 I, 4 h of exposure to 4 °C significantly increased the expression of Arg1, Il10, Clec10a,
and Mrc1 to a similar extent in both WT and Lcn2−/− BAT. In the second experiment, we cultured WT and Lcn2−/− stromal-vascular cells isolated from WT and Lcn2−/− BAT and induced them to differentiate into brown adipocytes as will be further described later. We examined the effect of IL-4 on the induction of M2 macrophage markers in differentiated brown adipocytes. Consistently, WT and Lcn2−/− brown adipocytes had a similar response to IL-4 stimulation of Arg1, Il10, and Mrc1 expression (Fig. 2f).

**Figures**

**Figure 1.** Cold-induced activation of BAT thermogenesis is impaired in Lcn2−/− mice. A, body temperature in Lcn2+/+ mice during acute cold exposure (n = 9–12). B, Lcn2 protein expression in BAT of normal mice by cold stimulation (each lane represents an individual animal). C, serum glucose levels (n = 5–8); D, lactate levels (n = 5–8); E, liver glycogen content (n = 5–7); F, serum catecholamine levels (n = 7–9). G, HSL phosphorylation at Ser-563 in BATs and WATs during cold exposure (each lane represents an individual animal). Glycerol release from BAT explants (H) and WAT explants (I) are in response to NE or Iso, respectively. The data are presented as mean ± S.E. Experiments were repeated on two to four independent sets of mice, yielding similar results. *, p < 0.05; **, p < 0.01. * indicates comparison between genotypes.
FIGURE 2. Cold- and HFD-induced thermogenic activation of BAT in Lcn2−/− mice. A, cold-induced UCP1 protein expression in BAT (each lane represents an individual animal); B, cold-induced mRNA expression of UCP1; C, PGC-1α (n = 4–5 mice); D, PRDM16 (n = 4–5 mice); E, rectal temperature of HFD-fed WT and Lcn2−/− mice (n = 14); F, UCP1 protein expression in BAT of Lcn2−/− mice (each lane represents an individual animal); G, mRNA expression of PGC-1α and PRDM16 in BAT of Lcn2−/− mice; H, H&E staining of BAT of Lcn2−/− mice; I, mRNA expression of M2 macrophage markers in BAT of cold-induced mice (n = 4–5 mice); J, mRNA expression of M2 macrophage markers in differentiated brown adipocytes with IL-4 treatment. The data are presented as means ± S.E. The experiments were repeated 2–3 times with different sets of animals and SV cell cultures, yielding similar results. *, p < 0.05; **, p < 0.01. * indicates comparison between genotypes.
showed that mitochondria have fewer cristae in Lcn2−/− brown adipocytes (Fig. 3A). Additionally, large lipid droplets accumulated (Fig. 3A) in Lcn2−/− brown adipocytes, whereas small lipid droplets were observed in WT brown adipocytes after 4 h of cold exposure, which is in line with the higher triglyceride contents of BAT (Fig. 3B) in Lcn2−/− mice compared with WT controls. Moreover, Lcn2−/− brown adipocytes have an increased lipid area (data not shown) and decreased mitochondria number per cell area (Fig. 3C), which may result from the hypertrophy of brown adipocytes in Lcn2−/− mice as the diameter of Lcn2−/− brown adipocytes was markedly increased when compared with that of WT cells (Fig. 3D). Consistent with this result, the expression of genes controlling mitochondrial biogenesis and oxidative function such as Pgc1a (Fig. 2C), Nrf1, CoxIV, Cox8b, and Cidea was significantly decreased in Lcn2−/− BAT under the cold-stimulated condition (Fig. 3F). This suggests that Lcn2 deficiency does not affect muscle mitochondrial oxidation.

**Lcn2 Deficiency Reduces Peroxisomal Metabolism of Very Long-chain Fatty Acids in BAT during Cold Exposure**—To fully explore the potential defects that contribute to the development of cold-induced hypertrophic and thermodynamically functional brown adipocytes in Lcn2−/− mice, we examined the fatty acid profile of TAG in BAT at 22 and 4 °C. Interestingly, we found that the portion of VLCFAs, i.e., C20:0, C20:1, and C22:1 fatty acids, was significantly higher in Lcn2−/− mice than in WT mice under the cold conditions at 4 °C (Fig. 4, A and B) but not at 22 °C (Fig. 4, C and D). This result suggests that there is a cold-associated defective adaptation in VLCFA metabolism in Lcn2−/− BAT, which is indicative of inefficient peroxisomal function of BAT during cold adaptation in Lcn2−/− mice. To provide additional supporting evidence, we examined the gene expression of peroxisomal acyl-CoA oxidase (Acox1), a key peroxisomal rate-limiting enzyme, in BAT during cold exposure. As illustrated in Fig. 4E, the mRNA expression levels of Acox1 were increased in BAT of WT mice after 4 h of cold exposure.
However, this cold-induced response was significantly diminished in Lcn2−/− mice.

Lcn2 Deficiency Impairs Thermogenic Activation and Oxidative Metabolism of Brown Adipocytes—As we have shown above, BAT Lcn2 protein levels were significantly increased during the 4-h cold exposure, suggesting that Lcn2 is important for the regulation of BAT thermogenic activation. Because mitochondrial remodeling and biogenesis occur during the differentiation of brown adipocytes (36), it is likely that Lcn2 is essential for the recruitment of functional brown adipocytes, thereby affecting the thermogenic capacity of BAT. To test this hypothesis, we assessed the cell autonomous role of Lcn2 in mitochondrial biogenesis and the recruitment of functional brown adipocytes. We isolated SV cells from BAT of WT and Lcn2−/− mice and induced them to differentiate into brown adipocytes, followed by the assessments of metabolic activities. As shown in Fig. 5A, the morphological differentiation of brown adipocytes was not significantly different between WT and Lcn2−/− SV cell cultures. To validate the brown adipocyte characteristics of these cells from WT mice, we examined the expression levels of brown adipocyte markers. Compared with undifferentiated SV cells at day 0, brown adipocytes at day 7 of differentiation showed markedly increased expression of brown adipocyte markers, including Ucp1, Cidea, Cox8b, Prdm16, CoxIV, and Pparg (data not shown). More importantly, differentiated brown adipocytes expressed significantly higher levels of Ucp1 and Pgc1a when compared with primary differentiated inguinal adipocytes from WT mice (data not shown). Interestingly, Lcn2−/− differentiated brown adipocytes had significantly lower expression levels of Ucp1, Pgc1a, Cidea, Prdm16, CoxIV, and Ppara than WT brown adipocytes (Fig. 5B).

To provide the functional evidence for mitochondrial oxidation capacity in brown adipocytes, we performed the glucose uptake and oxidation of 14C-labeled fatty acids to assess the metabolic activity of differentiated brown adipocytes lacking Lcn2. As shown in Fig. 5C, the uptake of 2-deoxyglucose was significantly increased in the basal state in Lcn2−/− brown adi-
In agreement with increased glucose uptake, glucose levels were decreased in the culture medium of Lcn2−/− brown adipocytes (Fig. 5D). However, the lactate released from Lcn2−/− brown adipocytes was significantly increased when compared with WT cells (Fig. 5E), suggesting an increased anaerobic glycolysis of glucose. Moreover, Lcn2−/− brown adi-
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Adipocytes had significantly reduced capability to oxidize oleate from either exogenous (pulse) or endogenous (chase) sources (Fig. 5, F and G). Additionally, we measured the redox state (the ratio of NAD+/NADH), a good indicator of ETC activity. At day 8 of differentiation, NAD+ and NADH levels were detected in differentiated brown adipocytes. Compared with WT cells, the ratio of NAD+/NADH was significantly lower in Lcn2−/− differentiated brown adipocytes (Fig. 5H). All these data support the fact that Lcn2 has a critical role in regulating the recruitment of functional brown adipocytes in a cell autonomous fashion.

Lcn2 Deficiency Impairs Adrenergic Independent Activation of p38 MAPK Signaling in BAT—β-Adrenergic-PKA-p38 MAPK signaling is the major pathway that mediates cold-induced Pgc1α and Ucp1 expression (37). In particular, p38 MAPK is essential for β-adrenergic activation of Ucp1 gene expression (19). We then examined the activation status of p38 MAPK signaling in BAT of Lcn2−/− mice during cold exposure. Intriguingly, the results showed that cold-induced p38 MAPK phosphorylation in BAT was markedly attenuated in Lcn2−/− mice (Fig. 6A), suggesting the disruption of p38 MAPK pathway activation in BAT of Lcn2−/− mice. Consistent with that observation, the results from in vitro SV cell cultures showed that the phosphorylation of p38 MAPK was significantly decreased in Lcn2−/− brown adipocytes particularly under the insulin-stimulated condition when compared with WT control cells (Fig. 6B). As we showed earlier, cold-induced catecholamine release and HSL phosphorylation in WAT and BAT did not differ between WT and Lcn2−/− mice, suggesting that Lcn2 deficiency does not affect β-AR signaling.

It is known that β-AR signaling is the major activator of p38 MAPK. To specifically test whether Lcn2 is required for the adrenergic activation of the p38 MAPK signaling pathway, we determined the direct effect of NE on the phosphorylation of PKA, HSL, p38 MAPK, and AMPK as well as Pgc1α and Ucp1 expression in Lcn2−/− brown adipocytes. Differentiated brown adipocytes were treated with NE in the absence of insulin for 1 h for the protein phosphorylation or 24 h for the gene expression. As illustrated in Fig. 6, C–E, Lcn2 deficiency had no impact on NE stimulation of the thermogenic pathway. NE stimulated the phosphorylation of PKA, HSL, p38 MAPK, and AMPK (Fig. 6, C and D) as well as Ucp1 and Pgc1α expression (Fig. 6E) in Lcn2−/− brown adipocytes as efficiently as it did in WT cells. These results provide the strong evidence that Lcn2 regulates the nonadrenergic activation of the p38 MAPK signaling pathway and the transcription of PGC-1α and UCP1 in brown adipocytes. To provide the direct evidence for Lcn2 regulation of p38 MAPK phosphorylation, we treated brown adipocytes with recombinant Lcn2 for 24 h and examined the activation of p38 MAPK phosphorylation. Our results demonstrated that the treatment of recombinant Lcn2 led to a significant increase in p38 MAPK phosphorylation in both WT and Lcn2−/− brown adipocytes (Fig. 6F).

Activation of PPARγ Rescues Adaptive Thermodynamics in Lcn2−/− Mice—PPARγ is a key downstream effector of PGC-1α. PPARγ agonist has been known to be a potent inducer of UCP1 expression in brown adipocytes (38) as well as a regulator of mitochondrial biogenesis (39, 40). Our data have suggested that the disruption of the PGC-1α upstream (p38 MAPK) pathway activation is the primary defect affected by Lcn2 deficiency. To provide additional evidence supporting this conclusion, we determined whether directly activating PPARγ, which bypasses the p38 MAPK pathway, can rescue the BAT mitochondrial oxidation and cold-intolerant phenotype in Lcn2−/− mice. Mice were first treated with rosiglitazone (10 mg/kg/day), the synthetic ligand for PPARγ, via oral gavage for 25 days, followed by a 5-h exposure at 4 °C. As shown in Fig. 7, A and B, Rosi treatment did not change body temperature during cold exposure in WT mice, but it completely reversed cold intolerance in Lcn2−/− mice. Rosi treatment also led to the significant improvement of serum parameters in Lcn2−/− mice, such as increased liver glycogen content (Fig. 7C), serum glucose levels (Fig. 7D), decreased serum lactate (Fig. 7E), and free fatty acid levels (Fig. 7F). Moreover, the mRNA expression of Pgc1α, Prdm16, Nrf-1, and CoxIV was significantly up-regulated by Rosi treatment in Lcn2−/− BAT under cold conditions (Fig. 7, G and H). All these data suggest that the impaired p38 MAPK-PGC-1α-PPARγ activation could be the important contributor to the impaired metabolic activation of brown adipocytes and thermodynamics in Lcn2−/− mice.

DISCUSSION

Here, we show that Lcn2 has a critical new role in mitochondrial oxidative metabolism and BAT activation via a nonadrenergic mechanism. In mice lacking Lcn2, cold stress fails to efficiently increase Ucp1/Pgc1α expression and stimulate peroxisomal and mitochondrial oxidation of fatty acids in BAT. Additionally, Lcn2−/− mice have decreased BAT activity under the HFD-induced obesity. Moreover, the in vitro studies provide the direct evidence that Lcn2 regulates metabolic activation of brown adipocytes in a cell autonomous fashion; Lcn2 is essential for the adrenergic independent activation of p38 MAPK signaling pathway in brown adipocytes.

The SNS plays a key role in central regulation of adaptive thermogenesis and metabolic adaptation. However, Lcn2 deficiency does not seem to affect the SNS activation and adrenergic response of BATs and WATs to cold stimulation. This conclusion is made based on the following lines of evidence. Lcn2−/− mice had normal cold-stimulated catecholamine release. Lcn2−/− mice displayed normal cold-induced HSL phosphorylation in WATs and BATs. Although the basal levels of glycerol release from iWAT are slightly but significantly lower in Lcn2−/− mice, the NE- or Iso-stimulated lipolysis (glycerol release) in WATs and BATs was not evidently different between the two genotypes.

A high content of mitochondria and mitochondrial UCP1 is the unique characteristic of BAT that directly associates with the activation of BAT. During cold adaptation, mitochondrial biogenesis is increased to meet the increased energy oxidation and heat production in BAT. In addition to mitochondria, the role of peroxisome in energy metabolism and cold-stimulated thermogenesis in BAT has been gaining attention. The peroxisome is the primary site for VLCFA and branched-chain fatty acid catabolism. Cold exposure and thyroid hormone are known to stimulate peroxisome proliferation and peroxisomal β-oxidation (41) (42). The activity of peroxisomal ACOX1 in

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BAT can be increased by 10-fold in rats after exposure to 5 °C for 4 weeks, and it promotes thermogenesis (43) (44). PGC-1α has been known to play a central role in regulating mitochondrial biogenesis, thermogenic gene (Ucp1) expression, and energy expenditure. In a recent study, PGC-1α has also been shown to have a significant role in controlling peroxisome biogenesis in BAT (45). Our results showed that Lcn2 deficiency significantly reduces the response of Pgc1a and Ucp1 expression to the cold stimulation. The decreased mitochondrial number per cell area combined with a significant reduction in cold-induced expression of mitochondrial genes in BAT of Lcn2−/− mice suggest a reduced cold induction of mitochondrial biogenesis, which is one of the major contributors to the reduced thermogenic activation of Lcn2−/− BAT during cold exposure. Additionally, we discovered that Lcn2−/− mice had a higher proportion of VLCFA incorporated into TAG of BAT; this altered fatty acid profile was only observed under cold-stimulated conditions. These findings, along with the blunted

**FIGURE 6. Activation of p38 MAPK signaling in BAT and brown adipocytes.** A, cold-induced p38 MAPK phosphorylation in BAT; B, p38 MAPK phosphorylation in WT and Lcn2−/− primary differentiated brown adipocytes treated with or without insulin (INS) during the past 24 h. Phosphorylation of PKA and HSL (C) and p38 MAPK and AMPK (D) in brown adipocytes treated with NE for 1 h min in the absence of insulin. E, Ucp1 and Pgc1a gene expression in primary differentiated brown adipocytes treated with NE for 24 h; F, p38 MAPK phosphorylation in brown adipocytes treated with recombinant Lcn2 for 24 h. The data are presented as mean ± S.E. Experiments were repeated on two to four independent sets of mice, yielding similar results. *, p < 0.05; ##, p < 0.01. * indicates comparison between genotypes. ## indicates comparison between treatments.
cold induction of Acox1, a rate-limiting enzyme of the peroxisomal β-oxidation, in Lcn2−/− BAT suggest that Lcn2 deficiency also affects the peroxisomal function, which could be attributed to the dysregulation of PGC-1α. Moreover, the up-regulation of genes involved in fatty acid esterification such as Gpat1 and Gpat3 in Lcn2−/− brown adipocytes (data not shown) supports the fact that impaired peroxisomal function promotes nonmetabolized VLCFAs to incorporate into TAG leading to brown adipocyte hypertrophy in Lcn2−/− mice. Our results from the study in Lcn2−/− mice under the HFD condition provide further evidence supporting the role of Lcn2 as a thermogenic regulator of BAT function. In line with the changes in response to cold stimulation, Lcn2−/− mice upon HFD feeding had significantly lower body temperature, decreased Pgc1a and Ucp1 gene expression in BAT, and brown adipocyte hypertrophy when compared with WT mice.

Next, we sought to understand how Lcn2 affects BAT thermogenic activation. Acute cold exposure significantly up-regulates BAT Lcn2, suggesting that local Lcn2 is directly involved in the regulation of thermogenic signaling activation in BAT.
We hypothesized that Lcn2 is important for the functional activation of brown adipocytes, thereby determining the thermogenic capacity of BAT. To this end, we addressed whether Lcn2 has a cell autonomous effect and directly regulates the recruitment of functional brown adipocytes or the thermogenic activation of brown adipocytes. The ability of SV cells derived from BAT of Lcn2−/− mice to differentiate into functional brown adipocytes was assessed. We found that Lcn2 deficiency did not significantly alter the morphological differentiation of brown adipocytes in primary SV cell cultures. However, Lcn2−/−-differentiated brown adipocytes display much less metabolic activity compared with WT brown adipocytes, as evidenced by the decreased expression of brown adipocyte markers, including Ucp1, Pgc1α, Prdm16, CoxIV, and Ppara. Furthermore, we evaluated the mitochondrial oxidation of glucose and fatty acids in Lcn2−/− brown adipocytes. Interestingly, we found that Lcn2−/− brown adipocytes had a high activity of anaerobic glycolysis, suggesting a decreased mitochondrial oxidation capacity. Consistent with this observation, Lcn2−/− brown adipocytes had significantly decreased fatty acid oxidation and a reduced NAD+/NADH ratio. All of these data provide strong evidence that Lcn2 has a critical role in regulating UCP1-mediated thermogenesis of BAT by control of the functional activation of brown adipocytes. In the absence of Lcn2, BAT cannot be fully activated by thermogenic (cold) stimulation and displayed low levels of thermogenic activity. A recent report by Paton et al. (46) provides another line of evidence supporting that Lcn2 plays a positive role in mitochondrial oxidation function. In their study, recombinant Lcn2 promotes oleate oxidation in 3T3-L1 adipocytes; injection of Lcn2 increases energy expenditure in regular chow diet-fed mice.

β-AR signaling pathway is the major mediator of cold-induced activation of PGC-1α and UCP1 expression in BAT. Studies have demonstrated that p38 MAPK is a central signaling component of many pathways that regulate PGC-1α and UCP1 expression, including β3-adrenergic activation, exercise and cytokines, and insulin-signaling pathways (47). In this study, we found that cold-induced p38 MAPK phosphorylation was significantly attenuated in BAT of Lcn2−/− mice. In addition, our results from in vitro SV differentiated brown adipocytes demonstrated that p38 MAPK phosphorylation was significantly decreased in Lcn2−/− brown adipocytes under the insulin-stimulated condition (Fig. 6B). Insulin is known to play a role in regulating thermogenesis by enhancing the effect of thermogenic activator retinoic acid on Ucp1 gene expression (36), and our previous studies showed that insulin significantly stimulates Lcn2 expression and secretion (48). Therefore, it is reasonable that the effect of Lcn2 deficiency on p38 MAPK pathway activation is amplified in the insulin-stimulated state. Our data have provided the following lines of evidence strongly supporting that Lcn2 regulates p38 MAPK signaling through a nonadrenergic signaling pathway. First, we showed that the sympathetic and thyroid hormonal signaling in Lcn2−/− mice remains intact as supported by the normal levels of catecholamine release, HSL phosphorylation in WAT and BAT, and the normal mRNA expression of thyroid hormone receptor α (Thra) and thyroid hormone receptor β (Thrb). In BAT (data not shown) in response to cold stimulation. Second, our results from in vitro SV-differentiated brown adipocytes demonstrated that NE was able to stimulate the phosphorylation of HSL, PAK, and p38 MAPK as well as UCP1 and PGC-1α expression to a similar extent in WT and Lcn2−/− brown adipocytes (Fig. 6C–E). Third, we showed that Lcn2 was able to directly stimulate p38 MAPK phosphorylation in WT and Lcn2−/− brown adipocytes (Fig. 6F). Finally, we proved the hypothesis that Lcn2 deficiency affects the activation of upstream PGC-1α/UCP1 (p38 MAPK) pathway in Lcn2−/− mice. As we anticipated, Rosi, which is a synthetic ligand for PPARγ, was able to fully reverse the cold intolerant phenotype and defective BAT activation of Lcn2−/− mice. All these data strongly support that p38 MAPK is one of the important nonadrenergic signaling pathways that mediate the Lcn2 role in the regulation of PGC-1α/UCP1 and BAT thermogenic activation. However, we also believe that the activation of PGC-1α and UCP1 can be regulated through other p38 MAPK-independent mechanisms. For example, PGC-1α can be activated or deactivated by AMPK-mediated phosphorylation as well as SIRT1-mediated acetylation. Thus, it cannot be ruled out that other mechanisms are involved in thermogenic function of Lcn2.

Here, we discovered a novel role for Lcn2 in mitochondrial-peroxisomal oxidative metabolism and BAT activation as an important regulator of p38 MAPK-PGC-1α-UCP1 pathway activation. Mice lacking Lcn2 display impaired BAT activation in response to cold stimulation and in HFD-induced obesity, as demonstrated by decreased mitochondrial biogenesis and oxidation, peroxisomal oxidation, and UCP1-mediated uncoupling. Lcn2 deficiency reduces the p38 MAPK-PGC-1α-UCP1 pathway activation, independent of β-AR activation in brown adipocytes. The data from in vitro studies support a cell autonomous effect of Lcn2 on the functional activation of brown adipocytes. In conclusion, our studies provide the evidence supporting that Lcn2 is a novel and critical regulator of BAT thermogenic function via controlling the nonadrenergic activation of the p38 MAPK-PGC-1α-UCP1 pathway and functional activation of brown adipocytes. The detailed mechanism for how Lcn2 regulates p38 MAPK-PGC-1α-UCP1 activation warrants further investigation.

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