Lipocalin-2 deficiency attenuates insulin resistance associated with ageing and obesity

Running title: Lipocalin-2 and insulin resistance

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Objectives: The pro-inflammatory cytokines/adipokines produced from adipose tissue act in an autocrine and/or endocrine manner to perpetuate local inflammation and to induce peripheral insulin resistance. The present study investigates whether lipocalin-2 deficiency or replenishment with this adipokine has any impacts on systemic insulin sensitivity and the underlying mechanisms.

Methods and Results: Under ageing or dietary-/genetic-obese conditions, lipocalin-2 knockout (Lcn2-KO) mice show significantly decreased fasting glucose and insulin levels, and improved insulin sensitivity compared to their wild type littermates. Despite enlarged fat mass, inflammation and the accumulation of lipid peroxidation products are significantly attenuated in the adipose tissues of Lcn2-KO mice. Adipose fatty acid composition of these mice varies significantly from that in wild type animals. The amounts of arachidonic acid (AA, C20:4 n6) are elevated by ageing and obesity, and paradoxically further increased in adipose tissue, but not skeletal muscle and liver of Lcn2-KO mice. On the other hand, the expression and activity of 12-lipoxygenase, an enzyme responsible for metabolizing AA, and the production of tumor necrosis factor alpha (TNFα), a critical insulin resistance-inducing factor, are largely inhibited by lipocalin-2 deficiency. Lipocalin-2 stimulates the expression and activity of 12-lipoxygenase and TNFα production in fat tissues. Cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC), an arachidonate lipoxygenase inhibitor, prevents TNFα expression induced by lipocalin-2. Moreover, treatment with TNFα neutralization antibody or CDC significantly attenuated the differences of insulin sensitivity between wild type and Lcn2-KO mice.

Conclusions: Lipocalin-2 deficiency protects mice from developing ageing- and obesity-induced insulin resistance largely by modulating 12-lipoxygenase and TNFα levels in adipose tissue.
The prevalence of obesity increases dramatically and has attained the characteristics of an epidemic (1). Studies in both humans and animals demonstrate that obesity is a state of low-grade, chronic inflammation, characterized by elevated circulating pro-inflammatory molecules produced predominantly from enlarged adipocytes and activated macrophages in adipose tissue (2-4). In fact, chronic inflammation in adipose tissue per se plays a key role in the development of obesity and associated metabolic disorders, such as type 2 diabetes mellitus (T2DM). Various pro-inflammatory adipokines, including tumor necrosis factor alpha (TNFα), interleukin 6 (IL6), resistin, retinol-binding protein 4 (RBP4) and plasminogen activator inhibitor-1, directly antagonize the metabolic actions of insulin and causes decreased insulin sensitivity (5; 6).

Lipocalin-2, also called growth factor-stimulated superinducible protein (SIP24) (7), neutrophil gelatinase-associated lipocalin (NGAL) (8), 24p3 or oncogene neu-related lipocalin (9; 10), belongs to the lipocalin superfamily consisting of over 20 small secretory proteins, including RBP4, adipocyte fatty acid binding protein, apolipoprotein D and prostaglandin D synthase (11). Members of lipocalin family share a highly conserved structural homology (12). By forming a cup-shaped hydrophobic cavity, lipocalins bind and transport a variety of small lipophilic substances such as retinoids, arachidonic acid and various steroids. Although lipocalin-2 can bind weakly to some common ligands of lipocalins, including leukotriene B4 and platelet activating factor, its high affinity endogenous ligand(s) remain to be identified.

Lipocalin-2 is abundantly produced from adipocytes (13-15). The expression and secretion of this protein increases sharply after conversion of preadipocytes to mature adipocytes. Its expression can be induced by various inflammatory stimuli, including lipopolysaccharide (LPS) and IL-1β (16; 17). The pro-inflammatory transcription factor NF-κB transactivates lipocalin-2 expression through binding to the consensus motif within its promoter (16; 18). These evidence suggest that lipocalin-2 may participate in inflammation-related disorders. Expression of lipocalin-2 in adipose tissue is elevated in various experimental models of obesity and in obese humans (19-23). Moreover, this increase can be reversed by the insulin-sensitizing drug rosiglitazone. In human subjects, serum concentrations of lipocalin-2 are associated closely with obesity-related anthropometric and biochemical variables (20). The positive correlations of serum lipocalin-2 with fasting glucose, homeostasis model assessment of insulin resistance (HOMA-IR) index and the inflammatory marker high sensitivity C-reactive protein (hs-CRP) are significant even after adjustment for body mass index, suggesting that it is an independent risk factor for insulin resistance, diabetes and inflammation. The present study has used a knockout mouse model to evaluate the impact of lipocalin-2 loss-of-function on systematic energy homeostasis and insulin sensitivities under both basal and obese conditions. The results demonstrate that lipocalin-2 plays a causal role in the development of insulin resistance at least partly through modulating the inflammatory responses in adipose tissue.

MATERIALS AND METHODS

Experimental animals. Male mice were used for this study. C57BL/6J and C57BL/6J db/db diabetic mice were from the Jackson Laboratory, Bar Harbor, Maine. The lipocalin-2 knockout (Lcn2-KO) mice were generated as reported (24). The mRNA and protein levels of lipocalin-2 were undetectable in all tissues evaluated including liver, fat and
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muscle. The mice were backcrossed to C57BL/6J mice for more than 20 generations. Leptin receptor (Lepr)/lipocalin-2/- double knockout (DKO) mice were established by cross-breeding male C57BL/6J db/+ mice with female Lcn2-KO mice. The mice were housed in a room under controlled temperature (23 ± 1°C) and 12-hour light-dark cycle, with free access to water and standard chow (LabDiet 5053; Purina Mills, Inc., Richmond, IN). Dietary obesity was induced in wild type and Lcn2-KO mice by allowing free access to a high-fat diet (D12451, Research Diet, New Brunswick, NJ) from the age of 4-weeks onwards. The comparisons throughout this study are between wild type and knockout littermates from hetero-crosses. Intra-peritoneal glucose tolerance test (ipGTT) and insulin tolerance test (ITT) were performed using mice that were fasted for overnight and six hours, respectively, as described (25). For drug treatment, 8 mg/kg of CDC (Cinnamyl-3,4-Dihydroxy-a-Cyanocinnamate, BIOMOL Research Laboratories, Plymouth Meeting, PA) mixed with sesame oil were injected intraperitoneally three times per week for two weeks. The control mice were injected with diluent sesame oil. TNFalpha neutralization experiment was performed by injecting the TNFα-neutralizing antibody (50 µg/mouse/day ip; Sigma-Aldrich Corp., St. Louis, MO) or control IgG during the two-week treatment period. The animal experimental procedures were approved by the Committee on the Use of Live Animals for Teaching and Research, University of Hong Kong, and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Production of recombinant adenoviruses and lipocalin-2 for in vivo treatment. The adenovirus vector encoding FLAG-tagged murine lipocalin-2 was generated using the Adeno-X Expression System (Clontech, Mountain View, CA). The recombinant adenovirus was injected into the tail vein of mice two weeks prior to tissue collection (25). The amount of injected adenovirus [10^8 plaque-forming units (pfu)] caused no toxicity in the mice. The increased expression level of lipocalin-2 was confirmed by both Western Blotting and ELISA (Supplementary Figure 1 can be found in an online appendix at http://diabetes.diabetesjournals.org). Recombinant murine lipocalin-2 was expressed, purified and endotoxin removed as described (20). The purity of the protein was confirmed by SDS-PAGE and mass spectrometry analysis. No siderophore or iron was found to bind to the protein.

Measurement of insulin and lipid levels. Fasting serum insulin concentrations were determined with a commercial ELISA kit (Mercodia AB, Uppsala, Sweden). The amounts of triglyceride (TG), total cholesterol (TC), and free fatty acids (FFA) in tissues and serum samples were analyzed as described elsewhere (26). Fatty acid compositions of the epididymal adipose tissue, liver and muscle were analysed by gas chromatography-mass spectrometry (GC-MS) (27). Nonadecanoic acid C19:0 and tridecanoic acid methyl ester C13:0 were added as internal control during sample processing. The standard curve was generated using the fatty acid standard Supelco® 37 Component FAME mix (10 mg/ml).

ELISA quantification of lipocalin-2, adiponectin, TNFα and 12(S)-HETE. Total serum lipocalin-2 and adiponectin levels were measured using in-house ELISAs (20; 28). Serum TNFα concentrations were quantified using a high-sensitivity TNF-alpha Quantikine ELISA System (R&D Systems Inc., Minneapolis). Mouse adipose TNF-alpha levels were measured using immunoassay kit from Invitrogen (Camarillo, CA). Tissue membrane and soluble fractions were prepared as described (29). Equal amounts (500µg) of samples were used for analysis. 12(S)-HETE in different tissues was measured using an
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EIA (Assay Designs, Ann Arbor) as described (30).

Measurement of glucose uptake. Fat pads or skeletal muscle strips were stimulated with or without insulin and the glucose uptake determined as described (31).

Evaluation of in vivo insulin signaling. After overnight fasting, mice were anaesthetized and one IU per kg insulin (Novo Nordisk, Denmark) or an equal volume of vehicle was administered through the portal vein. Adipose tissue (epididymal fat pads), liver and soleus muscle was collected 120 seconds after the injection and immediately stored in liquid nitrogen for subsequent Western Blotting analysis.

Quantitative RT-PCR analysis. Quantitation of target genes was performed using SYBR Green PCR Master Mix (Qiagen) and an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster city, CA). The primer sequences are listed in Supplementary Table 1.

Western Blotting. Antibodies against total or phosphorylated Akt and insulin receptor beta (IRbeta) were purchased from Cell Signaling. 100 micrograms of proteins derived from cell or tissue lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The immune complexes were detected with the enhanced chemiluminescence reagents from GE Healthcare (Uppsala, Sweden).

Thiobarbituric acid reactive substance (TBARS) assays. The concentrations of the lipid peroxidation product malondialdehyde (MDA) were determined with a commercial TBARS assay kit (Cayman Chemical, Ann Arbor, MI). The results were calculated against the total protein contents.

Total body electrical conductivity (TOBEC) measurement. The TOBEC was measured in an EM-SCAN SA-3203-type chamber (EM-SCAN Inc., Springfield, IL). Briefly, mice were anaesthetized and placed in the middle of the plexiglass® cylinder. A 10-HMz oscillating magnetic field was applied and the energy dissipation was detected and expressed as E-value. At least 5 measurements were taken for each mouse each time. The fat-free body mass was calculated by the formula: -3.732+0.578*body weight (g) + 2.967*E^{0.5}.

Histological analysis. Paraffin sections (5 µm) were prepared for hematoxylin and eosin staining and analyzed under a microscope (Leica Microsystems, Bensheim, Germany). The sizes of adipocytes were measured using the software Image J. Histological staining of macrophage specific marker was performed as described (32; 33).

Data analysis. All results were derived from at least three sets of repeated experiments. The statistical calculations were performed with the SPSS 11.5 statistical software package (SPSS Inc). Differences between groups were determined by Student’s t-test. All values were presented as means ± SD. In all statistical comparisons, a P value less than 0.05 was used to indicate significant differences.

RESULTS

Improved systemic insulin sensitivity in mice without lipocalin-2 under conditions of ageing, dietary and genetic obesity. Mice lacking lipocalin-2 had similar growth rates and food intake compared to their wild type littermates (Figure 1, A and B). However, starting from the age of 11 weeks, the fasting glucose levels of Lcn2-KO mice were significantly lower than those of wild type mice (Figure 1C). Moreover, the fasting serum insulin levels were constantly lower by ~45% in Lcn2-KO mice compared to wild type mice at all time points (Figure 1D). At the end of the monitoring period, wild type mice were much more glucose intolerant and insulin resistant than Lcn2-KO mice (Figure 1E and F). In fact, the values of ipGTT area under curve (AUC) in Lcn2-KO mice at the age of 11, 15 and 21 weeks were significantly
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Reduced than those in wild type mice (Figure 1G). Similar results had also been observed for ITT, showing that insulin sensitivity was greatly improved in Lcn2-KO mice at 13 and 23 weeks (Figure 1H).

Dietary obesity was induced by feeding the mice with 18-week of high fat diet. Compared to wild type animal, the percentage body weight gain of Lcn2-KO mice was slightly lower (116.4±0.2 % and 96.8±0.12 %, respectively), despite a similar food intake (Figure 2, A and B). The fasting glucose levels of Lcn2-KO mice were lower (4.0±0.67–5.6±1.18 mmol/L) than those of the wild type littersmates (6.2±0.22–8.4±1.51 mmol/L) throughout the monitoring period (Figure 2C). Although hyperinsulinemia was observed in both types of animals, the values remained to be much lower in Lcn2-KO mice than those of the wild type littersmates (Figure 2D). At the end of the treatment, severe glucose intolerance and insulin resistance were developed in wild type mice (Figure 2, E and F). Lipocalin-2 deficiency significantly alleviated high fat diet induced insulin resistance, and the effect could be observed as early as five weeks after high fat diet feeding (Figure 2, G and H).

Next, leptin receptor–deficient db/db mice lacking the expression of lipocalin-2 (DKO) were generated. Both db/db and DKO mice showed early onset obesity (Figure 3A). The food intake of db/db mice was slightly higher when compared to DKO mice (Figure 3B). At 7-week of age, db/db mice developed hyperglycemia (fasting glucose levels: 10.16±2.67 mmol/L, Figure 3C). By contrast, both fasting and fed blood glucose levels (data not shown) of DKO mice were maintained at a much lower level throughout the observation period. The db/db mice developed a severe and progressive hyperinsulinemia during the course of the study (348.421±75.716, 420.826±94.706 and 516.778.421±73.225 µU/ml at 7-, 9- and 11-week, respectively) (Figure 3D), whereas DKO mice showed a significantly lower fasting plasma insulin levels (55.18±12.8, 60.48±26.21 and 97.67±35.63 µU/ml at 7-, 9- and 11-week, respectively). The results from both ITT and HOMA-IR calculations confirmed that systemic insulin sensitivity was significantly higher in DKO mice compared to those of db/db controls (Figure 3, E and F).

Recombinant adeno viruses were used for administration of exogenous murine lipocalin-2 into Lcn2-KO mice and the wild type littersmates. Overexpressing this adipokine for two weeks significantly elevated fasting glucose levels and HOMA-IR indexes in both types of animals (Supplementary Figure 1). The serum insulin levels were significantly augmented in Lcn2-KO mice, but only slightly increased in wild type controls, as compared to those treated with recombinant adeno viruses encoding luciferase. On the other hand, acute treatment with lipocalin-2 recombinant protein by intraperitoneal injection into both types of animals at various dosages had no effects on circulating glucose and insulin levels during the short period of treatment (up to 24 hrs, data not shown).

Despite enlarged mass, the fat tissues of Lcn2-KO mice show attenuated inflammation and increased insulin sensitivity. Circulating lipid profiles were analyzed in wild type and lipocalin-2 null mice under four different conditions (Supplementary Table 2). Although elevated serum FFA levels could contribute to the development of systemic insulin resistance, no significant changes were detected. Serum TC levels were reduced in lipocalin-2 deficient mice. However, overexpression of lipocalin-2 did not increase the circulating TC concentrations. Individual tissue sample analyses revealed that compared to wild type mice, the amount of all three major lipid species (TG, FFA and TC) were increased by 1.5- to 1.8-fold in epididymal fat of Lcn2-KO mice fed with either normal chow or high fat diet. Moreover, overexpression of lipocalin-2 significantly reduced the lipid content in fat.
In obese Lcn2-KO mice, an expansion of the epididymal adipose tissue by ~50% was observed compared to wild type mice (Figure 4A). In DKO mice, the net weight of epididymal fat pad was also increased by approximately ~25% compared to db/db mice (data not shown). Conversely, adenovirus-mediated overexpression of lipocalin-2 reduced the epididymal adipose tissue mass by ~55% in wild type mice and ~48% in Lcn2-KO mice. Compared to the wide type mice, lipogenesis was significantly increased and lipolysis decreased in the adipose tissues of Lcn2-KO mice (Supplementary Figure 4). Histological examination revealed that the average areas of adipocytes derived from epididymal fat pads of obese Lcn2-KO mice was about three fold larger than those of obese wild type mice (Figure 4B). When expressed on a per organ basis, the total lipid contents in epididymal fat pads of high fat diet-fed Lcn2-KO obese mice were even more markedly augmented (FFA: 44.8±8.29 mmol; TG: 137.174±25.39 mg; TC: 1.7±0.32 mg) compared with that of wild type obese mice (FFA: 19.8±4.12 mg; TG: 55.9±11.62 mg; TC: 0.76±0.16 mg). The average cell size of epididymal adipocytes of Lcn2-KO mice fed with normal chow was also significantly larger compared to wild type littermates (Figure 4B). Increased subcutaneous fat mass had also been observed for obese Lcn2-KO mice compared to wild type littermates (data not shown). Body composition analysis using TOBEC, which reflects total body fat mass (34), revealed that 15 weeks of high fat diet induced an increase of 40% and 24% fat mass in Lcn2-KO mice and wild type mice, respectively, whereas the values were not significantly different between those fed with normal chow (wild type mice: 12.32±2.921; Lcn2-KO mice: 13.42±1.8309).

Immunohistochemical staining revealed that a large number of F4/80 positive macrophages were accumulated in the epididymal fat tissues from high fat diet-fed wild type mice, whereas the macrophages were virtually undetectable in Lcn2-KO mice, despite the enlargement of the fat cells (Figure 4C). The concentrations of malondialdehyde (MDA), markers of oxidative stress, were lower by 50% in Lcn2-KO mice compared to wild type mice (Figure 4D). The total protein levels of IκBalpha were increased in the adipose tissues of Lcn2-KO mice (data not shown). Quantitative PCR analysis revealed that the expressions of TNFα, MCP-1, F4/80 and CD14 were significantly lower in high fat-fed Lcn2-KO mice compared to those of the wild type animals (Supplementary Table 3). Insulin-induced phosphorylation of insulin receptor (IR) and Akt was examined in adipose tissue. While high fat diet-fed mice showed a much lower magnitude of response to portal vein injection of insulin (Figure 4E), both IR and Akt phosphorylations were enhanced significantly in lean and obese Lcn2-KO mice compared to wild type animals. Moreover, the insulin-stimulated glucose uptake was significantly higher in epididymal fat pad of Lcn2-KO mice, under both normal and high fat diet conditions than those of wild type mice (Figure 4E). Compared to fat tissue, the phosphorylations of IR and Akt in skeletal muscle and liver tissues showed less prominent changes between mice with or without lipocalin-2. Insulin-stimulated glucose uptake was not significantly different in soleus muscle of Lcn2-KO mice from that of the wild type littermates (Supplementary Figure 2A). Of note is that the expressions of key genes involved in gluconeogenesis were much lower in obese Lcn2-KO mice (Supplementary Figure 2B).

Lipocalin-2 treatment stimulates TNFα expression in adipose tissue partly through up-regulating 12-lipoxygenase expression and activity. GC-MS analysis revealed that fatty acid composition in the epididymal adipose tissue of Lcn2-KO mice, but not in the liver...
and skeletal muscle, varied significantly from those of wild type littermates, under both standard chow and high fat diet conditions (Supplementary Figure 3). One of the significantly increased fatty acid species was arachidonic acid (AA, C20:4 n6) (Figure 5A). Ageing and high fat diet elevated AA contents in adipose tissues, which were found to be further elevated in Lcn2-KO mice. Quantitative real time PCR was performed to measure the expression levels of enzymes involved in AA metabolic pathways. The results demonstrated that while cyclooxygenase-1 and cyclooxygenase-2 were not obviously different between the two types of animals (data not shown), lipocalin-2 deficiency dramatically attenuated both ageing- and dietary obesity-induced upregulation of 12-lipoxygenase (Figure 5, B and C). The activity of 12-lipoxygenase, indicated by the total amount of its metabolite 12(S)-HETE, was also largely reduced in the adipose tissues of obese Lcn2-KO mice (Figure 5D). Note that in liver and skeletal muscle tissues, the gene expression (data not shown) and activity of 12-lipoxygenase were not different between mice with or without lipocalin-2.

The above results showed that lipocalin-2 deficiency decreased TNFα expression in adipose tissue (Supplementary Table 3). Further analysis using tissues derived from different ages of animals revealed that the increased TNFα mRNA levels associated with both ageing and obesity were blocked in Lcn2-KO mice, and the significant differences could be observed in animals as young as seven-week old (Figure 6A). Similarly, the protein levels of TNFα were also decreased in adipose tissues of Lcn2-KO mice, especially in the membrane fractions, with a reduction of ~70% (Figure 6B). Administration of recombinant adenovirus expressing lipocalin-2 promoted TNFα expression by ~5 and ~11 folds in Lcn2-KO mice fed with standard chow and high fat diet, respectively (Figure 6C). These effects were largely reversed by treatment with CDC, a small molecular inhibitor of 12-lipoxygenase. Furthermore, overexpression of lipocalin-2 resulted in a significant increase of 12-lipoxygenase expression (Figure 6C) and 12(S)-HETE production (data not shown) in adipose tissue. Acute treatment with lipocalin-2 significantly increased the mRNA levels of both 12-lipoxygenase and TNFα at one and two hours, respectively, in Lcn2-KO mice (Figure 7A), but not in those treated with CDC (data not shown). In the mean time, a transient but significant decrease of serum FFA was observed in mice treated with lipocalin-2 (Figure 7B). The 12(S)-HETE production was steadily elevated from two-hour after injection. These data indicated that arachidonate lipoxygenase pathway was involved in lipocalin-2 mediated TNFα production from adipose tissue. Note that a large amount of lipocalin-2 rapidly entered into the adipose tissues (Figure 7C). However, the levels of both serum and adipose lipocalin-2 gradually decreased and could not be detected at twelve hour after the treatment.

To investigate whether there was any relationship between the decreased 12-lipoxygenase activity/TNFα production and the improved insulin sensitivity in Lcn2-KO mice, CDC or specific TNFα neutralization antibody was administered into mice that were fed with high fat diet (Figure 8). Two weeks’ treatment with CDC significantly attenuated the progression of insulin resistance in both wild type and Lcn2-KO animals and abolished the differences between the two groups (Figure 8, A and B). On the other hand, similar treatment with TNFα neutralization antibody improved insulin sensitivity in wild type littermates, but had no significant effects on Lcn2-KO mice (Figure 8, C and D).

DISCUSSION

Although lipocalin-2 has been identified
for nearly two decades, its physiological function remains poorly understood. Studies have focused on its role in innate immune response to bacterial infection (24) and cancer progression (35). It has been considered as an early marker of acute kidney damage (36). In human obese subjects, like other insulin resistance-inducing adipokines and cytokines, circulating lipocalin-2 levels are markedly elevated (20-22). In db/db obese mice, increased serum levels of lipocalin-2 are mainly due to the selective augmentation of its expression in adipose tissue and liver (20; 21). Both stimulatory and inhibitory effects of lipocalin-2 on insulin sensitivities in 3T3-L1 adipocytes have been reported (21; 22). The present study has used a knockout mouse model to evaluate the physiological functions of lipocalin-2 on systematic energy homeostasis and insulin sensitivities. The results suggest that lipocalin-2 deficiency attenuates the development of ageing- and obesity-associated insulin resistance, hyperglycemia and hyperinsulinemia. Lipocalin-2 elicits its adverse effects at least partly by activating arachidonate 12-lipoxygenase metabolic pathway and stimulating adipose expression of TNFα, which may in turn magnify the local inflammation and cause impaired energy homeostasis and systemic insulin resistance.

TNFα has been proposed as a link between obesity and insulin resistance because it is highly expressed in adipose tissues of obese animals and humans and can directly impair insulin signaling in both cultured cells and experimental animals (37). Obese mice lacking either TNFα or TNFα receptors are protected against insulin resistance (38; 39). Infusion of TNFα to adult rats reduces systemic insulin sensitivity, which is associated with major changes of gene expression in adipose tissue (29; 40). Direct exposure of isolated cells to TNFα induces a state of insulin resistance in several systems, including adipocytes and myocytes (41). In addition to obesity and T2DM, insulin resistance is associated with many other pathological conditions including ageing, cancer and infections (42). A decline in fat-free mass and a relative increase in fat mass are common findings in aged subjects, which are associated with a rise in TNFα concentration and a deterioration of insulin action (43; 44). Neutralization of TNFα reverses age-induced impairment of insulin responsiveness (45). Although these pharmacological studies have attributed most of the action of TNFα to the pathogenesis of insulin resistance, the molecular basis underlying increased TNFα expression in obese state is largely unknown. The present study provides evidence suggesting that lipocalin-2 plays critical roles in regulating TNFα expressions in fat tissues, at least partly through upregulating 12-lipoxygenase expression and activity. First, increased levels of lipocalin-2 are found to be associated with both ageing (data not shown) and obesity (20) in wild type mice. Second, mice lacking lipocalin-2 are protected from ageing- and obesity-induced upregulation of TNFα and activation of 12-lipoxygenase in adipose tissue. Third, lipocalin-2 treatment increases TNFα levels, 12-lipoxygenase expression and activity. Fourth, blockage of arachidonate lipoxygenase pathway by CDC treatment prevents the induction of TNFα expression by both high fat diet (data not shown) and lipocalin-2 treatment. Taken together, the presence of lipocalin-2 may be indispensable for TNFα induction by various pathological conditions.

Consistent with the findings on TNFα production, insulin resistance is largely prevented in aged and obese Lcn2-KO mice. This improvement of insulin sensitivity is mainly correlated with attenuated inflammation in adipose tissues of mice lacking lipocalin-2. Both the total protein and adipose membrane fraction of TNFα are significantly decreased in obese Lcn2-KO
mice compared to wild type mice. Membrane
TNF-alpha is a precursor form of soluble
TNF-alpha and exerts pro-inflammatory
functions in a cell-to-cell contact manner. It
has been demonstrated that macrophages in fat
pads of obese mice and humans are localized
to dead adipocytes, and is often coincident
with increased TNFα expression (46). These
information suggest that lipocalin-2 may exert
adverse metabolic and inflammatory actions,
locally and systemically, partly through
up-regulating the expression of TNFα. This
has been further verified by introducing
neutralization antibodies to high fat-fed wild
type and Lcn2-KO mice. TNFα neutralization
attenuates insulin resistance in wild type mice,
while lipocalin-2 deficient mice do not show
reduced insulin sensitivity. Of note is that
CDC treatment, which attenuates TNFα
expression and 12-lipoxygenase activity
induced by lipocalin-2, improves insulin
sensitivity in both wild type and Lcn2-KO
mice. Since CDC at higher concentrations also
inhibits other lipoxygenases, it is highly
possible that some unidentified inflammatory
mediators may play a role in causing insulin
resistance in both wild type and Lcn2-KO
mice, which could not be prevented by
lipocalin-2 deficiency. In fact, our unpublished
observation suggests that CDC treatment
attenuates the expression of a wide range of
inflammatory adipokines, including TNFα,
IL6 and IL1β in adipose tissue of high fat fed
mice.

12-lipoxygenase has been linked to
inflammation and insulin resistance partly
through the production of biologically active
lipid species, such as 12(S)-HETE (30; 47).
Mice deficient with this gene are resistant to
inflammatory effects induced by Western diet.
Treatment with its product 12(S)-HETE
enhances the expression of proinflammatory
cytokine genes and impairs insulin signaling
in 3T3-L1 adipocytes. Stimulators of
12-lipoxygenase gene expression include
saturated fatty acids, such as palmitate (47). In
addition, the expression levels of this enzyme
can be upregulated by iron deficiency (48), in
which the overall effect is a perturbation of
lipid homeostasis. Using inductively coupled
plasma-mass spectrometry analyses, we have
found that lipocalin-2 deficiency is associated
with a higher level of iron contents in adipose
tissues of Lcn2-KO mice than in wide type
animals (Supplementary Figure 5). However,
the iron levels are decreased by high fat fed
feeding in both types of animals to a similar
extent, suggesting that other factors in
addition to iron may be involved in causing
the different expression levels of
12-lipoxygenases between mice with or
without lipocalin-2. Although lipocalin-2
belongs to a family of proteins that can bind to
lipids, its endogenous ligands have not been
identified. Acute lipocalin-2 treatment causes
a rapid but transient reduction of the
circulating FFA levels. It can also enhances
fatty acid uptake into fat tissue, suggesting
that the inducing effect of this adipokine on
12-lipoxygenase may also involve
transportation of lipid species into the
adipocytes.

Excessive ectopic lipid accumulation
plays an important role in inducing peripheral
insulin resistance (49). Note that lipid
accumulation in liver can be markedly
abolished by lipocalin-2 deficiency. Moreover,
the lipid contents in skeletal muscle are lower
in Lcn2-KO mice and can be augmented by
replacement with lipocalin-2, suggesting that
it may promote lipid remobilization from fat
to peripheral tissues. Indeed, irrespective of
obesity conditions induced by the diet or the
genetic mutations, the absence of lipocalin-2
enhances lipid storage in fat tissue and
treatment with this adipokine reduces the
adipose fat content, which may explain the
phenomenon that excess ectopic lipid
accumulation is attenuated in Lcn2-KO mice.
Nevertheless, whether or not lipocalin-2 could
promote peripheral insulin resistance through
its lipid-binding activities need to be further
addressed but is beyond the scope of this study.

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FIGURE LEGENDS:

Figure 1. Lipocalin-2 deficiency ameliorates age-associated deterioration of insulin sensitivity. Age-matched wild type and Lcn2-KO mice were fed with normal chow. Their body weight (A) and food intake (B) were monitored from five- to 24 weeks. Fasting blood glucose (C) and serum insulin concentrations (D) were measured in blood samples collected from tail vein. At the end of the period, Lcn2-KO mice showed significantly improved insulin sensitivity as evaluated by ipGTT (E) and ITT (F). The area under curve (AUC) of ipGTT (G) and ITT (H) were calculated for each set of experiment for demonstrating the progressive development of ageing-associated insulin resistance, which was attenuated by lipocalin-2 deficiency. *, P < 0.05 Lcn2-KO mice vs wild type controls, n=6-8.

Figure 2. Mice without lipocalin-2 are partly protected from high fat diet-induced insulin resistance. Age-matched wild type and Lcn2-KO mice were fed with high fat diet for 18 weeks. Body weight (A) and food intake (B) were monitored on a weekly basis. Fasting blood glucose levels (C) and serum insulin concentrations (D) were evaluated as in Figure 1. At the end of the treatment, mice deficient with lipocalin-2 showed greatly improved insulin sensitivity as demonstrated by ipGTT (E) and ITT (F). The AUC of ipGTT (G) and ITT (H) were calculated for monitoring the development of insulin resistance induced by high fat diet feeding. *, P < 0.05 Lcn2-KO mice vs wild type controls, n=6-8.

Figure 3. Insulin resistance caused by genetic obesity is attenuated in mice lacking lipocalin-2. Body weight (A) and food intake (B) were measured regularly for db/db and DKO mice between six to 12 weeks. Fasting blood glucose levels (C), fasting serum insulin concentrations (D), area under curve of ITT (E) and HOMA-IR indexes (F) were determined for 7-, 9- and 11-week old animals, respectively. *, P < 0.05 DKO vs db/db mice, n=3-6.

Figure 4. Increased adipocyte sizes, reduced inflammation and improved insulin sensitivity of the epididymal fat tissues derived from high fat diet-fed Lcn2-KO mice. A, The percentages of epididymal fat mass to body weights were shown for wild type and Lcn2-KO mice fed with 18 weeks of high fat diet (left panel), and those treated with recombinant adenovirus overexpressing luciferase or lipocalin-2 (right panel). *, P < 0.05 vs wild type controls; #, P < 0.05 vs those treated with recombinant adenovirus overexpressing luciferase, n=5-6. B, The sizes of adipocytes were measured under microscope (magnification, × 200) and calculated using image analysis software (Image J). Ten fields were randomly chosen and sizes of 20 cells in each field were measured. *, P < 0.05 vs wild type controls. C, Immunohistochemical analysis suggested that local inflammation occurred in epididymal fat tissues of high fat diet-fed wild type animals but not those of Lcn2-KO mice. Infiltrated macrophages were visualized by staining with a monoclonal anti-F4-80 antibody (magnification, × 400). D, MDA contents were significantly lower in epididymal fat tissue of Lcn2-KO mice than those of wild type mice, after high fat diet feeding for 18 weeks. * P < 0.05 vs wild type control group, n=5-6. E, Epididymal adipose tissues were collected from normal chow, or high fat diet fed wild type and Lcn2-KO mice, which were acutely injected with insulin as described in Methods. Both basal and insulin-stimulated phosphorylations of IRbeta and Akt were evaluated by Western Blotting analysis (upper panel). 100 µg of proteins were loaded for each sample and same membranes were stripped and blotted for monitoring total IRbeta and total Akt levels. Basal and insulin (100 nM)-stimulated glucose uptake was measured in isolated fat pads derived from wild type and
Figure 5. Ageing- and dietary obesity-associated upregulation of 12-lipoxygenase in adipose tissue is largely blocked by lipocalin-2 deficiency. A, GC-MS analysis revealed that the AA amounts in epididymal fat tissues of aged or obese Lcn2-KO mice were much higher than those of wild type mice. B, Quantitative PCR analysis of 12-lipoxygenase mRNA levels in adipose tissues showed significant difference between wild type and Lcn2-KO group. C, The protein expression of 12-lipoxygenase was much lower in Lcn2-KO mice (21 weeks old fed with standard chow or high fat diet) compared to the aged matched wild type controls. D, The 12(S)-HETE metabolites were reduced in the epididymal adipose tissues of mice without lipocalin-2. The amounts of 12(S)-HETE were not different in muscle or liver tissues when compared to those of wild type littermates (21 weeks old fed with standard chow or high fat diet). *, P < 0.05 vs five-week (A and B) or 21-week (D) old wild type mice fed with standard chow; #, P < 0.05 vs wild type mice of the same treatment group, n=3-6.

Figure 6. Lipocalin-2 deficiency prevents ageing- and obesity-induced TNFα expression, whereas lipocalin-2 treatment stimulates TNFα expression in adipose tissues. A, Quantitative PCR analysis of TNFα mRNA levels in the adipose tissues derived from different ages of mice fed with normal chow (left panel) or high fat diet (right panel). *, P < 0.05 vs Lcn2-KO mice, n=5. B, The protein levels of TNFα were assayed in membrane and soluble fractions of adipose tissues using the commercial ELISA kit. *, P < 0.05 vs wild type mice, n=5. C, Quantitative PCR analysis of TNFα (left panel) and 12-lipoxygenase (right panel) levels in epididymal fat pad collected from Lcn2-KO mice (six-week old) treated with adenovirus overexpressing luciferase (rAd-luciferase) or lipocalin-2 (rAd-Lcn2). The latter group was injected with vehicle or CDC as described in Methods. Vehicle treatment did not cause any changes on TNFα or 12-lipoxygenase expression (data not shown). The fold changes were calculated by comparing with rAd-luciferase treated standard chow-fed wild type mice. *, P < 0.05 vs rAd-luciferase mice fed with standard chow; #, P < 0.05 vs rAd-Lcn2 group, n=5.

Figure 7. Acute lipocalin-2 treatment rapidly induces TNFα and 12-lipoxygenase expression in the adipose tissues of Lcn2-KO mice. Mice fed with high fat diet for six weeks were treated with lipocalin-2 (800 µg/mouse) or vehicle (a bacterial-expressed unrelated protein purified following the same procedure as lipocalin-2) by intraperitoneally injection. A, TNFα and 12-lipoxygenase mRNA levels were evaluated by quantitative PCR and the 12(S)-HETE metabolites measured by EIA. Vehicle treatment had no effects on these parameters (data not shown). *, P < 0.05 vs time zero, n=6. B, The circulating lipid levels (TG, FFA and TC) were measured using the serum collected at different time points before and after injection. C, Lipocalin-2 contents in serum and adipose tissue were quantified using an in-house ELISA. * P < 0.05 vs vehicle, n=6. Not that the vehicle treatment had similar results as those mice injected with PBS (data not shown).

Figure 8. Both CDC treatment and neutralization of TNFα abolish the differences of insulin sensitivity between wild type and Lcn2-KO mice. Mice had been fed with high fat diet for six weeks before starting the treatment with CDC (A and B) or TNFα neutralization antibody (C and D) for another two weeks. ipGTT (A and C) or ITT (B and D) were performed at the end of experiment. AUC was calculated and displayed at the bottom of each panel. *, P < 0.05 vs all
other groups; #, P < 0.05 vs CDC treated mice (A and B) or Lcn2-KO mice (C and D), n=3.

Figure 1

- **Figure 1A**: Body weight (g) over weeks.
- **Figure 1B**: Food intake (g/day) over weeks.
- **Figure 1C**: Fasting blood glucose (mmol/L) over weeks.
- **Figure 1D**: Serum insulin (mU/mL) over weeks.
- **Figure 1E**: Blood glucose (mmol/L) over time after injection.
- **Figure 1F**: Blood glucose (mmol/L) over time after injection.
- **Figure 1G**: AUC ITT over weeks.
- **Figure 1H**: AUC ITT over weeks.
Figure 3

A. Body weight (g) vs. Age (week)

B. Food intake (kcal/mouse/day) vs. Age (week)

C. Resting blood glucose (mmol/L) vs. Age (week)

D. Serum insulin (mU/l) vs. Age (week)

E. AUC ITT vs. Age (week)

F. HOMA-IR vs. Age (week)
Figure 4

A

[Graph showing the effect of high fat diet on wild type and Lcn2-KO mice.]  
- Bars labeled as 'High fat diet' and 'Normal chow' for both wild type and Lcn2-KO mice.

B

[Graph showing the effect of diet on adipocyte size.]  
- Adipocyte size comparison between NC and HF diets for both wild type and Lcn2-KO mice.

C

[Image showing histological sections of adipose tissue.]  
- Sections labeled as 'Wide type' and 'Lcn2-KO' for both NC and HF diets.

D

[Graph showing the effect of insulin on Akt phosphorylation.]  
- Bars labeled as 'Total Akt', 'P-Akt', and 'P-IRbeta' for both basal and insulin conditions.

E

[Table showing protein expression levels for both wide type and Lcn2-KO mice under normal chow and high fat diet conditions.]  
- Proteins include 'Total IRbeta', 'P-IRbeta', 'P-Akt', and 'Total Akt'.

Figure 5

A

[Graph showing the effect of diet on mRNA expression.]  
- mRNA expression levels for '5-lipoxygenase' over time for both normal and high fat diets.

B

[Graph showing the effect of diet on mRNA expression.]  
- mRNA expression levels for '12-lipoxygenase' over time for both normal and high fat diets.

C

[Table showing protein expression levels for both wide type and Lcn2-KO mice under normal chow and high fat diet conditions.]  
- Proteins include 'Actin' and '12-lipoxygenase'.

D

[Graph showing the effect of diet on protein expression.]  
- Protein expression levels for '12-lipoxygenase' and 'Adipose', 'Liver', and 'Muscle' under normal and high fat diet conditions.
Figure 6

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)
Figure 7

A

- TNFα
- 12-lipoxygenase
- 12(S)-HETE

B

- TG
- FFA
- TC

mRNA levels (fold change) vs. Time after Injection (hr)
Figure 8