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Global deletion of lipocalin 2 does not reverse high-fat diet-induced obesity resistance in stearoyl-CoA desaturase-1 skin-specific knockout mice

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ABSTRACT

Over the past century, obesity has developed into a paramount health issue that affects millions of people worldwide. Obese individuals have an increased risk to develop other metabolic disorders, such as insulin resistance and atherosclerosis, among others. Previously we determined that mice lacking stearoyl-CoA desaturase-1 (SCD1) enzyme specifically in the skin (SKO) were lean and protected from high-fat diet induced adiposity. Additionally, lipocalin 2 (Lcn2) mRNA was found to be 27-fold higher in the skin of SKO mice compared to control mice. Given reports suggesting that Lcn2 plays a role in protection against diet-induced weight gain, adiposity and insulin resistance, we hypothesized that deletion of Lcn2 alongside the skin-specific SCD1 deficiency would diminish the obesity resistance observed in SKO mice. To test this, we developed mice lacking SCD1 expression in the skin and also lacking Lcn2 expression globally and surprisingly, these mice did not gain significantly more weight than the SKO mice under high-fat diet conditions. Therefore, we conclude that Lcn2 does not mediate the protection against high-fat diet-induced adiposity observed in SKO mice.

1. Introduction

Stearoyl-CoA desaturase 1 (SCD1) is a Δ9 desaturase that catalyzes the formation of *cis*-monounsaturated fatty acids (MUFA) from saturated fatty acids. Palmitic acid (16:0) and stearic acid (18:0) are the preferred substrates of SCD1 and their desaturation yields palmitoleic acid (16:1n7) and oleic acid (18:1n9), respectively [1]. In mice, SCD1 is expressed at relatively high levels in skin and white adipose tissue [2]. Within the skin, in situ hybridization experiments demonstrated that SCD1 is expressed exclusively in the sebaceous gland [3]. Mice with a naturally occurring mutation or targeted deletion of the SCD1 gene (GKO) exhibit a severe deleterious skin phenotype that includes alopecia, dysfunctional sebaceous glands, atrophic meibomian glands and narrow eye fissures [3] [2]. In addition, GKO mice have increased energy expenditure, resistance to genetic and diet-induced adiposity, as well as protection against the development of hepatic steatosis and insulin resistance [4,5].

Due to the complex and extensive phenotypes of GKO mice and to understand the tissue-specific roles of SCD1, we generated a number of tissue-specific SCD1 knockout mouse models using Cre-loxP technology. We have subsequently demonstrated that while skin-specific knockout mice (SKO) are resistant to high-fat diet (HFD) induced obesity and associated metabolic disorders [6], liver-specific knockout, adipose-specific knockout and liver/adipose double knockout mice are not [7,8]. In addition, SKO mice exhibit all of the hair, skin and eye phenotypes of GKO mice [6]. Taken together, the results from these metabolic studies have emphasized the importance of skin SCD1 in mediating protection against HFD-induced adiposity. In follow-up experiments, we discovered that retinol metabolism is dysregulated in the skin of

Abbreviations: DKO, Lcn2−/−;SCD1 SKO; HFD, high-fat diet; SCD1, stearoyl-CoA desaturase 1; GKO, SCD1 global knockout; MUFA, monounsaturated fatty acids; RAR, retinoic acid receptor; SKO, SCD1 skin knockout; TG, triglycerides; WAT, white adipose tissue.

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SKO mice, as retinol, retinoic acid and retinyl esters were all significantly increased, and microarray analysis of skin mRNA revealed that the altered retinol metabolism in SKO mice is associated with upregulated expression of retinoic acid receptor (RAR) target genes [9]. Lipocalin 2 (*Lcn2*) was one of the most highly induced genes in skin of SKO mice identified by microarray analysis and has been shown by others to be upregulated in human sebocytes upon retinoic acid treatment [10]. Prolonged treatment (72 h) of human SEB-1 cells with retinoic acid and A939572, an SCD1 inhibitor, resulted in 9-fold upregulation in Lcn2 mRNA and protein and similar upregulation in other retinoic acid-induced target genes; these changes in expression and protein synthesis implicate SCD1 as an important mediator of retinol metabolism, at least *in vitro* [9]. In SKO mice, retinoic acid-regulated genes were significantly upregulated in the skin within 23 days of birth despite the fact that skin SCD1 expression typically remains low in the skin until around 8 days after birth. These early changes demonstrated that irregularity in skin retinoic acid metabolism precede adulthood and suggest the possibility that poorly controlled retinoic acid levels may underlie the systemic metabolic changes observed in SKO mice [9].

Lcn2 is a 25 kDa secreted protein [11] and a member of the lipocalin family of proteins that bind and transport a number of small molecules [12]. Lcn2 has been shown to have low binding affinity for retinoic acid [13] while it is unresolved whether Lcn2 binds retinol [13,14]. Lcn2 also binds iron-containing siderophores and possesses anti-microbial properties [12]. In mouse models, Lcn2 has been proposed to play a prominent role in obesity-associated inflammation as well as related metabolic phenotypes including insulin sensitivity and level of adiposity, although the directionality of the involvement of Lcn2 in these events remains unclear. Results from Lcn2 knockout mouse models have been conflicting, where Lcn2 has been demonstrated to promote [15], protect against [16] or have minimal effect on insulin resistance [17]. In addition to effects on insulin sensitivity, Guo et al. also demonstrated that Lcn2 knockout mice have significantly greater body weight and adipose tissue weight as compared to wild type mice [16].

With the dramatic 27-fold upregulation in skin Lcn2 in SKO mice [9] and the suggested role of Lcn2 in protecting against diet-induced weight gain, adiposity and insulin resistance, we sought to determine if Lcn2 might mediate the metabolic state of SKO mice. To address our objective, we crossed Lcn2 knockout mice with SKO mice and fed a high-fat diet (HFD) for up to 15 weeks. The results from this study reveal that Lcn2 does not mediate the protection against HFD-induced weight gain, adiposity, hepatic steatosis and glucose tolerance in SKO mice.

2. Materials and methods

2.1. Animals and diets

SCD1$^{flox/flox}$ (Lox) mice were generated as described previously [7] and were used as control mice for all experiments. Skin-specific SCD1 knockout mice (SKO) were generated by crossing Lox mice with transgenic mice expressing Cre recombinase under the control of the human keratin 14 promoter, as previously described [6]. Lcn2−/− mice were previously described [16]. To generate mice with global deficiency of Lcn2−/− and skin-specific deficiency of SCD1 (double knockout mice, DKO), Lcn2−/− mice were crossed with Lox mice to generate Lcn2$^{+/-}$;SCD1$^{flox/+}$. Male and female Lcn2$^{+/-}$;SCD1$^{flox/+}$ mice were then crossed to generate Lcn2$^{-/-}$;SCD1$^{flox/flox}$ mice. Subsequently, male SKO mice were crossed with female Lcn2$^{-/-}$;SCD1$^{flox/flox}$ mice to generate Lcn2$^{+/--}$;SCD1$^{flox/flox}$;Ker-Cre/+ mice. DKO mice were then generated by crossing male Lcn2$^{+/-}$;SCD1$^{flox/flox}$;Ker-Cre/+ mice with female Lcn2$^{-/-}$;SCD1$^{flox/flox}$ mice.

All mice were maintained on a 12 h light–dark cycle (6PM–6AM) and had free access to food and water unless specified otherwise. Breeders were fed Purina 5015 diet. Offspring were weaned at 21 days and fed a chow diet (Purina #5008). Mice were individually caged at 7 weeks of age and fed a lard-based, high-fat diet (HFD; 60% kcal from fat; Research Diets #D12492) starting at 8 weeks of age. Male mice were fed the HFD for 10–15 weeks while females were fed the diet for 15 weeks. All mice were nonfasted at euthanization by isoflurane overdose. Blood was collected via cardiac puncture, tissues were weighed and immediately snap-frozen in liquid nitrogen and stored at −80 °C. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison.

2.2. Western blot analysis

Whole tissue lysate from liver and microsomal protein from skin was used for immunoblot analysis of Lcn2, SCD1 and GAPDH or CREB as loading controls. Whole tissue was homogenized in RIPA buffer and protein was quantified by BCA protein assay (Pierce). Microsomal protein was prepared as previously described [8]. For immunoblots, 50 μg of protein per sample was separated by 12% SDS–PAGE and transferred to PVDF membrane (Millipore). Membranes were incubated overnight with anti-SCD1 (Santa Cruz Biotechnology, #sc-14720), Lcn2 (R & D Systems), GAPDH or CREB (Santa Cruz Biotechnology, #sc-58) primary antibodies, followed by IgG-horseradish peroxidase-conjugated secondary antibody. Proteins were visualized with ECL Prime Detection Reagent (Amersham).

2.3. Quantitative real-time PCR

Liver total RNA was extracted using TRI reagent. Subsequently, total RNA was treated with Turbo DNase (Ambion) and then reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Relative mRNA expression levels were quantified by cDNA amplification with gene-specific forward and reverse primers and Power SYBR Green PCR Master Mix on an ABI 7500 Fast RT PCR system. Data were normalized to Arbp using the ΔΔCt method. Primer sequences available upon request.

2.4. Hepatic triglyceride analysis

Total lipids were extracted from 10 mg liver tissue. Liver TG were measured using a colorimetric enzymatic assay (Wako Chemicals, USA).

2.5. Glucose tolerance tests

Glucose tolerance tests were performed with male mice after 9 weeks of HFD consumption. Lox, SKO and DKO mice were dosed with a 20% glucose solution at 2 g/kg body weight via intraperitoneal injection. Blood was collected from the tail vein and glucose was measured at 0, 30, 60, 90 and 120 min post-injection using a blood glucose meter and glucose test strips (One Touch Ultra, Diabetic Express).

2.6. Statistical analyses

All results are expressed as mean ± SEM. Variables that did not follow a Gaussian distribution were log transformed for statistical

analyses. Weight gain trends and glucose tolerance test results were analyzed using repeated measures ANOVA. To account for correlated errors, we used AR(1) error structure. Repeated measures analyses were performed using SAS 9.3 software (SAS Institute Inc). All other data were analyzed using one-way ANOVA with Tukey's post hoc test and were performed using GraphPad Prism 6.0c (GraphPad Software). Results with P-value < 0.05 were considered statistically significant.

3. Results

3.1. Generation of Lcn2−/−;SCD1 SKO mice

To address the question of whether Lcn2 mediates the effects of SCD1 skin-specific knockout (SKO) mice, we crossed mice with a global deficiency of Lcn2 with SKO mice to generate Lcn2−/−;SCD1 SKO, which we refer to as double knockout (DKO) mice hereafter. DKO mice were visibly indistinguishable from SKO mice at birth and throughout the duration of the studies. As previously reported, SKO mice exhibit dry skin, alopecia and close eye fissure phenotypes [6] and all of these phenotypes were present in DKO mice (images not shown). Immunoblot analysis was performed to assess skin and hepatic tissue levels of SCD1 and Lcn2 protein. Microsomes were prepared from skin tissue to enrich for SCD1 protein and immunoblot analysis revealed that SCD1 was expressed in skin of Lox mice but was dramatically reduced in skin of SKO and DKO mice (Fig. 1A). The residual SCD1 protein expression may be due to subcutaneous adipose tissue. Analysis of skin tissue lysate revealed that Lcn2 protein expressed at a low level in Lox but highly induced in SKO mice (Fig. 1A). As expected, Lcn2 protein was not detected in skin tissue of DKO mice (Fig. 1A). Lox, SKO and DKO mice all expressed SCD1 in the liver (Fig. 1B). Lcn2 is absent in liver tissue of DKO mice but dramatically upregulated in SKO mice (Fig. 1B). The upregulation of Lcn2 in SKO mice was particularly evident when compared to Lox controls, in which hepatic Lcn2 was not detected (Fig. 1B).

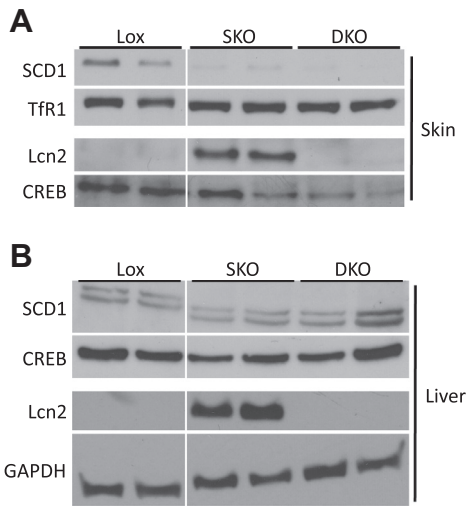

Fig. 1. Immunoblot analysis of SCD1 and Lcn2 protein in liver and skin tissue of high-fat diet fed Lox, SKO and DKO mice. Whole tissue lysates were used for liver SCD1, liver Lcn2 and skin Lcn2 blots. Microsomes were used for skin SCD1 blot. (A) SCD1 is present in skin tissue of Lox mice but absent in SKO and DKO mice. Lcn2 is highly induced in skin of SKO mice, expressed at a low level in Lox mice and absent in DKO mice. (B) SCD1 protein is detected in liver of Lox, SKO and DKO mice. Lcn2 is highly induced in liver of SKO mice compared to Lox mice and is absent in DKO liver. CREB, GAPDH and TfR1 were used as loading controls. SKO, SCD1 skin-specific knockout; DKO, Lcn2−/−;SCD1 SKO.

3.2. Lcn2 deficiency does not restore protection against high-fat diet induced obesity in SKO mice

To determine if the upregulation of Lcn2 in SKO mice mediates the protection against high-fat diet induced obesity, we fed 8 week old male and female Lox, SKO and DKO mice a lard-based, HFD (60% kcal from fat) for 10–15 weeks. Body weights and food intake were measured weekly. In agreement with our previous study [6], male and female SKO mice were dramatically protected against HFD-induced weight gain, while Lox mice gained weight steadily throughout the study (Fig. 2A and B). Similar to SKO mice, male and female DKO mice were protected against weight gain and were not different from the SKO groups at any time point (Fig. 2A and B). In male mice, the divergence in body weight of SKO and DKO mice from the Lox controls became significant at week 5. In females, the SKO and DKO mice were significantly lighter than Lox mice at week 6. SKO and DKO mice were hyperphagic when fed the HFD (Fig. 2C and D). The mean weekly food intake was increased by 50% in SKO and DKO mice compared to Lox mice in both males and females.

3.3. Lcn2 deficiency does not increase adiposity in high-fat diet-fed SKO mice

Total liver weight was not significantly different among the genotypes in male mice (Fig. 3A). However, the mean liver weight of female SKO and DKO mice was significantly greater than that of the female Lox (Fig. 3B). Consistent with significantly reduced body weight, adiposity of HFD-fed SKO and DKO mice was significantly reduced as compared to Lox mice. Gonadal (Fig. 3B and E) and subcutaneous (Fig. 3C and F) white adipose depots were significantly reduced in male and female SKO and DKO mice. Taken together with the body weight data, these results demonstrate that Lcn2 does not mediate the protection from HFD-induced weight gain and associated adiposity in SKO mice.

3.4. Liver triglyceride accumulation and glucose tolerance are not influenced by Lcn2 deficiency in HFD-fed SKO mice

We previously demonstrated that SKO mice are also protected from hepatic steatosis that accompanies long-term consumption of HFD [6]. We used a biochemical assay to measure hepatic TG levels in mice fed the HFD for 15 weeks. While Lox mice accumulated hepatic TG to a high level, male and female SKO and DKO mice remained protected from hepatic lipid accumulation (Fig. 4A and B). SKO mice are insulin sensitive under both chow and high-fat dietary conditions [6]. To assess the potential role of Lcn2 in this phenotype, we performed glucose tolerance tests in male mice after feeding the HFD for 9 weeks. 4 h fasted blood glucose levels were not different among Lox, SKO and DKO mice (Fig. 4C). However, following intraperitoneal glucose injections, SKO and DKO cleared the glucose bolus more rapidly than Lox and had significantly lower blood glucose levels at 60, 90 and 120 min post-injection as compared to the Lox mice (Fig. 4C).

4. Discussion

Previous findings in SCD1 SKO mice along with results from studies in Lcn2 knockout mice suggested a potential role for Lcn2 in mediating the resistance of HFD-induced obesity and related complications in SKO mice [6,16]. However, in the current study we demonstrate that Lcn2 does not account for the protection against HFD-induced adiposity and hepatic steatosis in SKO mice, as global deletion of Lcn2 in SKO mice did not reverse these phenotypes. This work suggests that alternative cellular mechanism(s)

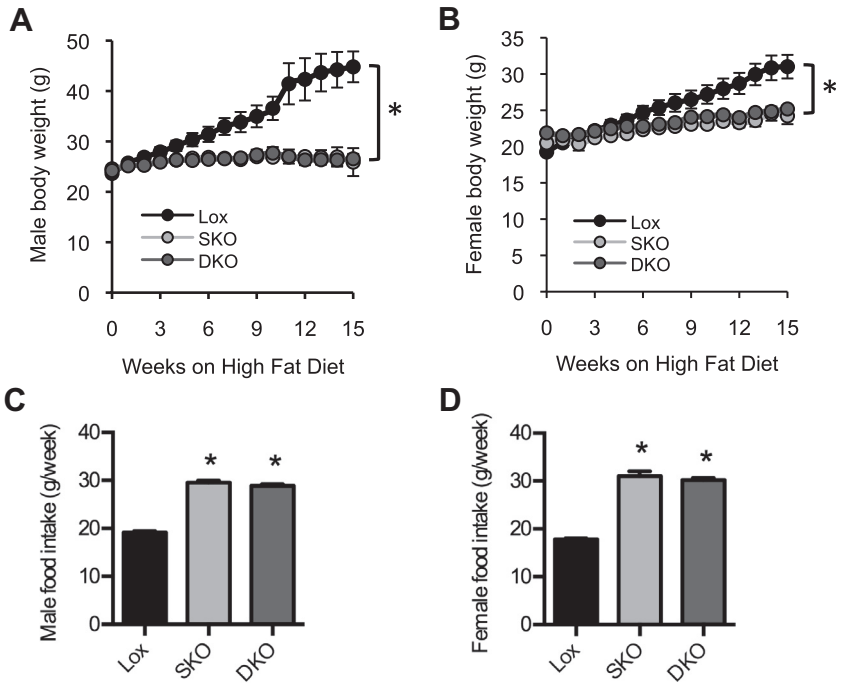

Fig. 2. Global Lcn2 deficiency in SCD1 SKO mice does not reverse the resistance to high-fat diet-induced obesity. At 8 weeks of age, (A) male and (B) female mice were fed a high-fat diet for 10–15 weeks. Male and female SKO and DKO gained significantly less weight than their Lox counterparts. The mean weekly food intake for (C) males and (D) females was also determined. SKO, SCD1 skin-specific knockout; DKO, Lcn2−/−;SCD1 SKO. Values are mean ± SEM. *$P < 0.05$ vs. Lox; repeated measures ANOVA.

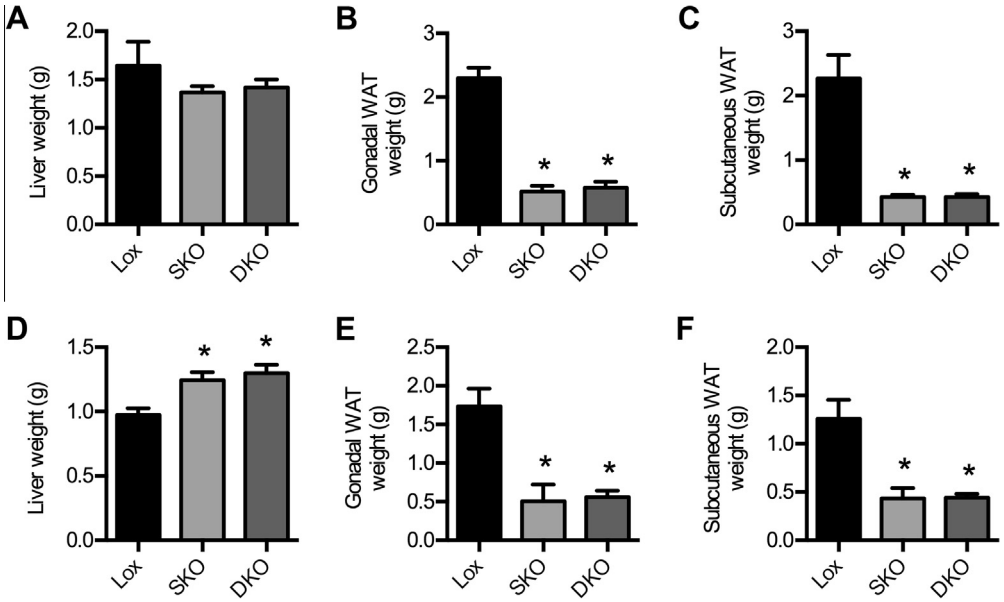

Fig. 3. Global Lcn2 deficiency does not restore white adipose tissue mass in SCD1 SKO mice fed a high-fat diet for 15 weeks. (A) Liver, (B) gonadal white adipose tissue (WAT) and (C) subcutaneous WAT weights in male mice ($n = 5–6$/group). (D) Liver, (E) gonadal WAT and (F) subcutaneous WAT weights in female mice ($n = 6–11$/group). SKO, SCD1 skin-specific knockout; DKO, Lcn2−/−;SCD1 SKO. Values are mean ± SEM. *$P < 0.05$ vs. Lox; one-way ANOVA with Tukey's post hoc test.

underlie these HFD-induced phenotypes, including HFD-induced obesity, hepatic steatosis and glucose tolerance in SKO mice.

One potential reason for the observed Lcn2 upregulation in SKO liver and skin tissue is the skin inflammation that develops in the absence of SCD1 expression while minimally impacting the SKO metabolic phenotype. However, the possibility that Lcn2 mediates aspects of the SKO metabolic phenotype cannot be wholly discounted through this research alone. *In vitro* studies with adipocytes and macrophages as well as *in vivo* studies in Lcn2 KO mice suggest Lcn2 may suppress the production of certain chemokines and function as an anti-inflammatory [16,18]. Additionally, a recent study has provided more direct evidence demonstrating that Lcn2 plays a role in activating the polarization of anti-inflammatory M2 macrophages [19]; though another study showed that Lcn2 promotes pro-inflammatory M1 macrophages [20]. Despite the debated directionality of the role of Lcn2 in inflammatory state, elevated Lcn2 has remained an accepted marker for the pro-inflammatory response associated with obesity [21].

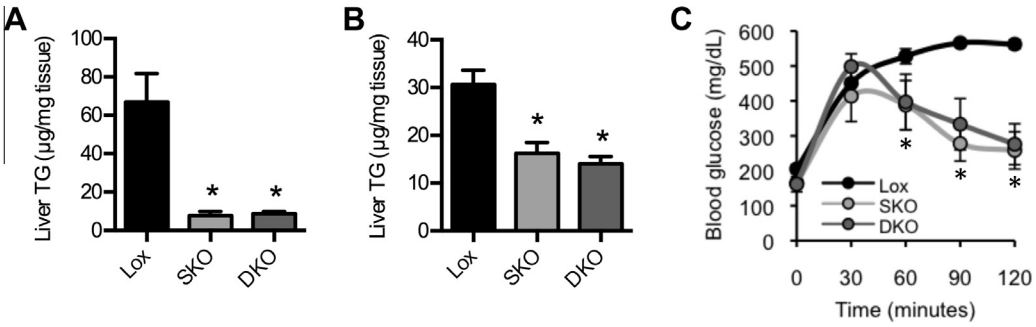

Fig. 4. Global Lcn2 deficiency does not rescue liver TG or glucose clearance in high-fat diet fed SCD1 SKO mice. Male and female mice were fed a high-fat diet for 15 weeks beginning at 8 weeks of age. Mice were nonfasted at euthanization. Liver lipids were extracted and TG mass was determined in (A) males (n = 4–6/group) and (B) females (n = 6–8/group) using a biochemical assay. (C) Glucose tolerance tests were performed in male mice after 9 weeks of high-fat diet (n = 3–4/group). Mice were fasted for 4 h and then intraperitoneally injected with 2 g glucose/kg body weight. Blood glucose was determined at 0, 30, 60, 90 and 120 min post-injection using a glucometer. SKO, SCD1 skin-specific knockout; DKO, Lcn2−/−;SCD1 SKO. Values are mean ± SEM. A and B: $^{*}P < 0.05$ vs. Lox; one-way ANOVA with Tukey's post hoc test. C: $^{*}P < 0.05$ vs. Lox, significant with SKO and DKO comparisons at 60, 90, and 120 min; repeated measures ANOVA.

Modulation of the inflammatory response might play a role in the SKO metabolic phenotype and it has been previously speculated that a specific pro-inflammatory stimulus in the skin may be the primary mediator of SKO hypermetabolism [9]. The overlapping phenotypes of SKO and DKO mice in the current study suggests that if a pro-inflammatory stimulus is responsible for SKO hypermetabolism, it is unlikely to be an inflammatory response that is directly or indirectly modulated by Lcn2. Early work on the asebia mouse model, which has a naturally occurring mutation in *Scd1*, demonstrated reduced inflammation and restoration of hair growth when treated with cyclosporine A [22]. A similar study on SKO mice could facilitate similar restorative effects and potentially demonstrate a role for the inflammatory response in the metabolic phenotype of SKO mice.

Though Lcn2 deficiency has been shown to modulate the expression and activity of PPAR-γ in adipocytes *in vitro*, the global role of Lcn2 in modulation of insulin signaling is not fully understood [23]. Past work by others has implicated Lcn2 deficiency in the development of insulin resistance while other work has demonstrated a role for Lcn2 in promoting the development of insulin resistance [8,24]. The effect of Lcn2 on insulin sensitivity may be in part mediated through regulation of TNF-α, although whether Lcn2 promotes or represses TNF-α expression remains unresolved [15,16].

Lcn2 possesses multiple biological functions, ranging from siderophore sequestration to PPAR-γ modulation, which complicates the understanding of its potential role in mediating the phenotypes exhibited in SKO mice. Work in humans has demonstrated significantly elevated serum Lcn2 levels in psoriasis patients with no correlation to body mass index, suggesting that Lcn2 may function as an inflammatory marker outside of its known role in the pro-inflammatory state of obesity [24]. Previous microarray analysis of skin tissue indicated that SKO mice demonstrated significant upregulation of psoriasis-related genes outside of Lcn2, including a 26-fold upregulation in Tenascin C and an 11-fold upregulation in thrombospondin 1 [9]. Together, these data suggest that the gene expression response of skin-specific SCD1 deletion mirrors the psoriasis response; possibly a result of elevated retinoic acid in the SKO skin and subsequent upregulation of retinoic acid receptor signaling [9].

Surprisingly, feeding a lard-based HFD, of which the fat composition is approximately 30% 18:1n-9, to GKO or SKO mice does not compensate for their reduced synthesis of 18:1n-9 by SCD1 [6,7]. This underscores the difference between exogenously supplied and endogenously synthesized oleate, in which exogenous oleate cannot overcome the hypermetabolism of GKO and SKO mice. We previously reported that dietary oleate supplementation at a level of 5% (w/w) to a semipurified diet does not restore TG or wax ester synthesis in eyelids of GKO mice [2]. Additionally, past work also revealed that in GKO mice, triolein supplementation of a high-carbohydrate diet at a level of 20% (w/w) does not completely rescue the reduction in lipogenic gene expression and hepatic liver TG accumulation [25]. Together, these results suggest that endogenous 18:1n-9 exerts specific biological effects that dietary 18:1n-9, at least at the levels previously tested, cannot reproduce.

We previously demonstrated that the skin phenotype of SKO mice is consistent with characteristics of atopic dermatitis. Furthermore, the gene expression profile of SKO skin tissue revealed increased expression of interleukin cytokines and inflammatory mediators, including tumor necrosis factor and transforming growth factor beta, in the skin [9]. Many members of the interleukin family, including IL-18 and IL-33 were found to be significantly elevated in the skin of SKO mice as well [9]. Additionally, Lcn2 deficient mice have decreased expression of the anti-inflammatory markers IL-10 and arginase 1 in liver and adipose tissue; suggesting that Lcn2 may play a role in mitigating the pro-inflammatory response, at least in these tissues [16]. It is likely that elevated levels of Lcn2 in the skin of SKO mice are mediated through the skin's inflammatory response, possibly in a cellular effort to mitigate bacterial infection risk in the damaged skin of the SKO mice. Overall, the results from the current study reveal that the dramatic upregulation of Lcn2 in SKO mice does not mediate the protection against HFD-induced adiposity, hepatic steatosis and glucose tolerance, as these phenotypes were not altered by the global deletion of Lcn2 in SKO mice.

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