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The Role of Lipocalin 2 in the Regulation of Inflammation in Adipocytes and Macrophages

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Adipose tissue-derived cytokines (adipokines) are associated with the development of inflammation and insulin resistance. However, which adipokine(s) mediate this linkage and the mechanisms involved during obesity is poorly understood. Through proteomics and microarray screening, we recently identified lipocalin 2 (LCN 2) as an adipokine that potentially connects obesity and its related adipose inflammation. Herein we show that the levels of LCN2 mRNA are dramatically increased in adipose tissue and liver of *ob/ob* mice and primary adipose cells isolated from Zucker obese rats, and thiazolidinedione administration reduces LCN2 expression. Interestingly, addition of LCN2 induces mRNA levels of peroxisome proliferator-activated receptor-γ (PPARγ) and adiponectin. Reducing LCN2 gene expression causes decreased expression of PPARγ and adiponectin, slightly reducing insulin-stimulated Akt2 phosphorylation at Serine 473 in 3T3-L1 adipocytes. LCN2 administration to 3T3-L1 cells attenuated TNFα-effect on glucose uptake, expression of PPARγ, insulin receptor substrate-1, and glucose transporter 4, and secretion of adiponectin and leptin. When added to macrophages, LCN2 suppressed lipopolysaccharide-induced cytokine production. Our data suggest that LCN2, as a novel autocrine and paracrine adipokine, acts as an antagonist to the effect of inflammatory molecules on inflammation and secretion of adipokines. (*Molecular Endocrinology* 22: 1416–1426, 2008)

ADIPOSE TISSUE IS now clearly understood to be an endocrine organ (1), and a major site of chronic low-grade inflammation that is associated with obesity and insulin resistance, a hallmark of type 2 diabetes (2–6). Adipose tissue-derived adipokines and cytokines play important roles in energy homeostasis, inflammation, and insulin resistance. This function of adipose tissue is dysregulated in obesity, contributing to systemic insulin resistance and metabolic syndrome. However, the molecular and cellular mechanism underlying this link is not clear. This can be attributed to the lack of understanding of how a collection of adipokines/cytokines mediates obesity-associated inflammation and insulin resistance. To this end, we used high-throughput proteomic and microarray approaches to identify and characterize adipokines that are potential mediators of insulin resistance. Of the already identified new adipokines (7), lipocalin 2 (LCN2) seems to be an attractive candidate that potentially links obesity-associated inflammation.

LCN2 or neutrophil gelatinase-associated lipocalin belongs to the superfamily of lipocalins, and it was originally identified as a 25-kDa protein secreted from human neutrophils (8, 9). Retinol-binding protein 4 is another lipocalin superfamily member that has been recently identified as an adipokine that affects glucose metabolism and insulin sensitivity (10). Lipocalins possess common crystal structures of an eight-stranded continuously hydrogen-bonded antiparallel β-barrel and participate in various biological processes. This structure confers to the lipocalins the ability to bind and transport a wide variety of small hydrophobic molecules such as retinol, fatty acids, steroids, and thyroid hormone (11). Tissue distribution and expression of LCN2 in neutrophils and bone marrow, and in the tissues that are exposed to microorganisms such as trachea, lung, stomach, salivary gland, and colon (12), indicate its involvement in inflammatory responses. In neutrophils, LCN2 secretion is highly regulated by the activation of inflammation and infection (8); lipopolysaccharide (LPS) and TNFα are the two strong inducers of LCN2 production. LCN2 deficiency resulted in an increased susceptibility to bacterial infection in mice (13, 14). Most intriguingly, LCN2 promoter possesses the binding sites of two key transcription factors, nuclear factor-κB (NFκB)

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Abbreviations: FASN, Fatty acid synthase; FBS, fetal bovine serum; GLUT, glucose transporter; GFP, green fluorescent protein; GM-CSF, granulocyte macrophage colony-stimulating factor; IRS-1, insulin receptor substrate 1; KRH, Krebs Ringer HEPES; LCN2, lipocalin 2; LPL, lipoprotein lipase; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; NOS2, nitric oxide synthase 2; PPAR, peroxisome proliferator-activated receptor; RNAi, RNA interference; shRNA, short hairpin RNA; TZD, thiazolidinedione.

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and CCAAT enhancer binding protein (15), and glucocorticoid response element (16), suggesting that transcriptional activation of this gene in adipose tissue is associated with inflammation and obesity. These characteristics of LCN2 led us to study its function in inflammation, insulin resistance, and obesity.

Here, we investigated the role and mechanism of LCN2 in inflammatory activity and insulin action in adipocytes and macrophages. Our results demonstrate that LCN2 antagonizes the detrimental effects of inflammatory molecules on inflammation and metabolism in adipocytes and macrophages. While this manuscript was being prepared for submission, Yan *et al.* (17) published a report of a similar project using different approaches. However, our work focuses on the effect of LCN2 on inflammatory activity in both adipocytes and macrophages, a question not addressed in their study.

RESULTS

Adipose Tissue Expression of LCN2 in Obesity and Insulin Resistance

We have identified LCN2 as a new adipokine from the conditioned medium of primary adipose cells (7) and found that the levels of LCN2 mRNA by cDNA microarray were increased approximately 28-fold in isolated rat primary adipose cells after 24 h in culture (Fig. 1A). As reported in our previous study, isolated primary rat adipose cells spontaneously developed insulin resistance when cultured *in vitro* for 24 h (18). We then examined LCN2 expression in different fat depots and during adipocyte differentiation. LCN2 was selectively expressed in adipose tissue from the epididymal fat depot as compared with very low levels of expression in the inguinal fat depot in mice (data not shown). As illustrated in Fig. 1B, little or no LCN2 was expressed in undifferentiated

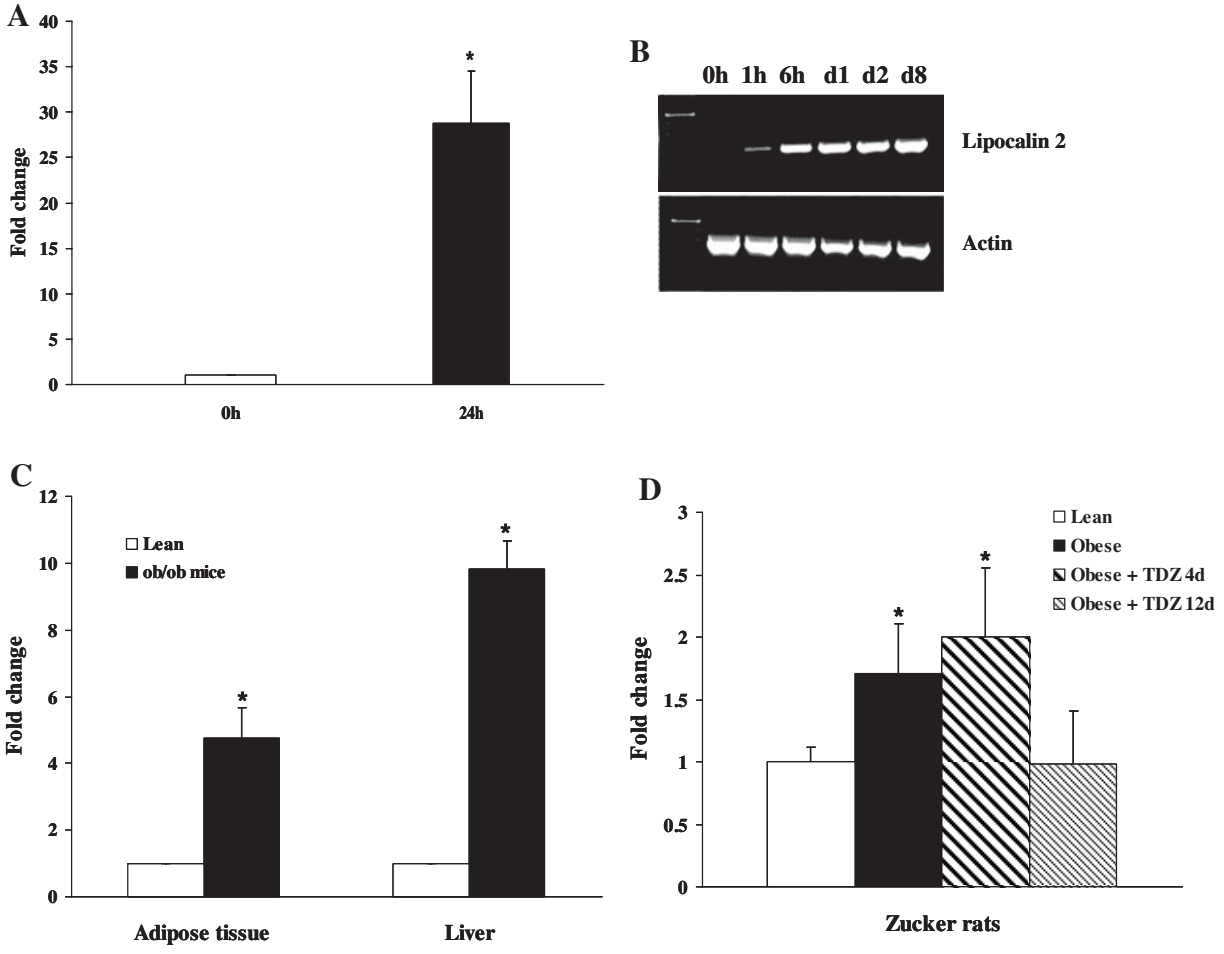

Fig. 1. Adipose Expression of LCN2 in Obesity and Insulin Resistance

LCN2 mRNA levels in cultured primary rat adipose cells after 24 h in culture (A), 3T3-L1 adipocytes during differentiation (B), and epididymal adipose tissue and liver of ob/ob and their control C57BL/6J mice (C) and isolated primary adipose cells of Zucker obese rats with or without TZD treatment (D). Levels of LCN2 mRNA were detected by real-time RT-PCR. The expression levels in each sample in panels A, C, and D were normalized to the level in 0 h or lean control and shown as fold changes. The results are presented as mean ± SE. The statistical *P* value and sample size are as indicated in *Results*.

3T3-L1 fibroblasts. However, LCN2 expression was markedly induced during adipocyte differentiation.

We next assessed the regulation of adipose expression of LCN2 in obesity and insulin resistance. As shown in Fig. 1C, LCN2 expression was dramatically increased in epididymal adipose tissue and liver of *ob/ob* mice ($P < 0.05$, n = 12 of individual animals) as compared with age-matched C57BL/6J controls fed on a normal chow diet. LCN2 expression also increased in adipose cells isolated from epididymal fat pad of obese Zucker *fa/fa* rats ($P = 0.047$; n = 6) relative to lean Zucker rats, and this increase was reversed by thiazolidinedione (TZD) administration for 12 d ($P = 0.04$, n = 6), but not 4 d ($P = 0.95$, n = 6) (Fig. 1D). TZD treatment for 12 d resulted in a significant improvement of insulin sensitivity, as evidenced by improved glucose tolerance and decreased plasma levels of insulin, triacylglycerols, and free fatty acids (data not shown).

Increasing LCN2 Action Induces Peroxisome Proliferator-Activated Receptor (PPAR)γ Expression and Protects against the Inhibitory Effect of TNFα on Metabolic Gene Expression in 3T3-L1 Adipocytes

To elucidate the potential role of LCN2 in inflammation and metabolism in adipocytes, we examined the effect of nonliganded mouse recombinant LCN2 on expression of PPARγ, a key antiinflammatory transcription factor and adiponectin, a PPARγ target metabolic gene in 3T3-L1 adipocytes. Interestingly, LCN2 treatment for 24 h significantly induced PPARγ protein (Fig. 2A) and mRNA expression (Fig. 2C) as well as adiponectin gene expression (Fig. 2C).

To test the potential role of LCN2 in antiinflammation and inflammation-mediated metabolic dysregulation we examined the interference of LCN2 with TNFα effect in 3T3-L1 adipocytes. As illustrated in Fig. 2B, control cells showed approximately 10-fold increase of glucose uptake in response to insulin, this response being reduced to about 1.5-fold in cells treated with TNFα for 24 h ($P = 0.0002$, Student's *t* test), partly due to an increase in basal levels of glucose uptake as previously reported (19, 20). In the cells with LCN2 (500 ng/ml) cotreatment for 24 h, TNFα-increased basal glucose uptake ($P = 0.038E03$, Student's *t* test) was significantly reduced (TNFα *vs.* LCN2 + TNFα; $P = 0.043$, Student's *t* test), and the insulin response was rescued to approximately 3.2-fold ($P = 0.012$, Student's *t* test), although LCN2 alone had no significant effect on insulin-stimulated glucose uptake (Fig. 2B). Treatment of the cells with LCN2 alone significantly

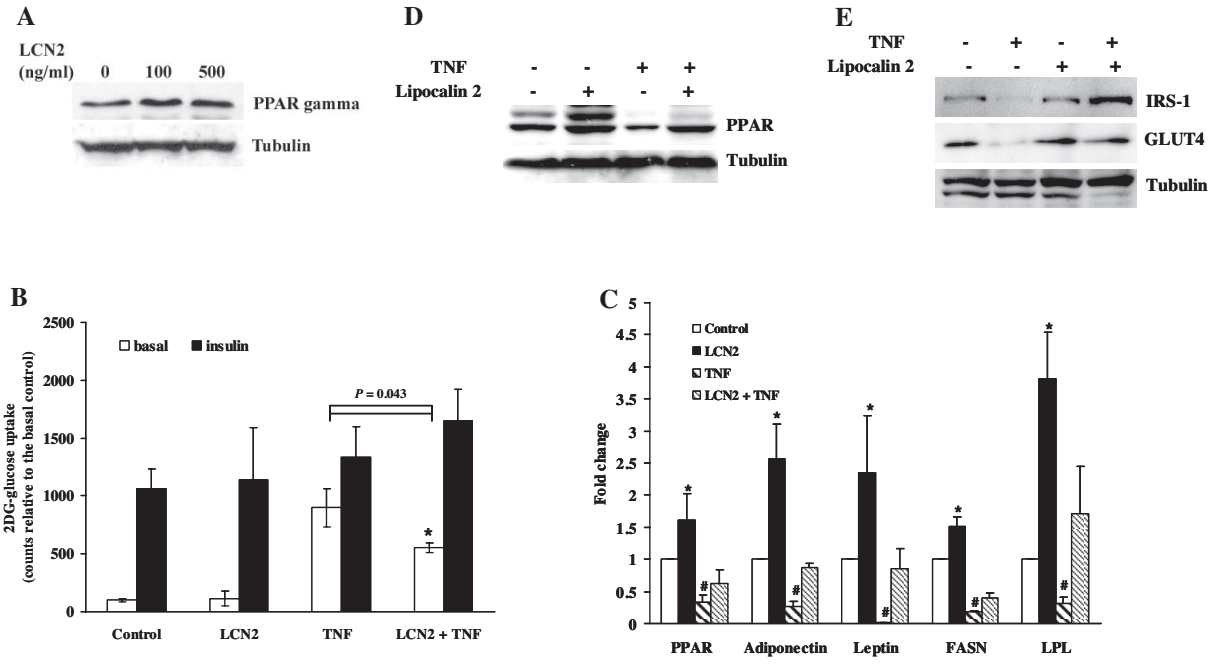

Fig. 2. LCN2 Enhances Expression of PPARγ and PPARγ Target Genes and Attenuates TNFα effect on Glucose Uptake and Expression of PPARγ and PPARγ Target Genes in 3T3-L1 Adipocytes

3T3-L1 adipocytes at d 8 of differentiation were treated with 0.5% FBS and 1 mg/ml glucose for 24 h, followed by various treatments as described in *Materials and Methods*. Cells with 24 h LCN2 treatment were harvested for immunoblotting of anti-PPARγ and tubulin (A). Day 9 differentiated serum-starved 3T3-L1 adipocytes with various treatments of LCN2, TNFα, or combined LCN2 and TNFα for 24 h were incubated with [^{3}H] 2-deoxy-D-glucose in the absence and presence of 100 nmol/liter insulin as described in *Materials and Methods* and glucose influx was determined (B). The results represent three to four independent experiments of mean ± SE. The statistical information is as indicated in the figures and *Results*. In addition, cells were harvested for gene expression of PPARγ, adiponectin, leptin, FASN, and LPL (C), and immunoblotting of anti-PPARγ (D), and GLUT4 and IRS-1 (E). Note: the values for leptin expression was scaled down 10-fold.

increased PPARγ (P = 0.02, Student's t test) and PPARγ target genes adiponectin (P = 0.01, Student's t test), leptin (P = 0.0005, Student's t test), fatty acid synthase (FASN) (P = 0.037, Student's t test), and lipoprotein lipase (LPL) (P = 0.021, Student's t test) (Fig. 2C). In addition, TNFα treatment for 24 h resulted in a significant reduction in PPARγ and adiponectin gene expression (Fig. 2C) and PPARγ protein levels (Fig. 2D). Cotreatment of LCN2 for 24 h reversed this inhibitory effect of TNFα on adiponectin (P = 0.0002, Student's t test) and partially on PPARγ (P = 0.021, Student's t test), leptin (P = 0.035, Student's t test), FASN (P = 0.026, Student's t test), and LPL (P = 0.003, Student's t test) gene expression (Fig. 2C) as well as protein levels (Fig. 2D). In addition, TNFα inhibition of insulin receptor substrate (IRS)-1 and glucose transporter (GLUT)4 protein expression was restored by cotreatment of LCN2 for 24 h (Fig. 2E).

Reducing LCN2 Expression Affects Expression of PPARγ, PPARγ Target Genes, and Insulin Action in Adipocytes

To better understand the role of LCN2 in adipocyte function, we studied the effect of reducing LCN2 expression in adipocytes on expression of PPARγ, PPARγ target genes, and insulin action. We used lentiviral short hairpin RNA (shRNA) technique to knock down LCN2 mRNA levels in 3T3-L1 cells. Undifferentiated 3T3-L1 fibroblast cells were infected with lentivirus vectors expressing LCN2 RNA interference (RNAi) targeting three different LCN2 sequences. Cells infected with lentiviral green fluorescent protein (GFP) served as controls. After 8 d of differentiation, infected cells were examined for the transfection as well as gene knockdown efficiency. GFP signal in LCN2 shRNA lentivirus-infected adipocytes was examined using fluorescent microscopy for validating the efficiency of lentiviral transfection (Fig. 3A). Lentiviral

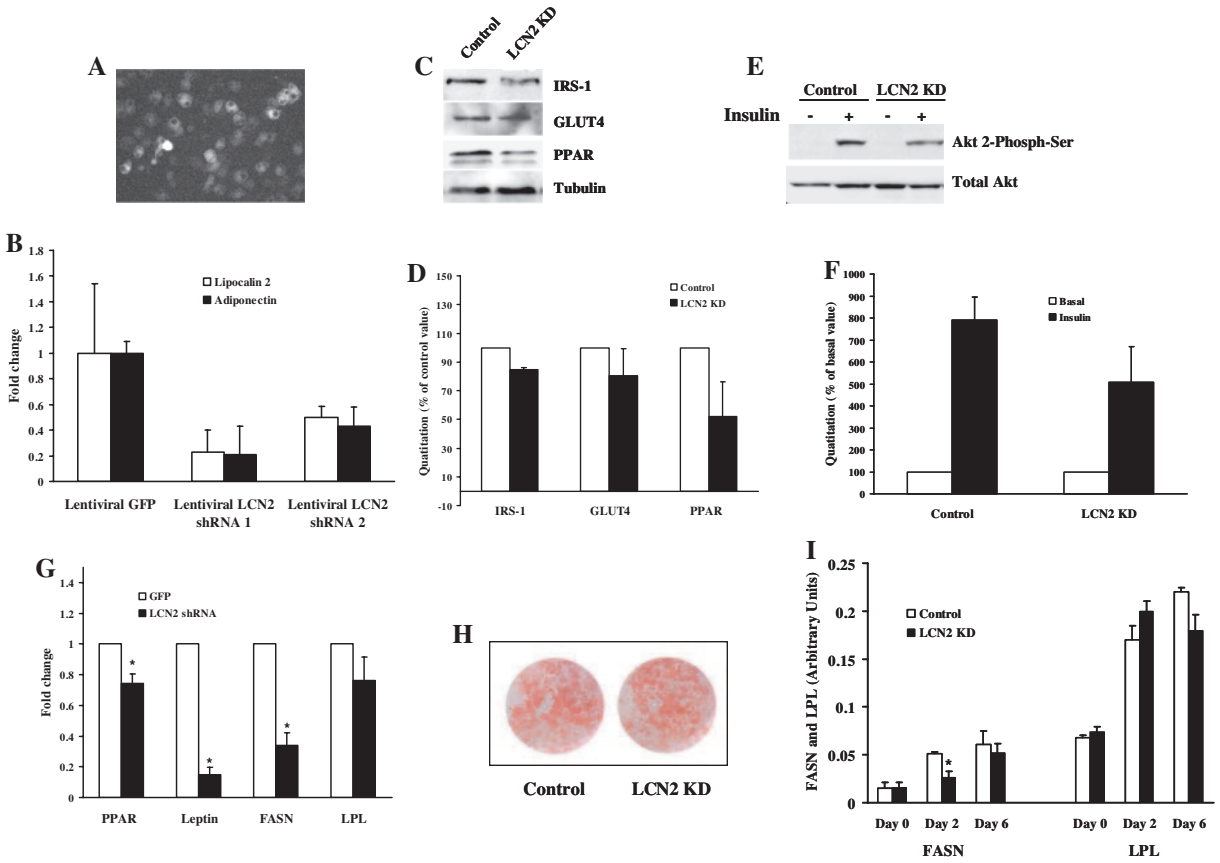

Fig. 3. Alterations in Gene Expression and Insulin Signal Transduction in LCN2 Knockdown Adipocytes

LCN2 shRNA lentivirus-infected 3T3-L1 fibroblasts were induced to differentiate into adipocytes. At d 8 of differentiation, cells were examined for determining transfection efficiency using fluorescent microscopy (A); the mRNA levels of LCN2 and adiponectin (B) were determined by real-time RT-PCR as well as lipid accumulation by Oil-red O staining (H). Cells were harvested for detecting protein levels of IRS-1, GLUT4, and PPARγ by Western blotting (C and D), insulin-stimulated Akt2 phosphorylation at Serine 473 (E and F), and mRNA levels of PPARγ, adiponectin, leptin, FASN, and LPL (G). At d 0, d 2, and d 6 of differentiation, cells were harvested for detecting mRNA levels of FASN and LPL gene expression (I). The results are presented as mean ± SE (B, G, and I), and the statistical information is as indicated in *Results*.

LCN2 shRNA 1 and lentiviral LCN2 shRNA 2, but not LCN2 shRNA 3 were able to stably knock down the gene expression of LCN2 by approximately 80% and 50%, respectively (Fig. 3B).

To determine whether decreased expression levels of LCN2 affect adipocyte metabolism and differentiation, expression of PPARγ, adiponectin, leptin, GLUT4, IRS-1, FASN, and LPL was examined in LCN2-knockdown adipocytes. As illustrated in Fig. 3, C–G, LCN2 knockdown adipocytes showed significantly decreased mRNA levels of PPARγ ($P = 0.05$, Student's *t* test), adiponectin ($P < 0.05$, Student's *t* test), leptin ($P = 5.779E-05$, Student's *t* test), FASN ($P = 0.001$, Student's *t* test), and LPL ($P = 0.19$, Student's *t* test) (Fig. 3G) and protein levels of PPAR γ, and only a slight decrease in IRS-1 protein levels (Fig. 3, C and D). The degree of decreased adiponectin gene expression by two different lentiviral LCN2 shRNA was correlated with their efficiency of reducing LCN2 gene expression (Fig. 3B). The examination of insulin sensitivity in LCN2 knockdown adipocytes showed that insulin-stimulated Akt2 phosphorylation at Serine 473 was slightly decreased (Fig. 3, E and F), whereas insulin-stimulated glucose uptake was not significantly different between control and LCN2 knockdown adipocytes (data not shown). These data indicate that the effect of down-regulation of LCN2 expression on insulin sensitivity is not significant in adipocytes. To assess the effect of decreased LCN2 expression on adipocyte differentiation, morphological changes in lipid accumulation and a time course of FASN and LPL gene expression was examined during the differentiation of LCN2 knockdown adipocytes. Oil-red O staining showed that lipid accumulation was not significantly different between GFP control and LCN2 knockdown adipocytes at d 8 of differentiation (Fig. 3H). As illustrated in Fig. 3I, the expression of FASN, but not LPL gene, was significantly decreased in LCN2 knockdown cells at d 2 of differentiation. At d 6, both FASN and LPL had a trend toward a decrease in the level of gene expression although the difference did not reach a statistically significant level. The data suggest that LCN2 knockdown affects adipocyte differentiation at the level of gene expression, but not the morphological change and lipid accumulation.

LCN2 Counters the TNFα Effect on the Production of Adipokines and Cytokines in 3T3-L1 Adipocytes and the LPS Induction of Cytokines in RAW264.7 Macrophages

To explore further the antiinflammatory role of LCN2 in adipose tissue, we examined the effect of LCN2 on cytokine production by 3T3-L1 adipocytes as well as cytokine gene expression by RAW264.7 macrophages. As shown in Table 1, in adipocytes, treatment of LCN2 alone for 24 h had no significant effect on the production of IL-6, monocyte chemotactic protein-1 (MCP-1), leptin, and adiponectin. However, as expected, TNFα treatment for 24 h significantly enhanced production of IL-6 and MCP-1 and inhibited production of leptin and adiponectin by adipocytes (Table 1). Cotreatment of LCN2 for 24 h attenuated TNFα-stimulated IL-6 and MCP-1 production and almost completely blocked TNFα inhibition of leptin and adiponectin production in adipocytes (Table 1).

To determine whether macrophages are one of the main target cells for LCN2 regulation in adipose tissue inflammation, the effects of LCN2 on cytokine gene expression were examined in RAW264.7 macrophages. LCN2 treatment for 24 h had no effect on gene expression of IL-1β, IL-6, MCP-1, TNFα, granulocyte macrophage colony-stimulating factor (GM-CSF), and nitric oxide synthase 2 (NOS2), and LPS treatment for 4 h significantly stimulated the expression of those genes in RAW264.7 macrophages (Table 2). Strikingly, LPS-stimulated gene expression of those six inflammatory molecules was almost completely blocked by 20 h pretreatment of LCN2 (Table 2). To further determine whether macrophages produce LCN2 and whether PPARγ is the important target of LCN2 effect in macrophages, we investigated LCN2 gene expression under the normal and inflammatory conditions and LCN2 effect on PPARγ gene expression in macrophages. As illustrated in Fig. 4A, LPS treatment for 4 h resulted in approximately 140-fold increase in LCN2 expression in macrophages. Figure 4B showed that macrophages had a relatively lower level of PPARγ gene expression as compared with adipocytes and, unlike in adipocytes, PPARγ gene expression was not significantly induced after LCN2 treatment for 24 h in macrophages (Fig. 4B).

Table 1. Effect of LCN2 on the Production of Adipokines and Cytokines in 3T3-L1 Adipocytes

Treatment	IL-6 (pg/ml)	MCP-1 (pg/ml)	Leptin (pg/ml)	Adiponectin (pg/ml)
Control	56.29 ± 22.83	587.50 ± 83.19	4,967.5 ± 1,069.53	39.63 ± 1.08
LCN2	88.07 ± 16.80	1,734.33 ± 604.51	4,930.44 ± 801.29	40.40 ± 1.07
TNFα	3,221.21 ± 563.85[a]	41,880.21 ± 3,729.99[a]	1,554.98 ± 497.62[a]	33.65 ± 1.72[b]
LCN2 + TNFα	2,291.9 ± 76.09	25,830.93 ± 5,188.09[c]	3,746.35 ± 1,260.12[c]	39.1 ± 0.56[c]

Results are mean ± SE (n = 6–8, duplicates of three to four independent experiments).
[a] $P < 0.01$ and [b] $P < 0.05$ compared with control.
[c] $P < 0.05$ compared with TNFα.

Table 2. Effects of LCN2 on the Expression of Cytokines in Macrophages

Treatment	IL-1b (fold change)	IL-6 (fold change)	MCP-1 (fold change)	TNFa (fold change)	GM-CSF (fold change)	NOS2 (fold change)
Control	1	1	1	1	1	1
LCN2	0.78 ± 0.04	1.14 ± 0.07	0.88 ± 0.09	1.03 ± 0.08	0.77 ± 0.05	0.63 ± 0.02
LPS	384.55 ± 48.32	232.4 ± 14.99	18.66 ± 0.33	1.42 ± 0.05	3.03 ± 0.06	3.04 ± 0.76
LCN2 + LPS	72.87 ± 4.32[a]	92.62 ± 4.04[a]	13.97 ± 0.32[a]	1.02 ± 0.01[a]	1.42 ± 0.04[a]	1.09 ± 0.03[a]

Results are expressed as fold change (treatment:control) and are mean ± SE (n = 9 from three independent experiments).
[a] $P < 0.01$ compared with LPS.

The above data from *in vitro* studies support the regulatory role of LCN2 in inflammatory activity in adipocytes and macrophages. However, paradoxically, LCN2 levels are increased in obesity. It raises a question whether increased LCN2 is a protective reaction secondary to inflammation. To test the hypothesis, we compared the timing of TNFα-induced dysregulation of metabolic gene and LCN2 expression in 3T3-L1 adipocytes. As hypothesized, expression of metabolic genes, namely, adiponectin, PPARγ, and GLUT4, was significantly reduced in adipocytes with less than 8 h of TNFα treatment ($P < 0.05$, Student's *t* test), but had no further reduction after 24 h. On the other hand, LCN2 expression was not significantly induced by 8 h treatment of TNFα ($P > 0.05$, Student's *t* test), but dramatically increased after 24 h treatment ($P < 0.05$, Student's *t* test) (Fig. 5).

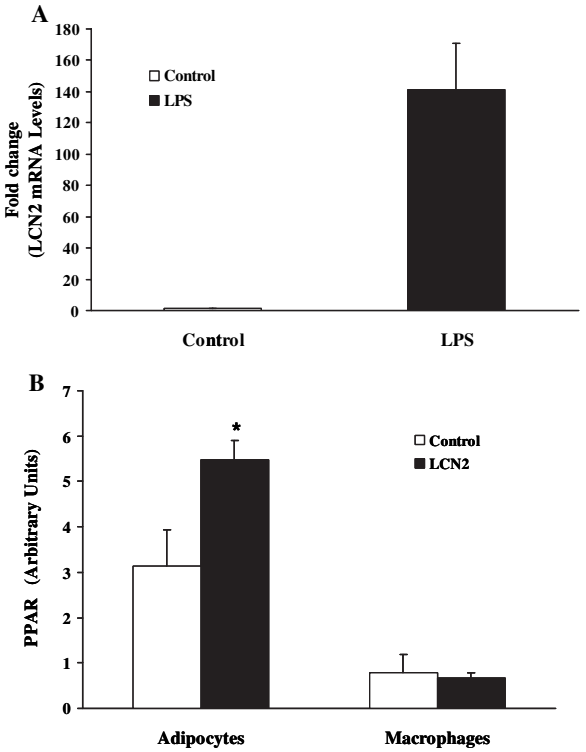

Fig. 4. LPS Induction of LCN2 Expression and LCN2 Induction of PPARγ Expression in Macrophages

RAW 264.7 cells were treated with or without LPS for 4 h or LCN2 (500 ng/ml) for 24 h. At the end of experiments, cells were harvested for mRNA extraction and LCN2 (A) and PPARγ(B) gene expression by real-time RT-PCR.

DISCUSSION

Adipose tissue synthesizes and secretes a large number of adipokines and cytokines that work as a network to regulate inflammation, insulin action, and glucose metabolism locally and systemically. This adipokine/cytokine networking system is altered in obesity, contributing to adipose tissue inflammation and impaired adipocyte metabolism. However, how adipokines and cytokines coordinately regulate inflammation and metabolism associated with obesity is not clearly understood. Herein, we characterize LCN2 as a new adipokine (7, 21) that plays an important role in the homeostatic regulation of inflammation in adipose tissue. LCN2 up-regulates PPARγ in adipocytes and antagonizes TNFα effects on inflammation and metabolic gene expression in adipocytes and macrophages.

Our results are consistent with a previous study, demonstrating that LCN2 is selectively expressed in epididymal fat depot and that its expression is highly induced during adipocyte differentiation (21). We showed that LCN2 mRNA is dramatically elevated in epididymal adipose tissue and liver of ob/ob mice. These results are in agreement with data from recent studies in obese animals and diabetic human subjects (17, 22). Furthermore, we found that LCN2 mRNA is also increased in primary adipocytes isolated from Zucker obese rats, and this increase is normalized by TZD administration. Our data from the investigation on the regulation of LCN2 in inflammation and metabolic gene expression in 3T3-L1 adipocytes demonstrate that the addition of LCN2 induces mRNA expression of PPARγ and its target genes, adiponectin, leptin, FASN, and LPL, and the physiological level of LCN2 (100 ng/ml) (13) clearly showed the effect on PPARγ protein expression. Conversely, knocking down LCN2 expression, using lentiviral shRNA gene silencing, results in decreased expression of PPARγ and its target genes, adiponectin, leptin, FASN, and LPL.

PPARγ exerts multiple functions in adipocytes, including the control of adipogenesis and the regulation of metabolism and inflammation. NFκB is the other

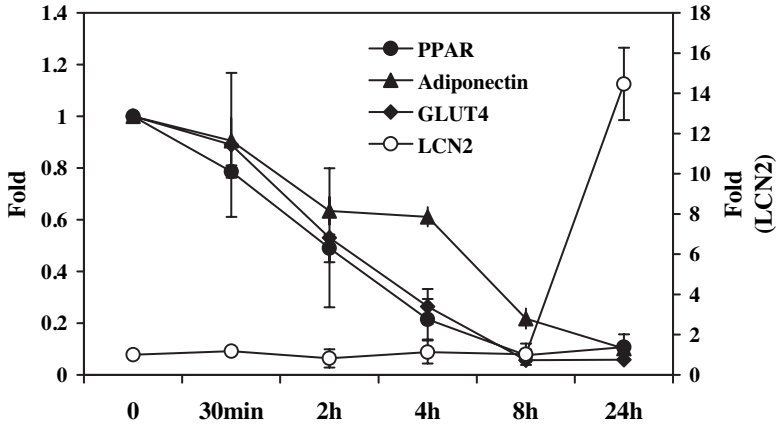

Fig. 5. The Time Course of TNFα Effect on Expression of LCN2 Gene and Genes Involved in Metabolism and Insulin Resistance in 3T3-L1 Adipocytes

3T3-L1 adipocytes at d 8 of differentiation were treated with 0.5% FBS and 1 mg/ml glucose for 24 h, followed by TNFα treatment. At the indicated time points, total RNAs were extracted for determining the mRNA levels of LCN2, PPARγ, adiponectin, and GLUT4 by real-time RT-PCR. The results represent three independent experiments of mean ± SE. The statistical information is as described in *Results*.

master transcription factor that controls inflammatory activities. PPARγ and NFκB mutually antagonize each other's activities. For instance, PPARγ antagonizes NFκB transcriptional activity and suppresses expression of NFκB controlled expression of proinflammatory genes (23). On the other hand, NFκB activation inhibits PPARγ activity, leading to subsequent insulin resistance (23). Our data show that LCN2 induces PPARγ expression in the absence of insulin in adipocytes, and the effect of LCN2 on insulin action is relatively small. This suggests that the antiinflammatory function of LCN2 is associated with its modulation of PPARγ activity via direct or indirect mechanisms by inhibiting NFκB activity. Thus, we then examined whether LCN2 counters the effect of TNFα on metabolism because TNFα is the strong inducer of NFκB signaling pathway activation. Strikingly, our results clearly show that LCN2 attenuates TNFα effect on glucose uptake as well as protein expression of PPARγ, GLUT4, and IRS-1. In particular, cotreatment of LCN2 significantly blocks TNFα inhibition of PPARγ expression at both protein and mRNA levels and expression of PPARγ target genes, adiponectin, leptin, FASN, and LPL, in 3T3-L1 adipocytes. This finding strongly suggests that antiinflammation is the primary role of LCN2 in adipose tissue, and the regulation of metabolic gene expression in adipocytes is likely the secondary effect of LCN2.

To further characterize the antiinflammatory properties of LCN2 and its possible role as an auto- or paracrine adipokine in the regulation of inflammation and its related metabolism, we examined and compared the impact of LCN2 on inflammatory responses of adipocytes and macrophages to TNFα and LPS stimulation. TNFα treatment for 24 h led to dramatically increased release of IL-6 and MCP-1 from 3T3-L1 adipocytes (Table 1). We also observed that LPS markedly stimulated the gene expression of IL-1β, IL-6, MCP-1, TNFα, GM-CSF, and NOS2 in Raw264.7 macrophages (Table 2), whereas treatment of LCN2 alone had no effect on either IL-6 and MCP-1 production in adipocytes or gene expression of IL-1β, IL-6, MCP-1, TNFα, GM-CSF, and NOS2 in Raw264.7 macrophages. On the contrary, LCN2 partially protects adipocytes from TNFα-induced production of IL-6 and MCP-1, and it completely reverses TNFα inhibition of leptin and adiponectin secretion from adipocytes (Table 1). More interestingly, LCN2 gene expression is markedly stimulated by LPS treatment in macrophages. LCN2 significantly attenuates the stimulatory effect of LPS on gene expression of cytokines in macrophages. These results strongly suggest that macrophages are the other important source of LCN2 production in adipose tissue, and the function of increased LCN2 is possibly to fight against LPS-induced inflammation. Unlike in adipocytes, the PPARγ gene is expressed at a relatively lower level, and its gene expression is not significantly induced by LCN2 treatment in macrophages. This suggests that antiinflammation is the direct effect of LCN2, and PPARγ-related metabolic effect in adipocytes could be secondary to the changes in inflammatory activity. The involvement of NFκB activity in LCN2 effects needs to be further characterized.

The effect of LCN2 on insulin sensitivity in adipocytes is not significant, but LCN2 markedly affects the adipocyte secretion of leptin and adiponectin, two key systemic metabolic regulators. It is likely that LCN2 affects insulin sensitivity via regulating the secretory function of adipocytes. Given the antiinflammatory role of LCN2, increased LCN2 in obesity and insulin resistance might be a protective mechanism against overactivation of inflammation. This hypothesis is supported by our data that the peak increase in TNFα-

induced LCN2 expression occurred after the appearance of dysregulation of metabolic gene expression in adipocytes. Moreover, the evidence from a previous study that hyperglycemia induces LCN2 expression in adipocytes *in vitro* as well as in adipose tissue *in vivo* (24) strengthens our observation. Taken together, our findings from *in vitro* studies, suggest a model that overproduction of proinflammatory factors during obesity triggers LCN2 release, which homeostatically regulates inflammatory responses in an autocrine or paracrine fashion. This hypothesis is supported by our preliminary data from the studies in LCN2-null mice that mice lacking LCN2 show decreased glucose tolerance and insulin insensitivity.

The mechanisms for LCN2 effects are probably related to its structural and physical characteristics, including binding of ligands and formation of macrocomplexes with other molecules. In addition, a receptor-mediated endocytosis mechanism for LCN2 intracellular effects has been indicated (25). The LCN2 ligands and receptors have not been well determined, and the potential ligands include formal peptides, cholesteryl oleate, retinoic acid, and retinal (26–30). Megalin/gp330, a member of the low-density lipoprotein receptor family, has been identified as a potential LCN2 receptor (31, 32). The role of LCN2 as an iron sequester in innate immunity has also been demonstrated in studies with LCN2-null mice (13, 14). LCN2 facilitates iron uptake by bacteria, promoting bacterial growth, and siderophores, small molecules secreted from bacteria, are required for LCN2 facilitation of iron uptake (33). LCN2 deficiency leads to an increased susceptibility to bacterial infection (13, 14). Because siderophores are produced only by bacteria, it is unlikely that iron sequestration could be a mechanism for LCN2 effects in mammalian cells. The experiments for defining the ligand binding and functional properties of LCN2 and its relation to LCN2 action are currently being undertaken.

Our findings are similar, although different, from a recently published study by Yan *et al.* (17). In their study they also found an increased expression of LCN2 in obese models and in adipocytes *in vivo* and *in vitro*. They found that agents that promote insulin resistance enhance expression, and TZDs inhibit expression of LCN2. Our observation that knocking down LCN2 gene expression had no significant effect on lipid accumulation in adipocytes is consistent with that in the study by Yan *et al.* (17). In addition, the results from both studies showed similarly decreased PPARγ in LCN2 knockdown 3T3-L1 adipocytes. In their study, however, an increase in insulin-stimulated glucose uptake was observed in LCN2 knockdown 3T3-L1 adipocytes, although this inconsistency was not discussed. Most importantly, our findings of the protective role of LCN2 on inflammation are novel and were not addressed previously.

In summary, we have found that levels of LCN2 expression are up-regulated in adipose tissue of obese animals, and up-regulation of LCN2 expression is reversed by TZD administration. In *in vitro* studies, LCN2 induces PPARγ expression and antagonizes TNFα effects on inflammation in adipocytes and suppresses LPS stimulation on cytokine expression in macrophages. Knocking down LCN2 expression causes a decrease in PPARγ and adiponectin expression in 3T3-L1 adipocytes. Our data suggest that LCN2 primarily acts as a negative regulator of inflammatory activity and inflammation-mediated adipocyte dysfunction.

MATERIALS AND METHODS

Animals

National Institutes of Health guidelines for animal handling were followed in this study, and experimental procedures were approved by animal care and use committee at the University of Minnesota.

Mice

Male C57BL/6J and *ob/ob* mice used in this study were obtained from The Jackson Laboratory (Bar Harbor, ME). Male C57BL/6J and ob/ob mice at 9–10 wk of age were maintained at 21 C on a 12-h light, 12-h dark cycle and fed *ad libitum*.

Rats

Lean and Zucker obese rats (Charles River Laboratories, Inc., Wilmington, MA) at 7–8 wk of age were on the normal rodent diet during the experiments. Zucker obese rats (total six rats per group) were treated with rosiglitazone (3 mg/kg/body weight) (GlaxoSmithKline, Philadelphia, PA) via gavage for 4 or 12 d. At the end of experiments, rats were euthanized and adipose tissue from epididymal fat pads was removed and adipose cells were isolated for RNA extraction.

3T3-L1 Cell Cultures

3T3-L1 cells were grown in DMEM with 100 IU/ml penicillin/streptomycin and 10% bovine calf serum until confluent. Cells were then differentiated with the differentiation cocktail for 2 d as described elsewhere (34). The cultures were then continued with DMEM (100 IU/ml penicillin/streptomycin, 10% fetal bovine serum, and 1 μg/ml insulin) for 6 d. On d 8 of differentiation, differentiated adipocytes were exposed to 0.5% fetal bovine serum (FBS) and 1 mg/ml glucose for 24 h, followed by the following four treatments: 1) control; 2) TNFα (3 nmol/liter) for 24 h; 3) LCN2 (500 ng/ml) for 24 h; and 4) cotreatment of LCN2 with TNFα for 24 h. Both mouse recombinant TNFα and LCN2 were purchased from R&D Systems, Inc. (Minneapolis, MN). Mouse recombinant LCN2 is prepared from murine myeloma cells and contains no siderophores and iron. At the end of the experiments, cells were prepared for 2-deoxy-D-[^{3}H]glucose uptake, RNA extraction for a quantitative real-time RT-PCR assay, and protein collection for Western blotting. The conditioned media were collected for cytokine assays.

Primary Rat Adipose Cell Isolation and Culture

Preparation of isolated rat epididymal adipose cells from normal male rats and lean and obese Zucker rats was per-

formed as described previously (35). Isolated cells were washed twice with DMEM containing 25 mM glucose, 25 mM HEPES, 4 mM L-glutamine, 200 nM N6-(2-phenylisopropyl)-adenosine, and 75 μg/ml gentamycin. After a final wash, adipose and stromal-vascular cells from lean and obese Zucker rats were immediately frozen in liquid nitrogen for RNA extraction. Adipose cells from normal rats were cultured for 24 h at 37 C, 5% CO_2 in DMEM containing 3.5% BSA. They were then harvested for mRNA exaction.

Murine Macrophage Cell Line RAW 264.7 and Culture

The murine macrophage cell line RAW 264.7 was kindly provided by Dr. David Bernlohr (University of Minnesota). RAW 264.7 cells were routinely maintained in DMEM supplemented with 10% FBS and pretreated with or without LCN2 (500 ng/ml) for 24 h. LPS (1 ng/ml) was added to the cells 4 h before harvesting with or without 20 h LCN2 pretreatment. At the end of the experiments, cells were harvested for mRNA extraction.

Quantitative Real-Time RT-PCR

Total RNAs from isolated rat adipose cells from epididymal fat depot, mouse adipose tissue, 3T3-L1 adipocytes, and macrophages with various treatments were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was treated with RQ1 DNase (Promega Corp., Madison, WI) at 37 C for 30 min, followed by extraction with phenol-chloroform and ethanol precipitation. The first-strand cDNA was generated using the oligo (dT) primer (Promega), and 10 μl of diluted cDNA (1:20) was used in each 25-μl real-time PCR reaction using the SYBR GreenER qPCR SuperMix Universal kit (Invitrogen) with an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Primers specific for the examined genes are listed in Table 3. β-Actin or 18s RNA was selected as an internal standard. Results were analyzed using the software supplied with the ABI 7500 system. All raw data are expressed as the ratio of the selected gene to β-actin or 18s. Statistical significance was determined by two-tailed Student's *t* test.

Glucose Uptake Assay

Uptake of 2-deoxy-D-[^{3}H] glucose (Amersham Biosciences, Piscataway, NJ) was measured in 3T3-L1 adipocytes as previously described (36). Briefly, cells were serum starved in Krebs Ringer HEPES (KRH) buffer supplemented with 0.5% BSA and 2 mmol/liter sodium pyruvate (pH 7.4) for 3 h and then incubated either with or without 173 nmol/liter insulin for 30 min at 37 C. Glucose uptake was initiated by the addition of [^{3}H] 2-deoxy-D-glucose to a final assay concentration of 100 μmol/liter at 37 C. After 5 min, 2-deoxyglucose uptake was terminated by three washes with ice-cold KRH buffer, and the cells were solubilized with 0.8 ml of KRH buffer containing 1% Triton X-100. The incorporated radioactivity was determined by scintillation counting. Nonspecific 2-deoxyglucose uptake was measured in the presence of 20 μmol/liter cytocholasin B and subtracted from the total glucose uptake assayed to obtain specific uptake.

Immunoblotting

Lysates of 3T3-L1 adipocytes were extracted in a solubilization buffer containing 25 mmol/liter Tris-HCl (pH 7.5), 0.5 mmol/liter EDTA, 25 mmol/liter sodium chloride, 10 mmol/liter sodium fluoride, 1 mmol/liter sodium vanadate, 1% Nonidet P-40, and protease inhibitor cocktails (Diagnostic Roche, Branchberg, NJ). Protein concentrations of lysates were detected with the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL). Equivalent amounts of proteins (50–70 μg of total proteins) were separated on SDS-PAGE and immunoblotted with anti-IRS-1 (Upstate Biotechnology, Inc., Lake Placid, NY), anti-Akt2 and Akt2-phospho Ser473 (Cell Signaling Technology, Inc., Danvers, MA), anti-PPARγ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-GLUT4 (kindly provided by Dr. Samuel Cushman at National Institute of Diabetes and Digestive and Kidney Diseases/National Institutes of Health) antibodies according to the recommendations of the manufacturers. After incubation with primary antibodies, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase. Antibody reactivity was detected by ECL Western Blotting Detection Systems (GE Healthcare BioSciences Corp., Piscataway, NJ).

Table 3. Sequences of Primers for Real-Time RT-PCR and RT-PCR

Accession No.	Gene	Primer Sequence	
		Forward (5′–3′)	Reverse (5′–3′)
NM_008491	Lipocalin 2	TGCCACTCCATCTTTCCTGTT	GGGAGTGCTGGCCAAATAAG
AY754346	Adiponectin	GCAGAGATGGCACTCCTGGA	CCCTTCAGCTCCTGTCATTCC
NM_008493	Leptin	CCACACACAGCTGGAAACTCC	GGCTTGCTTCAGATCCATCC
AY243585	PPAR γ	ACCACTCGCATTCCTTTGAC	AAGGCACTTCTGAAACCGAC
BC014282	GLUT4	CCATTCCCTGGTTCATTGTG	GTTTTGCCCCTCAGTCATTC
CT010344	LPL	TGAGAAAGGGCTCTGCCTGA	GGGCATCTGAGAGCGAGTCTT
BC059850	FASN	CTGGACTCGCTCATGGGTG	CATTTCCTGAAGTTTCCGCAG
NM_011333	MCP1	CTTCTGGGCCTGCTGTTCA	GAGTAGCAGCAGGTGAGTGGG
NM_013693	TNF α	CGTGGAACTGGCAGAAGAGG	CTGCCACAAGCAGGAATGAG
DQ788722	IL-6	TCTGCAAGAGACTTCCATCC	TTAGCCACTCCTTCTGTGAC
NM_008361	IL-1β	AAATACCTGTGGCCTTGGGC	CTTGGGATCCACACTCTCCAG
NM_009969	GM-CSF	GCCATCAAAGAAGCCCTGAAC	TCTTCAGGCGGGTCTGCAC
NM_010927	NOS2	CACAAGGCCACATCGGATTT	TCAATGGCATGAGGCAGGAG
NM_010548	IL-10	CATGGCCCAGAAATCAAGGA	GGAGAAATCGATGACAGCGC
NM_008360	IL-18	TCTTCTGCAACCTCCAGCATC	GACATGGCAGCCATTGTTCC
NT_039340.7	18S rRNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
AK145308	β-Actin	CCTAAGGCCAACCGTGAAAA	GAGGCATACAGGGACAGCACA

Generation of LCN2 Knockdown 3T3-L1 Adipocytes

A lentiviral-based RNAi vector pLentiLox 3.7 was kindly provided by Dr. Thomas Lanigan at the vector core laboratory, University of Michigan. The pLentiLox 3.7 vector contains the mouse U6 promoter for expression of hairpin RNAi, and a cytomegalovirus-GFP expression cassette for tracking RNAi expression. Oligos of RNAi stem loops for pLentiLox 3.7 directed against mouse LCN2 were synthesized and cloned into the *Hpa*I/*Xho*I site in pLentiLox 3.7. Three small interfering RNA sequence variants for LCN2 gene were synthesized, and recombinant lentiviruses were generated and tested for the efficiency. The three selected oligomers targeting the LCN2 nucleotide sequence were 5′-ggcctcaaggacgacaac-3′ (nucleotide positions 544–561) for lentiviral LCN2 shRNA 1, 5′-gactacaaccagttcgcc-3′ (nucleotide positions 428–445) for lentiviral LCN2 shRNA 2, and 5′-gcccaggactcacatcag-3′ (nucleotide positions 80–97) for lentiviral LCN2 shRNA 3 (third was less effective). A pLentiLox 3.7 expressing only GFP was used as a control. Lentiviruses were produced by the Vector Core Laboratory at the University of Michigan. 3T3-L1 fibroblasts at approximately 80% confluence were transduced with 1.25 ml of 1× viral supernatant ($\sim 10^6$ pfu) in UltraCULTURE (CAMBREX, Charles City, IA) supplemented with 8 μg/ml Polybrene for 12 h. Cells were then switched to DMEM with 10% bovine calf serum, and induced to differentiate into adipocytes. The infection efficiency was monitored by GFP expression after 48 h. Total RNA was extracted from infected and differentiated adipocytes for checking mRNA levels of LCN2 by quantitative real-time PCR.

Cytokine Assay

Cytokine/chemokine levels in the conditioned medium were evaluated by a multiplex method using the Luminex platform (Luminex, Austin, TX) and mouse-specific bead sets (R&D Systems) at the Cytokine Reference Laboratory, University of Minnesota. Values were interpolated from recombinant protein standards supplied by the manufacturer (R&D Systems).

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