THE ADIPOKINE LIPOCALIN 2 IS REGULATED BY OBESITY AND PROMOTES INSULIN RESISTANCE

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Running title: Lipocalin 2 is a novel adipokine

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Abstract

Objective: We identified lipocalin2 (Lcn2) as a gene induced by dexamethasone and TNF-α in cultured adipocytes. The purpose of this study was to determine how expression of Lcn2 is regulated in fat cells and to ascertain whether Lcn2 could be involved in metabolic dysregulation associated with obesity.

Research Design and Methods: We examined Lcn2 expression in murine tissues and in 3T3-L1 adipocytes in the presence and absence of various stimuli. We used quantitative Western blotting to look at Lcn2 serum levels in lean and obese mouse models. We used retroviral delivery of shRNA to reduce Lcn2 levels in 3T3-L1 adipocytes, in order to assess effects on insulin action.

Results: Lcn2 is highly expressed by fat cells in vivo and in vitro. Expression of Lcn2 is elevated by agents that promote insulin resistance and is reduced by thiazolidinediones. The expression of Lcn2 is induced during 3T3-L1 adipogenesis in a C/EBP-dependent manner. Lcn2 serum levels are elevated in multiple rodent models of obesity, and forced reduction of Lcn2 in 3T3-L1 adipocytes improves insulin action. Exogenous Lcn2 promotes insulin resistance in cultured hepatocytes.

Conclusions: Lcn2 is an adipokine with potential importance in insulin resistance associated with obesity.
The worldwide epidemic of obesity and type 2 diabetes has focused attention on adipocyte biology and the role of adipose tissue in the integration of systemic metabolism (1). The discovery of leptin more than a decade ago established a paradigm in which secreted proteins from adipocytes coordinate energy balance and glucose homeostasis (2; 3). Since that initial discovery, the number of adipocyte-derived signaling molecules has grown ever larger, and the term ‘adipokine’ was coined to reflect the fact that many of these molecules exert positive or negative actions on inflammation. Several adipokines promote insulin sensitivity, including leptin (2), adiponectin (4), and visfatin (5), while others induce insulin resistance, such as resistin (6) and retinol binding protein 4 (RBP4) (7).

Lipocalin 2 (Lcn2), also known as neutrophil gelatinase-associated lipocalin (NGAL), siderocalin, and 24p3, is a member of a large superfamily of proteins that includes RBP4. Lipocalins are small, generally secreted proteins with a hydrophobic ligand binding pocket (8). Known ligands for lipocalins include retinol, steroids, odorants, pheromones, and in the case of Lcn2, siderophores (9). Siderophores are small molecules used by bacteria to poach iron from their hosts, a necessary co-factor for the growth of some pathogens. Lcn2 is used by the mammalian innate immune system to sequester siderophore and thus deprive the bacteria of iron. Mice lacking Lcn2 appear normal but die when exposed to siderophore-requiring strains of bacteria in quantities that are cleared easily by wild-type mice (10; 11). Lcn2 can thus be considered an iron transport protein, and it has been implicated in the apoptotic induction of pro-B cells (12) and in the biology of the genitourinary system, both as a developmental factor and as a protective mechanism in renal ischemia (13).

In this study we identify Lcn2 as a factor dramatically induced by dexamethasone and by TNF-α in 3T3-L1 adipocytes, and show that adipose tissue is a dominant site of Lcn2 expression in the mouse. We also study the regulation of Lcn2 expression in adipocytes, and demonstrate that it is regulated by obesity. We also provide data suggesting that Lcn2 promotes insulin resistance in adipocytes.

**RESEARCH DESIGN AND METHODS**

**Cell culture and differentiation.** 3T3-L1 cells were cultured in DMEM with 10% BCS at 5% CO2. Once confluence was reached, cells were exposed to DMEM with 10% FBS containing a pro-differentiative cocktail including dexamethasone (1 μM), insulin (5 μg/mL), and isobutylmethylxanthine (0.5 mM). After 2 days, cells were maintained in medium containing insulin until ready for harvest at day 7. NIH-3T3 cells were maintained in the same conditions. In some experiments, 3T3-L1 cells were differentiated only with 5 μM rosiglitazone plus 10% FBS, changed every two days. H4IE rat hepatoma cells were cultured in αMEM medium (Invitrogen), supplemented with 10% FBS at 37 °C with 5% CO2. Cells were seeded in 24-well plates with 50% confluence. Before treatment, cells were washed twice with αMEM containing 0.2% FBS, and cultured overnight (18 hours) in medium supplemented with or without recombinant Lcn2 (10 nM), with Dex treatment (250 nM) in parallel as a positive control. The next day, cells were treated with 100 nM insulin for 30 min, then washed twice with KRH buffer plus lactate and pyruvate (10 mM HEPES, pH7.4, 96 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 20 mM Lactate, 2 mM Pyruvate). Cells were incubated in KRH buffer, supplemented with Lcn2, Dex, or carrier solution for another 6 hours at 37°C. Supernatants were collected for the glucose oxidase assay, and cells were harvested by TRIzol (Invitrogen) for RNA analysis. Recombinant Lcn2 was produced as described (13). Endotoxin was assayed in 100 ul of 1 nM recombinant Lcn-2 solution by means of a limulus amoebocyte lyse gel clot assay (0.125 EU/ml sensitivity, Cambrex/Lonza, Inc., Allendale, NJ), and found to be below the limits of detection for the assay.

**Animals.** All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For the high-fat diet studies, 3 to 4 week-old FVB male mice were obtained from Taconic. Mice were fed a standard chow diet (Formulab 5008) or high-fat diet (55% fat calories, Harlan-Teklad 93075). Animals were put on diet treatment at 4-5 weeks of age and plasma Lcn2 levels were measured at 12 weeks on diet (ad libitum fed state). 9 week-old db/db females and lean littermate controls (+/+ or db/+ ) were obtained from Charles River (n=8 each). Mice were fed a standard chow diet (Formulab 5008) and plasma Lcn2 levels were measured in the ad libitum fed state. Male ob/ob
or ob/+ mice were purchased from Jackson Labs and studied at 10 months of age.

**Northern blotting.** Cells were lysed in Trizol® and processed according to the manufacturer's instructions. Murine tissues were harvested from wild-type C57BL/6J mice. For each sample, 10 µg of total RNA was loaded onto formaldehyde-agarose gels, transferred onto nylon membranes, and hybridized with the appropriate 32P-labeled probe in Ultrahyb® (Ambion).

**Quantitative PCR.** First strand cDNA synthesis for quantitative PCR was performed using RETROscript® (Ambion). Total RNA (1.5 mg) was converted into first-strand cDNA, using oligo dT primers as described in the kit. cDNA was amplified and detected with the Brilliant® SYBR® Green QPCR master mix (Stratagene) according to the manufacturer's instruction. Real-time PCR was performed in a Mx3000P® thermocycler (Stratagene), and its software was used to calculate the cycle threshold of each reaction. Validation experiments were performed to demonstrate equal efficiencies of target Lcn2 and of internal control (18S rRNA for tissues and cyclophilin for 3T3-L1 cells). The relative amount of Lcn2 transcripts was determined using comparative Ct method with the expression level of untreated control as 1. Primer sequences are as follows: m18S-F, AGTCCCTGCCCTTTGTACACA; m18S-R, GATCCGAGGGCCTCACAAC; mCyclophilin-F, GGTGGAGAGAGAGCGTCCAAT; mCyclophilin-R, GCCGGAAGTCGACAATGATG; mLcn2-F, ACTTCCGGAGCGATCAGTT; mLcn2-R, CAGCTCCTTGTTCTCCTCAT; mFabp4-F, TGGAAGCTTGTCTCCAGTGA; mFabp4-R, TGGCATCTCTGTGTCAACCATG; mGlut1-F, ACTCATTACAGGCGGTGTC.

**Plasma Lcn2 measurement.** Plasma (1 µl) was diluted 30 times in 1x Laemml buffer, proteins were separated by SDS-PAGE on 15% gels and transferred to nitrocellulose membranes. A single band for Lcn2 protein was detected at about 23 kDa using anti-mouse lipocalin-2 specific goat IgG (Cat#:AF1857, R&D Systems). Bands were quantitated by densitometry with 3 control samples on each membrane providing standardization between membranes. Concentrations are arbitrary units per microliter of plasma with controls set at one.

**Isolation of adipocytes, macrophages, and non-macrophage stromal vascular cells (SVCs) from perigonadal adipose tissue.** Five week-old male C57BL/6J mice were obtained from Jackson Labs (Bar Harbor, ME) and were fed chow or high-fat diet (Research Diets D12331) beginning at six weeks of age (n=7 each group). At 26-34 weeks of age, fed mice were euthanized by CO2 inhalation and epididymal adipose tissue (~0.5g) was collected and placed in Krebs Ringer HEPEs buffer containing 10mg/mL fatty acid–poor BSA (Sigma-Aldrich, St. Louis, MI). The tissue was minced into fine pieces and centrifuged at 1000g for 10 min to remove erythrocytes and other blood cells. Minced tissue was then digested in 0.12 units/mL of low-endotoxin collagenase (Liberase 3; Roche Applied Science, Indianapolis, IN) at 37°C in a shaking water bath (80Hz) for 45 min. Samples were then filtered through a sterile 300µm nylon mesh (Spectrum Laboratories Inc., Rancho Dominguez, CA) to remove undigested fragments. The resulting suspension was centrifuged at 500g for 10 min to separate SVCs from adipocytes. Adipocytes were removed and washed with Krebs Ringer HEPEs buffer, then suspended in Trizol® for RNA isolation. The SVC fraction was incubated in erythrocyte lysis buffer (0.154mM NH4Cl, 10mM KHCO3, 0.1mM EDTA) for 2 min. Cells were then centrifuged at 500g for 5 min and resuspended in 100µL of FACS buffer (PBS containing 5mM EDTA and 0.2% fatty acid-poor BSA). The cells were incubated in the dark on a bidirectional shaker with FcBlock (20µg/mL; BD Pharmingen, San Jose, CA) for 30 min at 4°C. They were then incubated for 50 min with APC-conjugated primary antibody against F4/80 (5µg/mL; Caltag Laboratories Inc., Burlingame, CA) and PE-conjugated antibody against CD11b (Mac-1; 2µg/mL). Control aliquots of SVCs were incubated with APC-labeled (2µg/mL) and PE-labeled (5µg/mL) isotype control antibodies (Caltag Laboratories Inc., Burlingame, CA). After incubation, cells were washed and suspended in FACS buffer. F4/80+/CD11b+ macrophages and F4/80-/CD11b- non-macrophage SVCs were isolated with a MoFlo (DakoCytoamtion, Fort Collins, CO) fluorescence activated flow sorter. After sorting, F4/80+/CD11b+ and F4/80-/CD11b- cells were suspended in Trizol® for RNA isolation.
Retroviral infections. Retroviruses were constructed in pMSCV (Clontech) using either puromycin or hygromycin selectable markers. Viral constructs were transfected into 293T cells using CellPhect® transfection kit (GE Healthcare) along with plasmids expressing gag-pol and the VSV-G protein. Supernatants were collected after 48 h. After filtration to remove cell debris, supernatants were added to either 3T3-L1 or NIH 3T3 cells at 70% confluence; selection with puromycin (4 µg/mL) or hygromycin (175 µg/mL) was started 48 h later. Cells were selected and studied immediately or frozen for later use.

Promoter constructs, co-transfection and luciferase assay. Oligonucleotides were selected to amplify fragments of 1742, 731, 320, 222 and 131 base pairs specific to the 5'-untranslated region of the murine Lcn2 promoter and to include restriction enzymes cutting sites for facilitating cloning into the pA3-luc reporter vector. All constructs were confirmed by sequencing. The mutated -222 plasmids were constructed using the same procedure except the forward primer contained the desired mutation. NIH-3T3 cells were co-transfected by Lipofectamine® with the ratio of reporter: β-gal: C/EBP expression plasmid as 1: 0.1: 2. Cells were incubated for 48 hours, lysed, and assayed using the Luciferase Reporter Gene Assay kit (Roche). Luciferase activity was normalized to β-gal activity.

Chromatin immunoprecipitation (ChIP) assay. 3T3-L1 cells were treated with 1% formaldehyde for 15 min at room temperature to crosslink DNA with DNA binding protein complexes. The ChIP assay was performed using a kit from Upstate. Immunoprecipitation was carried out using 2 µg of the following antibodies: C/EBPα (sc-61), C/EBPβ (sc-150), and C/EBPδ (sc-636) from Santa Cruz Biotechnology. An aliquot of chromatin DNA prepared from the cells taken prior to immunoprecipitation was used as input DNA. Immunoprecipitated and input DNAs were assayed by PCR with primer pair 5'-CTGCTGACCCACAAGCAGT-3' and 5'-GGCAAGATTCTGTCCCTCTC-3' in the Lcn2 gene promoter region. Amplified PCR products were visualized on 2% agarose electrophoresis gels.

shRNA-mediated Lcn2 knockdown. Four independent hairpins targeted to murine Lcn2 were developed using software from Clontech. These hairpins were synthesized and cloned into a retroviral delivery vector (pSIREN-RetroQ; Clontech) and transduced 3T3-L1 pre-adipocytes as described (14), and infected cells were selected by 4 µg/ml puromycin 48 hours post-infection. Inhibition of Lcn2 expression was measured by Q-PCR as well as by Western blotting.

Glucose oxidase assay. For the glucose oxidase colorimetric method, we used the Amplex® Red glucose/glucose oxidase assay kit, following the manufacturer’s instruction. Absorption at 571 nm was measured in a PowerWave™ XS microplate Spectrophotometer (BioTek). This experiment was performed in triplicate (three wells for each condition).

Glucose uptake assay. 3T3-L1 cells were differentiated as above noted, except that cells were exposed to differentiation regimen (DMI) for three days. At day 3, cells were fed with DMEM containing 2% FBS. Fresh media were changed 24 hours before the assay. Before the assay, cells were starved for 3 hours in serum-free DMEM. Glucose uptake was determined as previously reported (15).

Statistical analysis. Statistics were generally performed with the T-test, except for comparisons of serum Lcn2 levels between lean and obese mice, for which the non-parametric Mann-Whitney test was employed because of non-normal distribution of data or small n.

RESULTS

Lcn2 expression in 3T3-L1 adipocytes is induced by dexamethasone and TNF-α. We performed a genomic screen to identify common mechanisms of insulin resistance, using Dex and TNF treatment of 3T3-L1 adipocytes as a model system. The major outcome of this study was the observation that genes associated with reactive oxygen species (ROS) were affected concordantly by these two highly disparate treatments (14). Lcn2 was another gene induced strongly by both TNF and Dex in the microarray experiment. This effect was confirmed by Q-PCR, which showed induction of Lcn2 mRNA of approximately 80-fold by Dex and 30-fold by TNF (Fig. 1A). The effect of TNF was also seen previously (16). The insulin-sensitizing agent rosiglitazone significantly attenuated Lcn2 mRNA expression by either agent.
**Lcn2 is highly expressed in adipocytes in vitro and in vivo.** Others have reported Lcn2 expression in fat (16-19), but there has been no attempt to compare adipose expression to other sites. Northern analysis showed that white adipose tissue was by far the dominant site of expression of Lcn2 in wild-type male mice (Fig. 1B). We also saw significant amounts of Lcn2 mRNA in lung and in testis/epididymis, both reported as major sites of expression (20). We next sought to determine whether Lcn2 expression is regulated during adipogenesis. 3T3-L1 pre-adipocytes were differentiated using a standard cocktail containing Dex, methylisobutylxanthine (Mix), and insulin and Lcn2 expression was assessed with Q-PCR at various time points. We noted an immediate and profound induction of Lcn2 mRNA within the first day of differentiation (Fig. 2A); levels remained elevated for at least seven days. The Ct for Lcn2 in mature 3T3-L1 adipocytes ranges from 21-23; the corresponding value in murine WAT is 18. We looked at the contribution of each component of the induction cocktail (Fig. 2B), and found that Dex was the dominant contributor to Lcn2 induction, as expected. Mix also had a significant effect on Lcn2 levels, however, and the combination of Dex and Mix was maximally potent, with no significant contribution from insulin. We were interested, however, to know whether Lcn2 expression in 3T3-L1 cells was dependent on the specific induction cocktail or whether it was linked to adipogenesis per se. This was addressed by differentiating 3T3-L1 cells in the absence of Dex, Mix, or insulin, using rosiglitazone only. Lcn2 expression rose more gradually than when Dex/Mix were present (Fig. 2C), but reached similar levels later during differentiation. The apparent contradiction between the effect of rosiglitazone in Fig. 1 and Fig. 2 is resolved by considering the developmental status of the cells; in undifferentiated cells, rosiglitazone promotes adipogenesis and thus indirectly promotes Lcn2 expression. In mature cells, however, the direct effect of rosiglitazone is suppression of Lcn2 expression.

**Expression of Lcn2 in adipocytes is C/EBP-dependent.** Many adipocyte genes are transcriptionally regulated by PPARγ and/or members of the C/EBP family of bZIP proteins (18). The ability of rosiglitazone to repress Lcn2 (Fig 1A) suggested that PPARγ was unlikely to be a direct inducer of Lcn2 expression. We thus tested whether C/EBP isoforms might serve this purpose. C/EBPα, β and δ, delivered by retroviral transduction, were all effective at inducing endogenous Lcn2 expression in PPARγ−/− fibroblasts (Fig. 3A). These cells were chosen to avoid the confounding effects of simultaneous adipogenesis; C/EBPs cannot induce differentiation in the absence of PPARγ (14). To identify C/EBP binding sites in the Lcn2 promoter, we performed a computational search (Fig. 3B). This revealed a possible C/EBP site with a high degree of conservation between mouse, rat and human at −218 of the murine promoter. Trans-activation assays in NIH-3T3 cells showed that the ability of C/EBPδ to induce expression of this construct dropped off sharply when deletions were made that eliminated this site (Fig. 3C). The same effect was seen with C/EBPα and β (data not shown).

Mutation of the core sequence of this site from TTGC to GGGA significantly decreased the ability of C/EBPδ to trans-activate the reporter (Fig. 3D). Finally, we used Chromatin Immunoprecipitation (ChIP) to demonstrate specific C/EBP isofrom binding to this element in living cells (Fig. 3E). Prior to differentiation, no C/EBP isofrom was bound to the site, but C/EBPβ and δ were highly bound by the first day after induction. By day 4, C/EBPα binds the site as well, consistent with the delayed appearance of this factor during 3T3-L1 adipogenesis (21), followed later by a reduction in C/EBPβ and δ binding that reflects their diminished expression.

**Lcn2 levels are elevated in obesity.** We next looked at whether Lcn2 expression is altered by obesity. Western blotting of lysates from the adipose tissue of obese (ob/ob) mice revealed a significant elevation of Lcn2 relative to lean controls (Fig. 4A). We also examined adipose tissue from mice fed either a chow or high-fat diet after fractionation into mature adipocytes, stromal-vascular cells, and macrophages (Fig 4B). Lean animals (30.3±0.4 g; n=9) had equivalent Lcn2 mRNA expression in the adipocyte and stromal-vascular fractions, while obese animals (53.0±0.10 g; n=8) shifted Lcn2 mRNA expression away from the stromal-vascular fraction and toward mature adipocytes. There was no significant expression of Lcn2 in adipose tissue resident macrophages in either the lean or obese state. We were somewhat surprised to find significant Lcn2 expression in the SVF of lean animals, given the low levels seen in cultured pre-adipocytes. There are two plausible explanations for this. First, the low-speed centrifugation method used to separate adipocytes from SVF may not separate cells that are early in the differentiation process (i.e. prior to significant lipid
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accumulation). Since Lcn2 appears to be induced early in differentiation, this could account for a higher-than-expected amount of Lcn2 in the SVF. Alternatively, there may be significant Lcn2 expression in other cell types in the SVF (e.g. endothelial cells or fibroblasts). Consonant with the expression in other cell types in the SVF (e.g. alternatively, there may be a significant amount of Lcn2 in the SVF.

In the fasted state (13.6-fold ± 4.2, p=0.01). Lcn2 levels are still elevated in obese db/db mice even in the fasted state (13.6-fold ± 4.2, p=0.01). The body weights of these mice were as follows: 31.6±0.8 g (chow) vs. 39.9±0.7 g (HFD); 21.0±0.45 g (db/+ vs. 41.3±0.85 g (db/db); 26.0±0.51 g (ob/+ vs. 47.7±3.51 g (ob/ob).

Lcn2 promotes insulin resistance in cultured adipocytes and hepatocytes. Several factors converge to suggest that Lcn2 may promote insulin resistance, including serum elevation in obesity, induction by TNF and Dex, repression by TZDs, and structural similarity to RBP4. We attempted to test this directly by adding purified Lcn2 to mature 3T3-L1 adipocytes and then measuring insulin-stimulated glucose uptake, but were unable to find a consistent change in glucose uptake in the presence of Lcn2, either as apo-Lcn2, or after the protein was incubated with a siderophore-iron complex (data not shown). We were concerned that this might reflect the fact that Lcn2 is not limiting in the culture medium of 3T3-L1 adipocytes, which produce and secrete large amounts of the protein. The amount of Lcn2 in conditioned medium is similar to that seen in the serum of obese mice (data not shown). We thus approached this issue from a different direction, by asking whether reducing Lcn2 levels leads to improved insulin action. This was accomplished through retroviral delivery of shRNA directed against Lcn2. We identified a hairpin that reduced expression of Lcn2 by >90%, as measured by Q-PCR (Fig. 5A) or Western blot (Fig. 5B). Importantly, cells expressing this shRNA were differentiated to the same degree as cells expressing a control hairpin, as determined by oil red O staining of lipid accumulation (Fig. 5C) and marker expression (Figs. 5A, B). Cells expressing the Lcn2 shRNA, however, showed elevated glucose uptake in both the basal and insulin-stimulated state (Fig. 5D). Importantly, the component of glucose uptake that reflects insulin action (i.e. the difference between the insulin-stimulated and the basal glucose uptake) was significantly elevated in cells expressing the Lcn2 shRNA (Fig. 5E).

We next tested whether exogenous Lcn2 could affect insulin sensitivity in cultured H4IIE hepatocytes. Lcn2 complexed to siderophore and to iron by itself had no discernible effect on either glucose production (Fig. 6A) or glucose-6-phosphatase expression (Figs. 6B, 6C). Liganded Lcn2 was able, however, to render insulin less able to suppress these parameters. No effect of Lcn2 was seen on PEPCK mRNA levels, either in the presence or absence of insulin (data not shown). Importantly, the magnitude of insulin resistance induced by Lcn2 in these cells was comparable to that achieved with Dex. Interestingly, apo-Lcn2 (i.e. not complexed with siderophore and iron) was unable to induce insulin resistance in cultured hepatocytes (Fig. 6D).

**DISCUSSION**

It is now appreciated that adipocytes secrete a wide array of proteins that influence systemic metabolism. These include factors that promote insulin sensitivity as well as others that induce insulin resistance(3). We show here that Lcn2 is highly expressed in adipocytes, that its expression is regulated by obesity, and that it induces insulin resistance. In this sense it behaves in a very similar fashion to RBP4, another member of the lipocalin superfamily and a close relative of Lcn2. While others have noted adipose expression of Lcn2 (16-19), our data are the first to demonstrate that adipocytes may be the dominant source of Lcn2 expression. Furthermore, we show that adipose-specific expression is dictated in large part by C/EBP-dependent trans-activation of a defined element in the Lcn2 promoter. The lack of Lcn2 in BAT is interesting, and implies that white adipose-specific factors besides C/EBP are required, or that BAT contains specific repressors of Lcn2 synthesis.

Lcn2 has been proposed to serve many functions, ranging from apoptosis to uterine involution to genitourinary development (12; 22; 23). Data
Lipocalin 2 is a novel adipokine obtained from knockout mice, however, suggests that Lcn2 serves as part of the innate immune system used as a non-specific defense against microbes (10; 11). In this capacity, Lcn2 expression occurs in inflamed epithelial tissues in direct contact with potential pathogens, such as respiratory and intestinal epithelium (24). Adipose tissue is not usually considered to be in direct contact with invading pathogens, but a large body of data has now accumulated suggesting that fat is intimately involved in immune activity and the acute phase response. Furthermore, obesity is considered to be a pro-inflammatory state with elevation of multiple markers of inflammation (25); increased Lcn2 seen in obese animals is consonant with this idea.

Based on the studies presented here, we propose that Lcn2 acts as an adipocyte-derived mediator of insulin resistance. This assertion is founded on several lines of evidence, both direct and indirect. First, agents that promote insulin resistance induce the expression of Lcn2, including glucocorticoids and TNF-α. Similarly, hyperglycemia, which also reduces insulin sensitivity in adipocytes, causes enhanced expression of Lcn2 in adipocytes (26). Second, insulin-sensitizing TZD compounds reduce the expression of Lcn2 in adipocytes (Fig. 4, and (27)). Third, Lcn2 is elevated in multiple murine models of obesity. Finally, reduction of Lcn2 in cultured adipocytes improved insulin sensitivity, demonstrating a direct link between this secreted molecule and cellular glucose homeostasis. The fact that exogenous Lcn2 did not affect glucose uptake in 3T3-L1 adipocytes (data not shown) is interesting, and suggested to us that Lcn2 levels in media conditioned by the cultured adipocytes are already so high that adding more has no incremental effect. Interestingly, data from db/db mice(16; 27) indicates that Lcn2 expression is elevated in the liver in this obese model; our own data suggests that liver Lcn2 expression trends lower in high-fat fed mice (data not shown). Thus, the contribution of extra-adipose sources of Lcn2 to the serum is unclear and may differ between obesity models.

How might Lcn2 act to induce insulin resistance? Lcn2 has been proposed to be an iron delivery protein (22), and there is a well-known association between iron accumulation and diabetes (28). Patients with hemochromatosis, for example, have insulin resistance in addition to reduced insulin secretory capacity (28), and iron intake in healthy women has been positively associated with the risk of developing type 2 diabetes (29). Most of this effect has been inferred to involve hepatic insulin sensitivity (28); iron-mediated dysregulation of insulin action in adipocytes has never been explicitly assessed. Adipocytes certainly express iron-regulatory proteins, however, and iron has been shown to mediate lipolysis in cultured fat cells (30). Consistent with the idea that iron is required for the effect of Lcn2 on insulin action, apo-Lcn2 was ineffective in causing insulin resistance in cultured hepatocytes. Iron may induce insulin resistance through the formation of specific reactive oxygen species, given the well-studied role of transition metals in catalyzing the Fenton reaction in cells. We have shown that ROS act causally in multiple forms of insulin resistance in mice and in cultured adipocytes (15).

Lcn2 may also signal through a specific receptor, which may or may not involve the subsequent delivery of iron. Green and colleagues cloned an organic cation transporter suggested to mediate Lcn2 internalization and downstream functions in multiple cell types (31). We have confirmed the expression of this molecule in brain and liver using Northern analysis and PCR, but do not detect it in adipose tissue from mice or in 3T3-L1 cells at any stage of development (data not shown). This indicates that Lcn2 may exert its effects in adipocytes via another mechanism.

A recent study found that serum Lcn2 levels are increased in obese humans, with excellent correlation between Lcn2 and measures of insulin resistance in this population (27). We have seen that cytokines induce Lcn2 expression in human subcutaneous adipocytes in a manner similar to that seen in 3T3-L1 cells (data not shown). These data suggest a possible role for Lcn2 in human insulin resistance.

Our data suggest that Lcn2 may be added to the growing list of secreted molecules that adipocytes use to modulate glucose homeostasis. We are now testing this hypothesis directly in vivo in mice using both gain-of-function and loss-of-function approaches.

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REFERENCES

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FIGURE LEGENDS

Fig. 1. Lcn2 is expressed in adipocytes and is regulated by Dex and TNF. A, mature 3T3-L1 adipocytes were treated with Dex (1 µM) or TNF (4 ng/µL) in the presence or absence of rosiglitazone (Rosi; 1 µM), and Lcn2 mRNA levels were measured by Q-PCR. Data presented as mean ± SD, *p<0.05, ***p<0.001 relative to no Rosi, n=3. B, Northern analysis of murine tissues heart (H), brain (B), spleen (S), lung, (Lu), liver (Li), skeletal muscle (Sk), kidney (K), testis (T), brown adipose tissue (Ba), and perigonadal white adipose tissue (Wa) from male C57Bl/6 mice.

Fig. 2. Expression of Lcn2 during 3T3-L1 adipogenesis. A, Time course of Lcn2 mRNA expression during 3T3-L1 adipogenesis. Data are presented as mean ± SD, n=3. B, Lcn2 mRNA expression in confluent 3T3-L1 pre-adipocytes treated with Dex (D), MIX (M), Insulin (I) or combinations thereof. Data are presented as mean ± SD, n=3. C, Lcn2 expression during 3T3-L1 differentiation induced by rosiglitazone, in the absence of DMI. Data are presented as mean ± SD, n=3. For B and C, the inset shows the corresponding amount of Fabp4 mRNA to mark the extent of differentiation.

Fig. 3. Lcn2 expression in adipocytes is C/EBP dependent. A, PPARγ-/- cells were infected with C/EBP-expressing retroviruses and endogenous levels of Lcn2 were measured by Q-PCR relative to cells transduced with empty vector. Data presented as mean ± SD, * p<0.05, **p<0.01, ***p<0.001, n=3. B, Alignment of mouse, rat and human Lcn2 promoter sequences reveals a putative C/EBP binding site. Boxed letters, core nucleotides essential for C/EBP binding. C, Deletion analysis of murine Lcn2 promoter fragments in transiently transfected NIH-3T3 cells in the presence (gray bars) or absence (white bars) of co-transfected C/EBPδ. Data represent mean ± SD, n=3. D, Mutation analysis of the core C/EBP-binding motif. NIH-3T3 cells were transfected with the wild-type -222 fragment-luciferase construct or with the same fragment after mutation of the core TTGC in the presence (gray bars) or absence (white bars) of co-transfected C/EBPδ. Data represent mean ± SD, n=6, # p=3.3e-10. E, Chromatin immunoprecipitation assay of the proximal Lcn2 promoter in 3T3-L1 cells at different time-points after differentiation.

Fig. 4. Lcn2 is elevated in obesity. A, Lcn2 protein levels in perigonadal white adipose tissue lysates (30 µg/lane) from male ob/+ (n=5) and ob/ob (n=7) mice. Mean ± SD, * p<0.05. B, Lcn2 mRNA expression in fractionated white adipose tissue from male C57BL mice given chow (n=7) or high-fat diet (n=7), relative to expression in chow macrophages. SVF= stromal vascular fraction, Ads= adipocytes, Macs = macrophages. Mean ± SD, * p<0.05, ***p<0.001. C, Lcn2 protein levels in serum from fed male ob/+ (n=6) and ob/ob mice (n=10), measured by Western blotting and expressed as fold relative to the mean of ob/+ controls. D, Lcn2 protein expression in serum from fed female db/+ (n=8) and db/db mice (n=8), expressed as fold relative to the mean of db/+ controls. E, Lcn2 protein expression in serum from chow (n=15) and high-fat fed male mice (n=18), expressed as fold relative to the mean of chow fed controls. Data for C, D, and E are shown as the mean for each group, with representative Western blots from three lean and three obese animals shown at top. For SD and statistical analysis see text.

Fig. 5. shRNA-mediated knockdown of Lcn2 improves insulin action in cultured adipocytes. A, mRNA expression of Lcn2 and markers in mature 3T3-L1 adipocytes expressing either control shRNA or shLcn2. B, protein expression of Lcn2 and other markers in mature 3T3-L1 adipocytes expressing either control shRNA or shLcn2. C, Oil Red O staining of mature 3T3-L1 adipocytes expressing either control shRNA or shLcn2. D, Basal (white bars) and insulin-stimulated (black bars) glucose uptake in mature 3T3-L1 adipocytes expressing either control shRNA or shLcn2. n=12, mean ± SD, * p<1e-5 relative to control shRNA, no insulin; # p=5e-5 relative to control shRNA, plus insulin. E, component of glucose uptake attributable to insulin, equivalent to the uptake in the presence of insulin minus the uptake in the absence of insulin. n=12, mean ± SD, * p<1e-4.
Fig. 6. Exogenous recombinant Lcn2 induces insulin resistance in H4IIE hepatocytes. **A**, **Left**, Glucose production induced by liganded Lcn2 (10 nM) or Dex (250 nM) in the presence or absence of insulin (100 nM). **Right**, effect of liganded Lcn2 (10 nM) or Dex (250 nM) on glucose-6-phosphatase mRNA expression in the presence or absence of insulin (100 nM). **B**, Dose response of liganded Lcn2 on glucose-6-phosphatase expression. **C**, Effect of apo-Lcn2 on glucose-6-phosphatase expression. For all panels, mean ± SD, * p<0.05, ** p<0.01, n=3.
Figure 1

A

![Bar chart showing relative expression levels under different conditions](chart.png)

B

![Image showing gel electrophoresis results](gel.png)

Lipocalin 2 is a novel adipokine
Figure 2

A

Relative Expression

Pread 0 1 2 3 4 5 6

B

Relative Expression

Ctrl D M I DI MI DMI

C

Relative Expression

Days post-Rosi

-2 0 1 3 5 7 9
Lipocalin 2 is a novel adipokine

Figure 3

A

B

C

D

E

Mouse  
-219  
CCTGGGTCAACCTGCACAGTCC

Rat  
CTGGGTCAACCTGCACAGTCC

Human  
TGCTGGCACCATCCTGACCAGGTGC

C/EBP Binding Site

C/EBPα

C/EBPβ

C/EBPδ

Relative Luc Activity

pcDNA

pcDNA-C/EBPδ

pA3Luc -131 -222 -320 -731 -1742

Relative Luc Activity

Day 0 1 2 4 7

C/EBPα

C/EBPβ

C/EBPδ

Input
Figure 4

Lipocalin 2 is a novel adipokine
Figure 5

A. Relative expression of Lcn2, Fabp4, PPARγ, Leptin, and Glut1 in Control and shLcn2 groups.

B. Western blot analysis showing Lcn2, IRS1, Akt2, and Glut4 expression in Control and shLcn2 groups.

C. Immunohistochemical staining for Lcn2 in Control and shLcn2 groups.

D. Relative glucose uptake in the absence or presence of insulin in Control and shLcn2 groups.

E. Insulin-stimulated component of glucose uptake in Control and shLcn2 groups.
Figure 6

A

B

C

Lipocalin 2 is a novel adipokine