High-saturated-fat diet-induced obesity causes hepatic interleukin-6 resistance via endoplasmic reticulum stress

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Abstract The relationship between liver interleukin-6 (IL-6) resistance following high-fat diet (HFD)-induced obesity and glucose intolerance is unclear. The purpose of this study was to assess the temporal development of hepatic IL-6 resistance and the role of endoplasmic reticulum (ER) stress in this process. We hypothesized that HFD would rapidly induce hepatic IL-6 resistance through a mechanism involving ER stress. Male C57BL/6N mice consumed chow or a HFD (60%) derived from lard (saturated) or olive oil (monounsaturated) for 4 days or 7 weeks before being injected intraperitoneally with IL-6 (6 ng/kg). Glucose, insulin, and pyruvate tolerance tests were used as proxies for systemic glucose metabolism and hepatic glucose production, respectively. Primary mouse hepatocytes were incubated with palmitate (saturated) and oleate (unsaturated) overnight, then treated with 20 nM IL-6. ER stress was induced via tunicamycin or prevented by sodium phenylbutyrate (PBA). Seven weeks of a saturated, but not monounsaturated, HFD reduced hepatic IL-6 signaling in conjunction with hepatic ER stress. Palmitate directly impaired IL-6 signaling in hepatocytes along with inducing ER stress. Pharmacologically induced ER stress caused hepatic IL-6 resistance, whereas PBA reversed HFD-induced IL-6 resistance. Chronic HFD-induced obesity is associated with hepatic IL-6 resistance due to saturated FA-induced ER stress.—Townsend, L. K., K. D. Medak, W. T. Peppler, G. M. Meers, R. S. Rector, P. J. LeBlanc, and D. C. Wright. High-saturated-fat diet-induced obesity causes hepatic interleukin-6 resistance via endoplasmic reticulum stress. J. Lipid Res. 2019. 60: 1236–1249.

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Historically, interleukin-6 (IL-6) was considered a proinflammatory cytokine contributing to the development of obesity-associated insulin resistance (1–3). However, accumulating evidence now suggests that IL-6 may contribute to the maintenance of normal systemic glucose metabolism (4–8) and that the increased circulating IL-6 concentrations during obesity may actually be an adaptive mechanism to resist obesity-associated glucose intolerance (9).

One of the first reports to challenge the dogma of IL-6 as a detrimental cytokine showed that IL-6-deficient mice develop late-onset obesity and systemic glucose intolerance (10). Subsequently, it was shown that a single high-dose injection of IL-6 (400 ng) improved glucose tolerance in lean, high-fat diet (HFD)-induced obese mice, streptozotocin-induced diabetic mice, ob/ob mice, and db/db mice (7). Moreover, chronic antagonism of IL-6 worsens glucose metabolism in db/db mice (7). Together, these data strongly support a more favorable role for IL-6 in the maintenance of systemic glucose homeostasis.

IL-6 may achieve some of its metabolic benefits via classical signaling in the liver, the tissue with one of the greatest concentrations of IL-6 receptors in the body, at least in mice and rats (8). This might explain why IL-6-deficient mice develop hepatic steatosis, inflammation, and insulin resistance, even when on a chow diet (11). Moreover, when the IL-6 receptor was genetically ablated specifically in the liver, mice developed hepatic inflammation, systemic glucose intolerance, and metabolic complications similar to those of genetic obesity (12). Thus, liver IL-6 resistance appears to be a key determinant of systemic glucose homeostasis.

Abbreviations: 4HNE, 4-hydroxynonenal; AKT, protein kinase B; Bip, binding immunoglobulin protein; Chop, CCAAT-enhancer-binding protein homologous protein; DAG, diacylglycerol; eIF2α, eukaryotic initiation factor-2α; ER, endoplasmic reticulum; G6pc, glucose 6-phosphatase; GGT, glucose tolerance test; HFD, high-fat diet; IL-6, interleukin-6; IP, intraperitoneal; PBA, sodium phenylbutyrate; Pck1, phosphoenolpyruvate carboxykinase 1; Ppib, peptidyl-prolyl cis-trans isomerase B; SHP2, pSH2-domain-containing tyrosine phosphatase; SOCS3, suppressor of cytokine signaling-3; STAT3, signal transducer and activator of transcription-3; TAG, triacylglycerol; WAT, white adipose tissue; Xbp1s, spliced x-box binding protein-1.
and insulin resistance, despite similar body weight and fat mass as wild-type mice (12). Hepatic IL-6 signaling likely regulates systemic glucose metabolism via effects on hepatic glucoregulatory enzymes, specifically G6Pase, and subsequent reductions in liver glucose output (4, 5, 13).

The endoplasmic reticulum (ER) is responsible for cellular protein synthesis and folding, among other roles (14). Certain conditions, including lipid accumulation and exposure to high concentrations of saturated fatty acids (15), cause an imbalance between protein-folding requirements and ER capacity, thereby disrupting ER homeostasis and activating the ER stress response [i.e., the unfolded protein response (14, 16, 17)]. ER stress is linked to the development of obesity-associated hepatic insulin resistance (14) and elevated hepatic glucose output in obesity (16), although the precise mechanisms remain unclear. ER stress has been shown to interfere with signal transducer and activator of transcription-3 (STAT3) phosphorylation, reducing its ability to suppress hepatic glucose output (4, 18). ER stress is also linked to leptin resistance (19), which signals via a similar STAT3-dependent pathway as IL-6. Interestingly, chronic HFD-induced obesity is associated with reduced hepatic STAT3 phosphorylation and suppressor of cytokine signaling-3 (Socs3) mRNA expression in response to exogenous IL-6 (20). To date, it is not clear whether the development of hepatic IL-6 resistance is a causal event in the development of impaired glucose homeostasis during diet-induced obesity, nor has the role of ER stress in mediating diet-induced liver IL-6 resistance been elucidated. Thus, the goal of the current project was to explore the temporal development of liver IL-6 resistance in response to HFD, its association with systemic glucose intolerance and markers of hepatic glucose output, and the potential mechanisms involved in this effect. We speculated that HFD-induced obesity would lead to hepatic ER stress, which would interfere with IL-6 signaling in association with markers of elevated hepatic glucose output.

MATERIALS AND METHODS

Male C57BL/6N mice (~7 weeks of age; Charles River) were individually housed on a 12/12 h light/dark cycle with free access to water and chow diet (Tekland catalog no. 7004; 3.5 kcal/g, 20%, 29%, and 51% kcal from protein, fat, and carbohydrate, respectively; supplemental Table S1) for 1 week prior to experimentation; all animals consumed chow unless otherwise stated. All animal protocols were approved by the University of Guelph Animal Care Committee and met the guidelines of the Canadian Council on Animal Care. Hepatocytes were isolated from mice in accordance with the University of Missouri Institutional Animal Care and Use Committee and the Truman VA Subcommittee on Animal Studies.

Four day HFD

Following 1 week acclimation, mice were switched to a HFD (60% kcal from lard, predominantly saturated fat; Research Diets catalog no. D12492) for 3 days (21, 22). On the third day, systemic glucose tolerance was assessed by an intraperitoneal (IP) glucose tolerance test (GTT) (23). Mice were fasted for 6 h and injected IP with glucose (2 g·kg⁻¹ body mass), and blood glucose was obtained from a tail vein using an automated handheld glucometer (Freestyle Lite, Abbott Laboratories) at baseline and 15, 30, 45, 60, 90, and 120 min postinjection. Food was returned after the final glucose measurement.

The morning following the GTT, fed mice were injected IP with recombinant IL-6 (6 ng·kg⁻¹ body mass; PeproTech, catalog no. 216-16) or an equal volume of sterile saline. The IL-6 dose was based on dose-response experiments where 3, 6, and 12 ng·kg⁻¹ body mass were injected IP into lean mice, and tissues were collected after 15 min (supplemental Fig. S1); these data showed maximal STAT3 phosphorylation at 6 ng·kg⁻¹ and that the liver is far more responsive, regardless of dose, compared with skeletal muscle. Importantly, 6 ng·kg⁻¹ seemed to elicit maximal STAT3 phosphorylation, while still remaining within a physiological circulating range (24). At 15 min after IL-6 injection, mice were anesthetized with a weight-adjusted bolus of sodium pentobarbital (~5 mg per 100 g of body mass). Livers were freeze-clamped in situ, and all tissues were immediately frozen in liquid nitrogen and stored at ~80°C.

In a separate cohort of mice, to assess whether short-term HFD affects in vivo hepatic glucose output, we performed an IP pyruvate tolerance test. Following 3 days of HFD, mice were fasted overnight (~2100–0900) and injected IP with pyruvate (2 g·kg⁻¹). Blood glucose concentrations were taken from a tail vein via handheld glucometer before and at 15, 30, 45, 60, 90, and 120 min after pyruvate. Finally, we performed an abbreviated insulin tolerance test, which allows for assessment of in vivo insulin action and measurement of tissue insulin signaling in the same animal (25, 26). Following 4 days of a HFD, fed mice were injected IP with insulin (1 U/kg), and blood glucose was measured immediately preinjection (i.e., 0) and then at 7 and 15 min postinjection, at which point tissues were collected for assessment of insulin signaling (25).

Seven week HFD

To determine whether more severe HFD-induced obesity and glucose intolerance were associated with hepatic IL-6 resistance, we placed mice on the same lard-based HFD for 7 weeks [Research Diets catalog no. D12492 (27)]. After 7 weeks, we performed an IP GTT, and the following day, fed mice were injected IP with recombinant IL-6 (6 ng·kg⁻¹ body mass) or an equal volume of sterile saline. Additionally, knowing that types of fat (e.g., saturated vs. unsaturated) have distinct metabolic outcomes in the liver (28), we tested whether there are differing effects on hepatic IL-6 signaling by feeding a separate cohort of mice a HFD consisting of primarily fat derived from olive oil (60% kcal/fat, predominantly MUFA s; Research Diets catalog no. D01112603) but otherwise matched to the previous HFD (detailed dietary breakdown is in supplemental Table S1).

Prolonged fast

Fasting mobilizes predominantly unsaturated fatty acids from adipose tissue, which acutely accumulate in the liver (29). Thus, to test whether hepatic fat accumulation could induce hepatic IL-6 resistance without accompanying obesity, we fasted lean mice for 30 h. Following the prolonged fast, mice were injected IP with recombinant IL-6 (6 ng·kg⁻¹ body mass) or an equal volume of sterile saline, and tissues were taken 15 min later.

Pyruvate tolerance test and hepatic gluconeogenic markers

In order to assess the effects of IL-6 on indices of in vivo hepatic glucose output, we performed IP pyruvate tolerance tests (23). Mice were fasted overnight (~2100–0900), IP injected with recombinant IL-6 (6 ng·kg⁻¹ body mass), and then 4 h later given an IP injection of pyruvate (2 g·kg⁻¹). Blood glucose concentrations were taken from a tail vein via handheld glucometer before and ~15, 30, 45, 60, 90, and 120 min postpyruvate. To assess the
cellular responses to IL-6 at this time point, we fasted a separate cohort of mice overnight, injected IP IL-6 (6 ng·kg⁻¹ body mass), and 4 h later collected liver (5). Finally, in another cohort of mice, we assessed insulin action following 7 weeks of both HFDs via brief IP insulin tolerance test. Following an overnight fast, mice were injected IP with either saline or IL-6 (6 ng·kg⁻¹ body mass). At 4 h after IL-6, mice were IP injected with insulin (1 U/kg), and blood glucose was measured by tail vein at baseline and 7 and 15 min postinsulin injection, at which point tissues were collected for assessment of insulin signaling.

Pharmacological induction and prevention of hepatic ER stress
Tunicamycin (Cayman Chemical, catalog no. 11089-65-9) is a commonly used drug that acutely causes ER stress by inhibiting the glycosylation of proteins, causing their accumulation in the ER lumen. Tunicamycin was dissolved in DMSO, diluted in sterile saline (0.9%), and injected IP at a dose of 1 μg·kg⁻¹ body weight (30, 31). At 48 h later (30, 31), mice were injected with IL-6, and livers were harvested after 15 min. We also used the chemical chaperone sodium phenylbutyrate (PBA; Cayman Chemical, catalog no. 1716-12-7) to reduce HFD-induced ER stress. To this end, mice were fed a traditional high-fat-based HFD (60% kcal from fat) beginning at 8 weeks of age for 5 weeks, and then half began daily treatment with PBA for 2 weeks while remaining on the HFD (16, 18). Mice received 2 gavages/day of PBA (500 mg·kg⁻¹ total of 1 g·kg⁻¹/day, at ~0800 and ~2000) for 1 week, then 1 gavage/day (total of 500 mg·kg⁻¹, ~0800) for the final week. Following 2 weeks of PBA (7 weeks of HFD), mice were either injected with IL-6 or saline, and tissues were collected 15 min later.

Isolated hepatocytes
We tested the effects of various FA combinations on in vitro IL-6 signaling by isolating primary murine hepatocytes. Hepatocytes were isolated from lean chow-fed mice using a two-step collagenase perfusion technique, described in detail previously (32). After isolation, approximately 2.5 × 10⁵ cells were plated on collagen-coated plates and incubated overnight in growth medium (Williams E medium, supplemented with 10% FBS, penicillin/streptomycin, 4 mM l-glutamine, 2 mg/ml epidermal growth factor, insulin-transferrin-selenium (ITS), 100 nM dexamethasone, 0.1% BSA, and 10 mM sodium pyruvate). The next day, medium was replaced with starvation medium (Williams E medium, supplemented with 0.1% 173 FBS, penicillin/streptomycin, 4 mM l-glutamine, and 0.1% BSA) (33). Starvation medium either contained control medium (matched for BSA and ethanol, which was used to dissolve palmitate), 250 μM palmitate + 250 μM oleate (32), 400 μM palmitate + 100 μM oleate, or 400 μM oleate + 100 μM palmitate, and cells were incubated for 24 h; oleate was included with the high dose of palmitate to prevent apoptosis (28, 34). It is important to emphasize that the total fatty acid concentration (500 μM) was identical for all conditions. Following 24 h FA incubation, medium was replaced by starvation medium with or without 20 ng/ml IL-6 for 90 min, as we have previously done (33) and similar to previous reports (13, 35).

Serum IL-6, NEFA, liver triacylglycerol, glycogen, liver histology, and lipidomics
Circulating IL-6 concentrations were determined using a commercially available ELISA kit (catalog no. RAB0308, Millipore-Sigma). Commercially available kits were also used to measure serum NEFAs (Wako Chemicals) and β-hydroxybutyrate (catalog no. MAK041, Millipore-Sigma). To quantify the hepatic content of triacylglycerol (TAG), snap-frozen liver (~30 mg) was homogenized in 1 ml of 1:2 methanol:chloroform and gently agitated overnight at 4°C (36, 37). One milliliter of 4 mM MgCl₂ was added to this mixture the following day, vortexed, and centrifuged for 1 h at 1,000 g at 4°C. The organic infranatant phase was extracted using a syringe to minimize contamination, evaporated overnight, and reconstituted in a 3:2 butanol-Triton X-114 mix. TAG content was measured with a commercially available kit (Sigma-Aldrich, catalog no. F6428). For the quantification of glycogen content, liver was freeze-dried and powdered, and glycogen was extracted with 0.5 M perchloric acid containing 1 mM EDTA, neutralized with 2.2 M KHCO₃, and quantified with kinetic assay (38, 39). Histological assessment was performed on samples initially stored in 10% formalin (~24 h) and then transferred to 70% ethanol. Images were taken at 40× magnification (37). Lipidomic analysis from frozen liver samples was performed by TLC and gas chromatography. Total lipids were extracted, and diacylglycerols (DAGs), TAGs, and ceramides from each sample were separated using TLC (22). Fatty acid composition of isolated ceramide, DAG, and TAG samples was analyzed by gas chromatography (22).

Western blotting
Protein was extracted as described previously (23, 25, 37). Equal amounts of protein were separated on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated at 4°C overnight with primary antibodies. The following day, membranes were incubated with secondary antibodies (Jackson Immuno-Research Laboratories) for 1 h at room temperature. Signals were detected using ECL and subsequently quantified by densitometry using ImageJ software. Phosphorylated and total protein content were expressed relative to a within-gel loading control (GAPDH or Ponceau). Phosphorylated proteins were then expressed relative to total protein content (23, 33, 37). An antibody against PGC1α was from Abcam (catalog no. 3242). Antibodies against IL-6 receptors (catalog no. AF-227-NA) and 4-hydroxynonenal (4HNE; catalog no. MAB3249) were from R&D Systems. Antibodies against pSH2-domain-containing tyrosine phosphatase (SHP2; catalog no. 5431), SHP2 (catalog no. 3792), protein inhibitor of activated STAT3 (PIAS3; catalog no. 4146), SOCS3 (catalog no. 2923), STAT3 (catalog no. 8768), pSTAT3 (catalog no. 9138), ERK (catalog no. 4695), pERK (catalog no. 9101), protein kinase B (AKT; catalog no. 9272), pAKT (41) (catalog no. 9271), pAKTThr308 (catalog no. 9275), eukaryotic initiation factor-2 (eIF2α; catalog no. 9792), pEIF2a (catalog no. 9792), pCREB (catalog no. 9198), and CREB (catalog no. 9197) were from Cell Signaling.

Real-time PCR
RNA was extracted using the TRIzol and RNeasy Mini Kits (Qiagen, catalog no. 74104) as we have described in detail previously (25, 37). cDNA was produced using the High-Capacity cDNA Reverse Transcription Kit (Thermo-Fisher Scientific, catalog no. 4368814), and PCR was run with the Sso Advanced Universal SYBR Green Supermix (Bio-Rad, catalog no. 1729271) using the PCR primers listed in Table 1 on a Bio-Rad CFX connect system. All markers are expressed relative to peptidylprolyl cis-trans isomerase B (Ppiib) (41), except for tunicamycin experiments (see Fig. 8), where Cyclophilin (Cyclo) (40) was used as an endogenous control because Ppiib is an ER protein (41) and tunicamycin induces supraphysiologic ER stress beyond that seen in obesity (42). Relative differences in mRNA expression were determined using the 2⁻ΔΔCt method and normalized to the respective control group (33, 37).

Statistical analysis
Statistical tests were completed using Graph Pad version 6.0. Normality was assessed by a Shapiro-Wilk's test and, if failed (P < 0.05), data were log₁₀ transformed. Data were then compared by unpaired two-tailed t test (e.g., tissue weights, GIT area under the curve (AUC), SocS3 mRNA expression delta, and liver TAGs) or two-way ANOVA with Tukey's post hoc test. A value of P < 0.05 was considered statistically significant.
ANNOVA (e.g., GTT, phosphoSTAT3 content, and Socs3 mRNA expression). Post hoc tests with Tukey’s post hoc correction were completed when a significant interaction was identified. All data are presented as mean ± SEM. Significance level was set at $P < 0.05$.

RESULTS

Short-term saturated HFD leads to systemic glucose intolerance, but not hepatic IL-6 resistance or ER stress

As little as 3–4 days of HFD can alter systemic (22) and hepatic glucose metabolism (21). Here, 4 days of HFD did not significantly affect body weight (Fig. 1A), but there was a slight increase in the size of epididymal white adipose tissue (eWAT) and inguinal WAT (iWAT) depots (Fig. 1B), and this was associated with modest systemic glucose intolerance (Fig. 1C) and elevated blood glucose following a pyruvate challenge [indicative of elevated hepatic glucose output (23); Fig. 1D]. This occurred independent of changes in whole body or liver insulin action (Fig. 1E). IL-6 injections led to similar circulating IL-6 concentrations in both groups (Fig. 1F) and equivalent increases in hepatic STAT3 phosphorylation (Fig. 1G).

Socs3 mRNA expression, another IL-6 signaling marker downstream of STAT3, was potentiated in HFD mice when expressed relative to chow-fed saline-treated mice (Fig. 1H). There was a 2-fold increase in hepatic TAG content (Fig. 1I), but no change in the expression of various ER stress genes (Fig. 1J). Thus, short-term HFD causes systemic glucose dysregulation, but this is not associated with changes in the response to exogenous IL-6 in the liver or evidence of ER stress. In other words, hepatic IL-6 resistance is not an early/causal event in the development of systemic glucose intolerance.

### Table 1. mRNA primer sequences used

| Gene (Reference) | Forward Sequence | Reverse Sequence |
|------------------|------------------|------------------|
| Bip (40)         | ACTGGGACACCATCTTCTT | ATCGCCAATCAGACGCTCC |
| Mcp1/Col2 (57)   | GGCTGGAAGCCTGACAGAGG | GGTCAAGCACGGCTTCC |
| Chop (60)        | CCACACACCTGAAAGAGCA | AGGTTGAAGCCGAGGAGCTCA |
| Cyclo (40)       | GGGTATCCAGTTGACGTGAG | TGGCAGGATCTGACAAATGCAT |
| iNOS (57)        | CCAAAGCTCTTCATCTTCCA | CGGTTTCCACAAAGAATCCATT |
| F4/80 (57)       | TGGTATCTTACAGGTTGACGGG | AGGCTTGGTGATGACAG |
| G6pc (58)        | AGGAAAGGCCTTCTATGTCGAT | GCGTTCGCCAACAGAATCCATT |
| Pck1 (58)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTCAGGACCTTGGAGAACAGTCC |
| Ppara (61)       | GAGGATGGCAAGAGGAAAGGAG | GAGGATGGCAAGAGGAAAGGAG |
| Chop (60)        | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |
| Mcp-1/Ccl2 (57)  | GGCTGGAGAGCTACAAGAGG | GGTCAGCACAGACCTCTCTC |
| G6pc (58)        | TGGTATCTTACAGGTTGACGGG | AGGCTTGGTGATGACAG |
| Chop (60)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTTCGCCAACAGAATCCATT |
| Ppara (61)       | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |
| Chop (60)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTTCGCCAACAGAATCCATT |
| Ppara (61)       | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |
| Chop (60)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTTCGCCAACAGAATCCATT |
| Ppara (61)       | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |
| Chop (60)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTTCGCCAACAGAATCCATT |
| Ppara (61)       | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |
| Chop (60)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTTCGCCAACAGAATCCATT |
| Ppara (61)       | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |
| Chop (60)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTTCGCCAACAGAATCCATT |
| Ppara (61)       | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |
| Chop (60)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTTCGCCAACAGAATCCATT |
| Ppara (61)       | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |
| Chop (60)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTTCGCCAACAGAATCCATT |
| Ppara (61)       | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |
| Chop (60)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTTCGCCAACAGAATCCATT |
| Ppara (61)       | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |
| Chop (60)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTTCGCCAACAGAATCCATT |
| Ppara (61)       | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |
| Chop (60)        | CGGAAAGGACCTTGGAGAACAGTCC | GCGTTCGCCAACAGAATCCATT |
| Ppara (61)       | GGGTGGCAAAGAAAAGGAGGT | GGGTGGCAAAGAAAAGGAGGT |

**Fig. 1.** Four days of HFD causes systemic glucose intolerance and hepatic TAG accumulation, but no change in IL-6 signaling. Mice were fed chow or a lard-based HFD for 3 days, after which time an IP GTT (2 g·kg$^{-1}$) was performed, and then the following day, mice were injected IP with recombinant IL-6 (6 ng·kg$^{-1}$), and tissues were collected after 15 min to assess IL-6 hepatic signaling. A: Body weight following 4 days of chow or HFD (n = 15 per group; unpaired t-test). B: Terminal tissue weights (n = 7 or 8 per group; unpaired t-test). C: Blood glucose during IP GTT and blood glucose AUC on the third day of HFD (n = 15 per group; two-way ANOVA and unpaired t-test, respectively). D: Blood glucose during IP pyruvate tolerance test (2 g·kg$^{-1}$) and blood glucose AUC on the third day of HFD (n = 6 per group; two-way ANOVA and unpaired t-test, respectively). E: Blood glucose during IP insulin tolerance test (ITT) (1 U/kg) and blood glucose AUC on the third day of HFD (n = 6 per group; two-way ANOVA and unpaired t-test, respectively). F: Circulating IL-6 concentrations determined by ELISA 15 min after exogenous IL-6 injection (n = 5 per group; no statistical test performed). ND, IL-6 not detected for that group. G: Hepatic STAT3 phosphorylation 15 min after IL-6 and representative Western blot images (n = 7 per group; two-way ANOVA). H: Hepatic Socs3 mRNA expression relative to chow-fed saline-injected mice 15 min after IL-6 (n = 6–8 per group; two-way ANOVA). I: Biochemical quantification of hepatic TAG content following 4 days of HFD (n = 15 per group; unpaired t-test). J: Hepatic mRNA expression of ER stress genes (n = 6–8 per group; two-way ANOVA). a.u., arbitrary units; BAT, brown adipose tissue; Ins, insulin; PTT, pyruvate tolerance test; SAL, saline. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Note that a line represents a main effect with the effect and corresponding $P$ value given above the line.
Chronic saturated HFD leads to severe glucose intolerance and hepatic IL-6 resistance in parallel with ER stress

Although IL-6 signaling was not impaired after 4 days, hepatic steatosis and glucose intolerance become progressively worse after prolonged HFD (21, 43). Following 7 weeks of HFD, mice gained substantial body weight (Fig. 2A) and became severely glucose intolerant (Fig. 2B). Chronic HFD reduced hepatic expression of gluconeogenic genes, glucose 6-phosphatase (G6pc) and phosphoenolpyruvate carboxykinase 1 (Pck1), and the lipogenic regulator Srebpl, whereas carnitine-palmitoyltransferase 1 (Cpt1) expression was increased (Fig. 2C); IL-6 only reduced Srebp1c expression, as shown previously (13). Again, IL-6 injections led to similar circulating IL-6 (Fig. 2D), but there was a significant blunting of hepatic STAT3 phosphorylation in HFD-fed mice (Fig. 2E). Downstream of STAT3, expression of Socs3 mRNA was similar when expressed relative to the chow-fed saline-injected (Chow-SAL) group, but because Socs3 expression was greater in high-fat diet-fed saline-injected (HFD-SAL) mice (~3-fold, P = 0.4), the change in Socs3 mRNA was blunted in HFD mice (Fig. 2F). The phosphorylation of STAT3 was similar in skeletal muscle and WAT of obese and lean mice (supplemental Fig. S1); other reports have shown that WAT and skeletal muscle retain their IL-6 signaling in obese db/db mice (18). Taken together, these data demonstrate that, in response to chronic HFD-induced obesity, the liver becomes resistant to acute IL-6.

Chronic HFD-induced hepatic TAG content and ER stress are associated with blunted IL-6 signaling

In an attempt to determine what might drive hepatic IL-6 resistance, we assessed various reputed inhibitors of the IL-6 signaling cascade in HFD-induced obese mice (44). There was no change in the content of either IL-6 receptor-α or glycoprotein 130 (GP130), an alternative IL-6 receptor (45) (Fig. 2G), but there was a decrease in phosphorylation of SHP2 in HFD mice, with a trend toward decreased activity (phosphorylation of ERK) and content (SOCS3 and PIAS3) of other inhibitors of IL-6 signaling (Fig. 2H). The content of 4HNE, a marker of lipid peroxidation and oxidative stress, was unchanged (Fig. 2I). Circulating NEFA and β-hydroxybutyrate were unchanged (Fig. 2J), whereas liver glycogen was slightly reduced in HFD mice (Fig. 2K). There was substantial (~4-fold) accumulation of total liver TAGs (Figs. 2L), particularly saturated TAGs, with no change in DAGs or ceramides (Table 2). Nevertheless, STAT3 phosphorylation was inversely correlated with total liver TAG content (Fig. 2M), implicating TAG accumulation in the development of hepatic IL-6 resistance. Importantly, despite TAG accumulation, various markers of inflammation remained similar between all groups, whereas ER stress markers, including binding immunoglobulin protein (Bip), CCAAT-enhancer-binding protein homologous protein (Chop), and spliced x-box binding protein-1 (Xbp1s), were significantly elevated in HFD-fed mice (Fig. 2N), along with phosphorylation of the translation initiation inhibitor eIF2α (Fig. 2O). In sum, these data show that the development of hepatic IL-6 resistance occurs quite early in steatotic development, occurring even before inflammation, and is associated with increased liver TAG content and markers of ER stress.

Prolonged high-monounsaturated-fat diet causes hepatic lipid accumulation, but not ER stress or hepatic IL-6 resistance

Saturated lipids, rather than total TAG content per se, are thought to be particularly damaging to the liver (46). The HFD used in the previous experiment (60% kcal from lard) is high in saturated fat; thus, we designed a diet with the same fat content but derived from olive oil, which is predominantly MUFA, to test whether the observed IL-6 resistance is dependent upon saturated fatty acids. Following 7 weeks of MUFA HFD, mice were obese and glucose intolerant, with reduced liver glycogen and liver TAGs similar (P = 0.19 by t-test) to what was observed with the traditional lard-based HFD (Fig. 3A–D). Prolonged MUFA feeding produced a similar phenotype to the traditional HFD, including body weight, fed hyperglycemia, glucose intolerance, and metabolic inflexibility based on respiratory exchange ratio (RER) (supplemental Fig. S2). Lipidomic analysis showed that this diet led to the preferential accumulation of MUFA compared with chow and traditional HFD, with a concomitant reduction in saturated and polyunsaturated TAGs and saturated DAGs (Table 2). Interestingly, impairments in the IL-6-induced phosphorylation of STAT3 (Fig. 3E) and the correlation between STAT3 phosphorylation and liver TAGs were absent in MUFA-fed mice (Fig. 3F). Socs3 mRNA expression in response to IL-6 was similar to chow-fed mice (Fig. 3G). Importantly, and in contrast to a traditional lard-based HFD, there was no sign of ER stress in MUFA-fed mice (Fig. 3H). These data provide support that saturated FAs drive ER stress and hepatic IL-6 resistance in obesity, whereas MUFAs are relatively benign in the liver.

Acute fasting causes hepatic lipid accumulation, but not ER stress or hepatic IL-6 resistance

Fasting activates lipolysis and the preferential breakdown and release of MUFA from adipose tissue, while simultaneously reducing circulating saturated fatty acids (47). Thus, we extended the previous findings to show that a 30 h fast, as expected, increased hepatic TAG content (Fig. 4A); total TAG was similar to chronic HFD, but with lower saturated fat content compared with lard-based HFD (Fig. 4B). Fasted mice lost ~4 g of body weight (data not shown). Fasting led to a 2-fold increase in hepatic DAG compared with chow-fed mice, which was greater than even chronic lard-based HFD (Table 2). There was no difference in IL-6-induced STAT3 phosphorylation (Fig. 4B), and Socs3 mRNA expression was actually potentiated in fasted mice (Fig. 4C) alongside reduced Xbp1s expression in fasted mice (Fig. 4D). These data provide further evidence that saturated fats, rather than TAGs per se, are the primary culprit behind hepatic IL-6 resistance in vivo.

Hepatic IL-6 resistance is associated with increased blood glucose following pyruvate challenge

Previous reports have shown that IL-6 signaling inhibits hepatic glucose production (4, 5) and decreases the expression of gluconeogenic genes (13, 33), so we assessed whether altered IL-6 signaling would affect blood glucose concentrations in response to exogenous IL-6 after pyruvate injections, a rough proxy of hepatic glucose production
Seven weeks of HFD causes hepatic IL-6 resistance associated with hepatic TAG accumulation and ER stress. Mice were fed chow or a lard-based HFD for 7 weeks, after which time an IP GTT (2 g·kg\(^{-1}\)) was performed, and then the following day, they were injected IP with recombinant IL-6 (6 ng·kg\(^{-1}\)), and tissues were collected after 15 min to assess IL-6 signaling. A: Body weight following 7 weeks of HFD (n = 15 per group; unpaired t-test). B: Blood glucose during GTT and blood glucose AUC (n = 4–7 per group; two-way ANOVA and unpaired t-test, respectively). C: Hepatic mRNA expression of gluconeogenic, oxidative, and lipogenic genes (n = 6 or 7 per group; two-way ANOVA). D: Circulating IL-6 concentrations 15 min after exogenous IL-6 injection (n = 4 or 5 per group; no statistical test performed). ND, IL-6 not detected for that group. E: Hepatic STAT3 phosphorylation relative to chow-SAL (left) with graph showing the change in STAT3 phosphorylation compared with saline-treated mice from each diet (center) and representative Western blot images (right) (n = 7 per group; two-way ANOVA and unpaired t-test, respectively). F: Hepatic Socs3 mRNA expression relative to chow-fed saline-injected mice (left) with graph showing the change in expression compared with saline-treated mice from each diet (right) (n = 6 per group; two-way ANOVA and unpaired t-test, respectively). G: Protein content of hepatic IL-6 receptor-α and GP130 with representative Western blot images (n = 7 per group; two-way ANOVA). H: Protein content of various reputed inhibitors of the IL-6 signaling cascade with representative Western blot images (n = 7 per group; two-way ANOVA). I: The 4HNE protein content, a marker of oxidative stress, in liver with Ponceau loading control (n = 7 per group; two-way ANOVA). J: Quantification of circulating NEFAs and β-hydroxybutyrate from lean and obese mice after 7 week HFD (n = 6–9 per group; unpaired t-test). K: Hepatic glycogen content (n = 6 per group; unpaired t-test). L: Biochemical quantification of hepatic TAG content (n = 7 per group; unpaired t-test) with histological images of livers stained with H&E (n = 4 per group; no quantification or statistical tests were performed). M: Correlation between hepatic TAG content and STAT3 phosphorylation in IL-6-injected mice (n = 14 per factor; Pearson correlation). N: Hepatic mRNA expression of inflammatory and ER stress genes (n = 6–8 per group; two-way ANOVA). O: Hepatic protein content of ER stress marker eIF2α with representative Western blot images (n = 7 per group; two-way ANOVA), a.u., arbitrary units; SAL, saline. * P < 0.05; ** P < 0.01; *** P < 0.001. Note that a line represents a main effect with the effect and corresponding P value given above the line.
IL-6 attenuated pyruvate-induced increases in blood glucose in lean mice, but failed to do so in mice that had consumed the predominantly saturated HFD for 7 weeks (Fig. 5A). However, unlike the saturated HFD, IL-6 was still able to attenuate pyruvate-induced increases in blood glucose in obese mice that consumed the MUFA diet (Fig. 5B), again indicating that saturated fat is likely driving IL-6 resistance rather than the simple accumulation of liver TAGs. Reflecting this, hepatic G6pc expression was reduced by IL-6 in chow- and MUFA-fed mice, but not in HFD-fed mice (Fig. 5C); G6pc, compared with other gluconeogenic genes, like Pck1 or Ppargc1a, appears to be most affected by IL-6 (4, 5, 13). Despite reduced blood glucose following pyruvate in chow- and MUFA-fed mice, there was no difference in the content of various gluconeogenic proteins (Fig. 5D). Glucose output was not associated with differences in insulin signaling, based on AKT phosphorylation, either endogenously (Fig. 5E) or in response to exogenous

Following each intervention, liver samples were assessed via TLC and gas chromatography (22). Absolute fatty acid contents (nmol/g wet weight) were compared by one-way ANOVA. Unlike letters indicate a significant ($P < 0.05$) difference between groups. A row without letters indicates that there were no significant differences between groups. SFA, saturated fatty acid.

Table 2. Hepatic lipidomic analysis

|                | Chow           | HFD            | Fasting        | MUFA           |
|----------------|----------------|----------------|----------------|----------------|
| TAGs (nmol/g wet weight) |                |                |                |                |
| Total          | 5.47 ± 1.49$^a$ | 28.41 ± 2.93$^b$ | 21.80 ± 3.06$^b$ | 24.48 ± 1.98$^b$ |
| SFA            | 1.65 ± 0.86$^a$ | 9.29 ± 1.11$^b$  | 6.30 ± 0.86$^b$  | 5.06 ± 1.11$^b$  |
| MUFA           | 2.02 ± 0.42$^a$ | 12.34 ± 1.44$^{a,e}$ | 8.95 ± 1.26$^b$  | 16.45 ± 0.80$^{a,e}$ |
| PUFA           | 1.82 ± 0.71    | 6.76 ± 0.47    | 6.57 ± 0.96    | 2.77 ± 0.61    |
| n3            | 0.09 ± 0.04    | 0.22 ± 0.01    | 0.17 ± 0.03    | 0.06 ± 0.01    |
| n6            | 1.73 ± 0.67$^a$ | 6.54 ± 0.47$^b$ | 6.40 ± 0.93$^b$ | 2.71 ± 0.60$^b$ |
| DAGs (nmol/g wet weight) |                |                |                |                |
| Total          | 0.18 ± 0.03$^a$ | 0.21 ± 0.05$^c$ | 0.37 ± 0.07$^a$ | 0.34 ± 0.07$^a$ |
| SFA            | 0.07 ± 0.01    | 0.09 ± 0.02    | 0.12 ± 0.02    | 0.10 ± 0.03    |
| MUFA           | 0.06 ± 0.01$^c$ | 0.07 ± 0.02$^a$ | 0.14 ± 0.05$^b$ | 0.17 ± 0.03$^b$ |
| n3            | 0 ± 0          | 0 ± 0          | 0 ± 0          | 0 ± 0          |
| n6            | 0.11 ± 0.02$^a$ | 0.04 ± 0.01$^c$ | 0.11 ± 0.02$^b$ | 0.07 ± 0.02$^c$ |
| Ceramides (nmol/g wet weight) |                |                |                |                |
| Total          | 0.01 ± 0.0022  | 0.0111 ± 0.0016| 0.01 ± 0.0018  | 0.0208 ± 0.0106|
| SFA            | 0.0028 ± 0.0016| 0.0034 ± 0.0019| 0.0084 ± 0.0018| 0.0087 ± 0.0019|
| MUFA           | 0.0005 ± 0.0002| 0.0087 ± 0.0069| 0.0008 ± 0.0006| 0.0034 ± 0.0019|
| PUFA           | 0 ± 0          | 0 ± 0          | 0 ± 0          | 0 ± 0          |
| n3            | 0 ± 0          | 0 ± 0          | 0.0005 ± 0.0005| 0 ± 0          |
| n6            | 0.0003 ± 0.0002| 0.0087 ± 0.0069| 0.0003 ± 0.0003| 0.0087 ± 0.0069|

Fig. 3. Seven weeks of a predominantly monounsaturated HFD causes obesity and hepatic TAG accumulation, but not hepatic IL-6 resistance or ER stress. Mice were fed a chow or a HFD derived predominantly from olive oil for 7 weeks, after which time a IP GTT (2 g·kg$^{-1}$) was performed and then the following day injected IP with recombinant IL-6 (6 ng·kg$^{-1}$), and tissues were collected after 15 min to assess IL-6 signaling. A: Body weight following 7 weeks of MUFA HFD (n = 14 per group; unpaired t-test). B: Blood glucose during GTT and blood glucose AUC (n = 7 per group; two-way ANOVA and unpaired t-test, respectively). C: Hepatic glycogen content (n = 6 per group; unpaired t-test). D: Biochemical quantification of hepatic TAG content (n = 7 per group; unpaired t-test). E: Hepatic STAT3 phosphorylation relative to chow-SAL and representative Western blot images (n = 7 per group; two-way ANOVA). F: Correlation between hepatic TAG content and STAT3 phosphorylation in IL-6-injected mice (n = 14 per factor; Pearson correlation). G: Hepatic Socs3 mRNA expression relative to Chow-SAL and representative Western blot images (n = 6 per group; two-way ANOVA and unpaired t-test, respectively). H: Hepatic mRNA expression of ER stress genes following 7 weeks of MUFA HFD (n = 6 per group; two-way ANOVA). a.u., arbitrary units; MFA, MUFA; SAL, saline. * P < 0.05; ** P < 0.01; *** P < 0.001. Note that a line represents a main effect, with the effect and corresponding P value given above the line.
insulin injection (supplemental Fig. S3). These data show that impaired IL-6 signaling in obesity is associated with markers of increased hepatic glucose output.

**Saturated fatty acids directly inhibit STAT3 phosphorylation associated with ER stress in isolated murine hepatocytes**

Next, we wanted to determine whether hepatic IL-6 resistance is due to systemic factors or is liver-specific. Thus, we isolated hepatocytes from lean mice and incubated them with a combination of saturated (palmitate) and unsaturated (oleate) fatty acids, in differing ratios but at the same concentration (500 μM), before acutely treating with IL-6 (33). We observed a dose-dependent reduction in STAT3 phosphorylation in response to increasing palmitate. High-dose (400 μM) palmitate completely blocked STAT3 phosphorylation, whereas the same concentration of oleate did not (Fig. 6A). Differences in STAT3 phosphorylation did not impact hepatic glucose output (Fig. 4).

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**Fig. 4.** Acute fasting in lean mice causes liver TAG accumulation, but not IL-6 resistance. Lean chow-fed mice were fasted for 30 h before being injected IP with recombinant IL-6 (6 ng·kg⁻¹). Tissue was collected 15 min later to assess hepatic IL-6 signaling. A: Biochemical quantification of hepatic TAG content after 30 h fast (n = 7 per group; unpaired t-test). B: Hepatic STAT3 phosphorylation relative to fed-SAL and representative Western blot images (n = 7 per group; two-way ANOVA). C: Hepatic Socs3 mRNA expression relative to chow-fed saline-injected mice (left) with graph showing the change in expression compared with saline-treated mice from either the fed or fasted group (right) (n = 7 per group; two-way ANOVA and unpaired t-test, respectively). D: Hepatic mRNA expression of ER stress genes following 30 h fast (n = 7 per group; two-way ANOVA). a.u., arbitrary units; SAL, saline. * P < 0.05; ** P < 0.01; *** P < 0.001. Note that a line represents a mean effect, with the effect and corresponding P-value given above the line.

**Fig. 5.** Seven weeks of lard-based HFD, but not monounsaturated HFD, impairs IL-6’s ability to suppress blood glucose following pyruvate challenge. Mice consumed either a standard chow diet, lard-based HFD, or a HFD derived from olive oil for 7 weeks. Mice were fasted overnight and then injected IP with IL-6 (6 ng·kg⁻¹), then 4 h later given an IP pyruvate tolerance test (2 g·kg⁻¹). A separate cohort received the same treatment, but tissues were collected 4 h after IL-6 to assess hepatic cellular responses. A: Blood glucose (left) with graph showing blood glucose AUC (right) following pyruvate injection in mice previously fed chow or a lard-based HFD for 7 weeks and injected IP with IL-6 (n = 6 per group; two-way ANOVA). B: Blood glucose (left) with graph showing blood glucose AUC (right) following pyruvate injection in mice previously fed a chow or olive oil-based HFD for 7 weeks and injected IP with IL-6 (n = 6 per group; two-way ANOVA). C: Hepatic mRNA expression of G6pc 4 h after IL-6 relative to Chow-SAL group (left) with graph showing the relative change in expression compared with saline-injected (SAL) mice from each diet (right) (n = 6 per group; two-way ANOVA); note, a line represents a significant difference within that diet. D: Protein content of hepatic gluconeogenic proteins following 7 weeks of HFD and 4 h after IL-6 (n = 4–6 per group; two-way ANOVA). E: Protein content of AKT (T308) and AKT (S473) in livers 4 h after IL-6 and representative Western blot images (n = 4–6 per group; two-way ANOVA). MFA, MUFA; PTT, pyruvate tolerance test; SAL, saline. * P < 0.05; ** P < 0.01; *** P < 0.001.
ER stress and hepatic IL-6 resistance are not resolved by reduced liver TAGs (Fig. 7F). These data demonstrate that ER stress gene expression was not improved despite repression, compared with mice consuming only a HFD (Fig. 9A–D). Following 2 weeks of daily PBA treatment, liver TAG content was similar to control (Fig. 9A). PBA treatment did not affect body weight or food intake, tissue weight, systemic glucose tolerance, or fed blood-glucose concentrations (Fig. 9A–D). Following 2 weeks of daily PBA treatment, liver TAG content was similar to control (Fig. 9E), but ER stress markers were reduced (Fig. 9F).

Acute pharmacologically induced ER stress causes, whereas the reduction of ER stress prevents, hepatic IL-6 resistance

To determine whether acute ER stress, in the absence of obesity, could cause hepatic IL-6 resistance, we injected lean mice with tunicamycin for 48 h. Tunicamycin, which induces ER stress by inhibiting the posttranslational glycosylation of folded proteins in the ER, led to substantial weight loss associated with smaller liver, iWAT, and brown adipose tissue depots (Fig. 8A, B). There was also substantial liver TAG accumulation (Fig. 8C). Bip, Chop, and Xbp1s expression were markedly increased, indicative of profound ER stress (Fig. 8D), with concomitant impairment of hepatic IL-6 signaling, evidenced by both IL-6-induced STAT3 phosphorylation and Socs3 mRNA expression being significantly blunted (Fig. 8E, F). Importantly, Socs3 expression was elevated by tunicamycin, but this had not yet translated into increased SOCS3 protein content (Fig. 8G); another report has shown that tunicamycin actually reduces SOCS3 protein content in hepatocytes, strongly supporting that SOCS3 is not responsible for reduced IL-6 signaling in this model (18).

Tunicamycin induces ER stress beyond physiological responses (42), so we utilized daily treatment with the chemical chaperone PBA during the final 2 weeks of a saturated HFD as a means to reduce ER stress (16, 18). Daily PBA treatment did not affect body weight or food intake, tissue mass, systemic glucose tolerance, or fed blood-glucose concentrations (Fig. 9A–D). Following 2 weeks of daily PBA treatment, liver TAG content was similar to control (Fig. 9E), but ER stress markers were reduced (Fig. 9F). This
Development of hepatic IL-6 resistance

reduction in ER stress was associated with a strong trend (P = 0.09) for greater STAT3 phosphorylation following IL-6 injection (Fig. 9G), which was reflected by potentiated IL-6-induced Socs3 mRNA expression compared with control-treated obese mice (Figs. 9H). These data strongly implicate ER stress in the development of high-saturated-fat diet-induced hepatic IL-6 resistance.

DISCUSSION

Hepatic IL-6 signaling is important for the maintenance of systemic glucose metabolism (7, 9, 12). Here, we show that in a model of chronic saturated HFD-induced obesity and glucose intolerance, the liver becomes resistant to exogenous IL-6, exhibiting reduced IL-6-induced STAT3 phosphorylation and Socs3 mRNA expression. We also provide evidence that hepatic IL-6 resistance is likely the result of ER stress induced specifically by saturated fatty acids, both in vivo and in vitro. Ultimately, this hepatic IL-6 resistance is associated with elevated markers of hepatic glucose output, including G6pc expression and blood glucose, following pyruvate challenge.

Obesity is associated with hepatic steatosis, inflammation, and ER stress (16, 17, 46, 50, 51). Similar to past reports (21), we found that as few as 4 days of HFD leads to a 2-fold increase in liver TAGs and modest systemic glucose intolerance, although early TAG accumulation was not associated with increased expression of ER stress markers or signs of IL-6 resistance. By 7 weeks of lard-based HFD, mice were obese and severely glucose-intolerant, in conjunction with substantial liver TAG accumulation; perhaps surprisingly, 7 weeks of high saturated and monounsaturated diets led to similar hepatic TAGs, DAGs, and ceramides. Interestingly, despite obesity and steatosis, there were no signs of hepatic inflammation [Tnfa, inducible NO synthase (iNOS), monocyte chemoattractant protein (Ccl2), or F4/80 mRNA expression], although evident ER stress (Bip, Chop, Xbp1s mRNA expression, and eIF2α phosphorylation). Inflammation (17, 52) and ER stress (14, 16) are both strongly linked to the development of obesity-associated hepatic insulin resistance, but our data suggest that hepatic ER stress develops early in obesity, occurring even before inflammation. Moreover, hepatic ER stress is related to increased hepatic glucose output in obesity and diabetes (16), which is one of the primary causes of early hyperglycemia in obesity, so it is possible that ER stress-induced IL-6 resistance may be at least partly responsible for this.

Rather than simple TAG accumulation, the type of fatty acid seems to be more important in regard to hepatic cellular responses (46, 53). For example, in isolated hepatocytes, exposure to the saturated fatty acid palmitate causes accumulation of ceramides, induction of reactive oxygen species, and apoptosis, but if the MUFA oleate is also present, then hepatocytes are protected (28). ER stress was potently induced when we exposed hepatocytes to a high dose of palmitate, whereas the same dose of oleate had no effect on ER stress markers. Indeed, palmitate demonstrated...
a dose-response effect on impairing IL-6-induced STAT3 phosphorylation. Importantly, earlier work showed that both tunicamycin- and palmitate-induced ER stress inhibit IL-6 signaling via dephosphorylation of STAT3 in isolated db/db mouse hepatocytes (18). Moreover, when ER stress was reduced in obese db/db mice by the chemical chaperone PBA, IL-6’s ability to suppress hepatic G6pc and Pck1 was partly rescued (18). Along these lines, we found that palmitate increased G6pc gene expression and rendered G6pc insensitive to IL-6-induced suppression, none of which occurred when palmitate and oleate were present at the same concentration or high-dose oleate.

**Fig. 8.** Pharmacologically induced ER stress inhibits hepatic IL-6 signaling. Mice were injected IP with tunicamycin (1 µg·g⁻¹) and 48 h later injected IP with IL-6 (6 ng·kg⁻¹), and tissues were collected 15 min later to assess hepatic IL-6 signaling. A: Weight change following 48 h of tunicamycin (n = 7–14 per group; unpaired t-test). B: Tissue weights following 48 h of tunicamycin (n = 14 per group; unpaired t-test). C: Biochemical quantification of hepatic TAG content (n = 7 per group; unpaired t-test). D: Hepatic mRNA expression of ER stress markers following 48 h of tunicamycin (n = 6 per group; two-way ANOVA). E: Hepatic STAT3 phosphorylation 48 h after tunicamycin relative to SAL-SAL (left), with graph showing the change compared with saline-treated mice from the same group (center) and representative Western blot images (right) (n = 7 per group; two-way ANOVA). F: Hepatic Socs3 mRNA expression 48 h after tunicamycin relative to SAL-SAL, with inset showing the change in expression compared with saline-treated mice from each group (n = 6 per group; two-way ANOVA and unpaired t-test, respectively). G: Hepatic SOCS3 protein content 48 h after tunicamycin relative to SAL-SAL group with representative Western blot images (n = 6 per group; two-way ANOVA). a.u., arbitrary units; BAT, brown adipose tissue; SAL, saline; TUN, tunicamycin. * P < 0.05; ** P < 0.01; *** P < 0.001. Note that a line represents a mean effect, with the effect and corresponding P value given above the line.

**Fig. 9.** Pharmacological reductions in HFD-induced ER stress improves hepatic IL-6 signaling, independent of changes in body mass and glucose tolerance. Mice consumed a lard-based HFD, then received daily gavages of PBA for 2 weeks (500 mg to 1 g·kg⁻¹) for a total of 7 weeks of HFD. Mice were then injected IP with IL-6 (6 ng·kg⁻¹), and tissues were collected 15 min later to assess IL-6 signaling. A: Body weight and food intake of mice during 2 week PBA treatment (n = 13 per group; two-way ANOVA). B: Tissue weights from mice following 2 week PBA or saline treatment (n = 6 per group; two-way ANOVA). C: Blood glucose during IP GTT on the 12th day of PBA treatment (n = 8 per group; two-way ANOVA). D: Fed blood glucose concentrations taken the morning (0800) of terminal experiments (n = 12 per group; unpaired t-test). E: Biochemical quantification of hepatic TAG content (n = 6 per group; unpaired t-test). F: Hepatic mRNA expression of ER stress genes following 2 weeks of PBA treatment in HFD-induced obese mice (n = 6 per group; two-way ANOVA). G: Hepatic STAT3 phosphorylation relative to SAL-SAL (left), with graph showing the change in STAT3 phosphorylation compared with saline-treated mice of each group (center) and representative Western blot images (right) (n = 6 per group; two-way ANOVA and unpaired t-test, respectively). H: Hepatic Socs3 mRNA expression 48 h after tunicamycin relative to SAL-SAL (n = 6 per group; two-way ANOVA). a.u., arbitrary units; SAL, saline. * P < 0.05; ** P < 0.01; *** P < 0.001. Note that a line represents a mean effect, with the effect and corresponding P value given above the line.
At the same time, when mice consumed a HFD derived predominantly from MUFAs (olive oil), we observed a similar metabolic phenotype to the traditional saturated fatty acid-based HFD, including weight gain, glucose intolerance, metabolic inflexibility, and hepatic TAG and DAG accumulation. However, despite a similar metabolic profile, there was a unique hepatic cellular response where neither ER stress nor hepatic IL-6 resistance developed. We showed similar results by acutely fasting mice, which mobilized predominately MUFAs from adipose tissue and reduces circulating saturated fatty acids (47). Similar to more prolonged exposure to MUFAs, fasting also produced hepatic TAG accumulation, but Xbp1s mRNA expression was actually reduced, with no impairment in IL-6 signaling; in fact, this reduction in Xbp1s was associated with augmented IL-6-induced Socs3 expression. Similar to other reports (15, 28), only high concentrations of palmitate led to ER stress in isolated hepatocytes, whereas the same concentration of oleate did not. Overall, this indicates that the induction of hepatic ER stress in obesity depends on the supply of saturated fatty acids.

A role for IL-6 in suppressing hepatic glucose production is fairly well-established (4, 5). On the other hand, IL-6 has much more modest effects on hepatic lipid metabolic genes (4, 13), consistent with our data; RER was similar between MUFAs- and HFD-fed groups, whereas β-hydroxybutyrate was similar between 7 week chow- and HFD-fed mice, supporting that IL-6 resistance is not related to alterations in lipid metabolism. IL-6 suppresses hepatic glucose production by phosphorylating and activating STAT3, which subsequently inhibits gene expression of gluconeogenic genes, most notably G6pc (4, 5, 13). It is worth mentioning that we observed IL-6-induced reductions in blood glucose following pyruvate challenge with concomitant reductions in G6pc mRNA expression, despite no difference in the protein content of G6Pase or any other gluconeogenic protein, similar to other reports of IL-6-associated reductions in hepatic glucose output (4, 5, 13). There is contention surrounding the relationship between substrate flux, gene transcription, and protein content with hepatic glucose production (48, 54), but they likely all have unique temporal relationships with hepatic glucose output (54). Nevertheless, oscillations in the expression of gluconeogenic enzymes has been suggested to reflect hepatic gluconeogenic capacity (48). Moreover, there is a striking correlation between G6pc and Pck1 mRNA expression and net gluconeogenic flux (54), despite no change in PEPCK or G6Pase protein content, at least in fasted dogs during hyperinsulinemic infusion (55). Thus, that IL-6-induced phosphorylation of STAT3 was reduced in saturated HFD-induced obese mice could explain why IL-6 also failed to suppress hepatic G6pc mRNA expression and blood glucose after pyruvate challenge.

Considering that both hepatic glucose output and circulating IL-6 are elevated in obesity, early work suspected that IL-6 was negatively affecting glucose regulation (1–3), possibly by causing hepatic insulin resistance (35, 56). Recently, this is being challenged, and it now seems that IL-6 is required for maintaining normal systemic glucose metabolism (4–8). We have previously observed that IL-6 signaling is similar between lean and obese mice, but that report used a much higher IL-6 dose (400 ng) and longer duration (90 min) with comparisons made to a low-fat diet (10% kcal/fat)-fed group, which might indicate that HFD-diet-induced IL-6 resistance can be overcome under some experimental conditions (33). It should also be emphasized that systemic glucose intolerance was dissociated from hepatic IL-6 resistance in various models, including 4 day HFD and chronic MUFA HFD. Nevertheless, our data support that increased circulating IL-6 may actually be a compensatory response in an attempt to reduce hepatic glucose output in obesity (9).

Taken together, our data demonstrate that, in response to saturated fatty acids, the liver and hepatocytes exhibit signs of ER stress, which inhibits IL-6 signaling, ultimately impairing IL-6’s ability to suppress markers of hepatic glucose production. ER stress is known to contribute to the development of obesity-associated hepatic insulin resistance (14) and elevated hepatic glucose output in obesity (16). Here, we show that ER-stress-induced IL-6 resistance may be a link between ER stress and the elevated glucose output seen during obesity.

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