XBP1s Is an Anti-lipogenic Protein*

Received for publication, March 24, 2016, and in revised form, June 12, 2016. Published, JBC Papers in Press, June 20, 2016, DOI 10.1074/jbc.M116.728949

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The increasing worldwide prevalence of obesity is considered one of the most serious public health problems of the 21st century (1, 2). Obesity is a predisposing factor for various metabolic disorders, including type 2 diabetes, cardiovascular disease, and non-alcoholic fatty liver disease (NAFLD)3 (3, 4). A subset of NAFLD patients develops non-alcoholic steatohepatitis, liver cirrhosis, and liver failure and eventually requires a liver transplantation (5–7). Additionally, NAFLD is highly associated with development of insulin resistance, and the vast majority of obese and type 2 diabetic patients are diagnosed with NAFLD (8, 9). Despite extensive efforts in the field, the underlying molecular mechanisms for NAFLD in obesity are poorly understood.

Endoplasmic reticulum (ER) stress, a condition in which ER homeostasis is disturbed by pathologies that include, but are not limited to, accumulation of misfolded proteins and overt nutrient supply, has been identified as an important player in the pathogenesis of metabolic diseases such as obesity, insulin resistance, and type 2 diabetes (10). ER stress leads to activation of the unfolded protein response (UPR), a complex signaling network that aims to resolve ER stress and restore ER homeostasis (11, 12). Inositol-requiring enzyme 1 (IRE1) is one of the three master modulators of UPR signaling. Together with PKR-like endoplasmic reticulum kinase and activating transcription factor 6 (ATF6), IRE1 senses ER luminal status and, accordingly, activates one of the most crucial signaling arms of the UPR. Activated IRE1 cleaves X-box binding protein 1 (XBP1) mRNA, leading to the formation of the spliced form of XBP1 (XBP1s), a highly active transcription factor and central regulator of ER homeostasis.

We have shown previously that nuclear translocation of XBP1s is severely impaired in the liver of obese and insulin-resistant mice (13). Reinstituting the hepatic activity of XBP1s reduced ER stress and improved glucose homeostasis through mechanisms that are both dependent (14, 15) and independent of XBP1s transcriptional activity (16). This indicates that XBP1s is indispensable for the maintenance of glucose homeostasis in obesity. Indeed, XBP1 haploinsufficiency led to the development of glucose intolerance and insulin resistance in HFD-fed BALB/c mice, a strain that is otherwise resistant to diet-induced insulin resistance (17).

Fatty livers of both obese rodents (17) and humans (18) have increased levels of ER stress. Importantly, alleviation of ER stress by chemical chaperone treatment (19) or overexpression of ER chaperone genes (20) reverses hepatic steatosis. Furthermore, pharmacological activation of ER stress results in hepatic steatosis in mice deficient in ER stress-sensing or ER quality control pathways (21, 22). Liver-specific IRE1α-null mice, which are deficient in XBP1s activity, have enhanced development of fatty liver upon pharmacological induction of ER stress (23). This suggests that XBP1s potentially serves a protective role in the development of hepatic steatosis. In contrast to these reports, which all indicate that increased ER stress promotes hepatic steatosis, it has been suggested that XBP1s, which is known to reduce ER stress and increase ER adaptive capacity, is a lipogenic transcription factor (24). Considering this discrep-
ancy and also taking into account that XBP1s activity is reduced in mouse models of obesity and NAFLD (13), we sought to investigate the role of XBP1s in the development of NAFLD.

**Results**

**XBP1s Reduces Hepatosteatosis in DIO Mice**—To investigate the effect of XBP1s on hepatic steatosis, first C57Bl/6J mice were fed an HFD (45% kcal) for 12 weeks and injected with adLacZ, adXBP1s, or ΔDBD-XBP1s (8*10E7 pfu/g) through the tail vein. A, blood glucose levels were assessed after 6 h of fasting on post-adenovirus injection day 9. B, a GTT with 1 g/kg glucose (i/p. injection) was performed on day 5 post-adenovirus injection. C, an ITT was performed on day 7 post-adenovirus injection by intraperitoneal insulin (0.75 IU/kg) injection. D, plasma insulin levels after 6-h fasting on day 9 post-adenovirus injection. E, homeostatic model assessment for insulin resistance as calculated from 6-h-fasted glucose levels and corresponding plasma insulin levels on day 9 post-adenovirus injection. F, average daily food intake (g/mouse/day) and G, body weight (g), H, relative hepatic mRNA levels of Xbp1s, Srebp1c, FasN, Acc1, Acc2, and Scd1 in livers of adLacZ and adXBP1s-injected mice. Gene expression was normalized using the 18s gene as a housekeeping gene. I and J, liver triglyceride content (I) and plasma triglyceride levels (J) were determined in 6 h-fasted mice on post-injection day 9. K, immunoblotting of XBP1s and FoxO1 in the nucleus and FasN in total homogenates of livers of LacZ and XBP1s-expressing mice. NUP98 was used as a loading control for nuclear lysates, whereas Tubulin served as a loading control for total liver lysates. Error bars are represented as mean ± S.E., and *p values were determined by Student’s t test or two-way analysis of variance testing with Bonferroni post-test for multiple comparison analysis (for GTT and ITT). *, p < 0.05; **, p < 0.01; ***, p < 0.001.
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improved in XBP1s-injected mice (Fig. 1C). Circulating plasma insulin levels were significantly lower than those of the control group (Fig. 1D), and homeostatic model assessment for insulin resistance was reduced by $>60\%$ (Fig. 1E) in adXBP1s-injected mice compared with the controls. Notably, these beneficial changes in glucose homeostasis were established independent of changes in food intake and body weight (Fig. 1, F and G). These results indicate that, in addition to the genetically obese and diabetic ob/ob mice (13–16), reinstatement of XBP1s action in the liver of DIO mice significantly improves glucose homeostasis.

We then assessed the parameters of hepatic lipid metabolism in XBP1s-overexpressing mice versus the controls. Surprisingly, expression of key lipogenic genes, including sterol regulatory element-binding protein 1c (Srebp1c), fatty acid synthase (Fasn), and stearoyl-CoA desaturase 1 (Scd1), were significantly reduced in the livers of adXBP1s-injected mice compared with the adLacZ-injected controls (Fig. 1H). Quantitative analysis revealed a 30% reduction in TG content in livers of XBP1s-injected mice compared with LacZ-injected controls (Fig. 1I). XBP1s expression, however, led to a significant increase in circulating TG levels (Fig. 1I), probably because of resolution of lipids from the liver to the circulation. Plasma and hepatic cholesterol levels were unaffected by XBP1s expression (Fig. S1, A and B). Protein levels of fatty acid synthase, a central enzyme in de novo lipogenesis, were significantly reduced (Fig. 1K). In line with our previous report in ob/ob mice (16), XBP1s expression led to exclusion of FoxO1 from the nucleus in the DIO model too (Fig. 1K).

XBP1s Reduces Hepatic Diacylglycerol Levels and Inhibits PKCe Translocation—TG in the liver are primarily formed through esterification of fatty acids (25). Diacylglycerol (DAG), which consists of a glycerol backbone with two fatty acyl groups, is an intermediate of the TG synthetic pathway and a DAG precursor molecule. In addition, DAG is a potent second messenger signaling molecule with the potential to activate PKC family members (26). Although, in obesity and NAFLD, many lipid species accumulate ectopically (e.g., in the liver), there has been specific attention on hepatic DAG accumulation and intracellular DAG localization following reports that DAGs can directly facilitate insulin resistance in NAFLD by activating the novel PKC isoform $\epsilon$ (PKCe) (27–29). DAG-activated PKCe subsequently binds to the insulin receptor, thereby preventing downstream insulin-mediated signaling. Among the PKC isoforms, PKCe has been shown to be highly increased in NAFLD and to correlate strongly with hepatic DAG content both in rodents (26, 28) and humans (30). Furthermore, intracellular DAG levels and subsequent activation of PKCe have been put forward as reliable predictors of insulin resistance in fatty livers of obese patients (30, 31).

Considering that TG content was significantly reduced in the livers of adXBP1s-injected DIO mice, we investigated whether XBP1s could also reduce hepatic DAG levels. For this purpose, we first analyzed total DAG levels in the livers of adXBP1s- and adLacZ-injected DIO mice. We found that XBP1s expression led to a slight but not significant ($p = 0.099$) decrease in total hepatic DAG levels (Fig. 2A). However, when the DAG content of hepatic cytosolic and membrane compartments was analyzed, we observed a significant reduction in DAG levels in the cytosolic compartment of the livers of XBP1s-injected mice (Fig. 2A).

Although gene expression levels of Pkce in livers of adXBP1s-injected mice were reduced compared with controls (Fig. 2B), total PKCe protein levels remained unaffected (Fig. 2C). Cellular localization of PKCe, which can be analyzed by the ratio of cytosolic to membrane PKCe, is an important determinant for PKCe activity (32, 33). Thus, we next analyzed PKCe levels in cytosolic and membrane compartments of livers of adXBP1s- and adLacZ-injected mice. Although PKCe levels were similar in cytosolic compartments of livers of adXBP1s-injected mice and their controls (Fig. 2D), the membrane content was significantly reduced in adXBP1s-injected mice (Fig. 2D). Therefore, the ratio of membrane/cytosolic PKCe was significantly reduced in adXBP1s-injected mice (Fig. 2E), indicating a decrease in PKCe activity.

Next we analyzed the levels of 13 different DAG species both in the cytosolic and membrane compartments by using LC/MS/MS in adLacZ- and adXBP1s-injected DIO mice. 10 of 13 analyzed DAG species were decreased in the cytosolic fraction of livers of XBP1s-expressing mice (Fig. 2F). 4 of 13 analyzed membrane DAG species were moderately but significantly increased in livers of XBP1s-expressing mice (Fig. 2G). Total ceramide levels remained unaffected; however, we observed differential regulation of various ceramide species in livers of XBP1s-expressing mice (Fig. S2, A and B).

These results establish a novel role for XBP1s in the regulation of hepatic DAG levels and PKCe activation. In addition, the results are suggestive of a regulatory role of XBP1s in glucose homeostasis through inhibition of PKCe activity as a result of reduced DAG content.

XBP1s Reduces the Hepatic Fatty Acid Synthesis Rate and Increases Macrolipophagy in ob/ob Mice—We next investigated whether the effects of XBP1s on the lipogenic gene expression profile seen in the DIO mouse model could be reproduced in obese and insulin-resistant ob/ob mice, which have severe accumulation of lipids in the liver. For this purpose, we injected adXBP1s or adLacZ into the tail vein of 10-week-old ob/ob mice. On post-injection day 9, we sacrificed the mice and extracted the livers for further investigation. Quantitative PCR analysis revealed significant reductions in the expression of lipogenic genes such as Acc1, Acc2, and Fasn (Scd1 levels were reduced but without reaching statistical difference) in XBP1s-expressing ob/ob mice compared with the controls (Fig. 3A).

The above findings indicate that XBP1s is able to reduce lipogenic gene expression in a diet-induced and a genetic model of obesity and insulin resistance in mice. Most of the effects of XBP1s on metabolic homeostasis have been attributed to the ability of XBP1s to act as a transcription factor by directly binding to the DNA, thereby regulating gene expression. To assess whether the DNA-binding capacity of XBP1s was a prerequisite to mediate the reduction in lipogenic gene expression, we tested whether a DNA-binding-defective mutant XBP1s (ADBD-XBP1s) could recapitulate the effects on lipogenic gene expression. Using an adenovirus-mediated approach, we expressed LacZ, wild-type XBP1s, and ADDBD-XBP1s, in which
the DNA-binding domain was replaced with an artificial nuclear localization signal (16), in the liver of ob/ob mice. As anticipated, expression of wild-type XBP1s led to a significant induction of XBP1s-mediated UPR genes such as Erdj4, Grp58, and Grp78 (Fig. S3). However, as we reported previously, ΔDBD-XBP1s was unable to increase expression of these genes.

FIGURE 2. XBP1s reduces hepatic diacylglycerol content and PKC activity. C57Bl6/J mice were fed an HFD (45% kcal) for 12 weeks (starting at 3 weeks of age) and injected with adLacZ or adXBP1s (8*10^7 pfu/g) through the tail vein. A, DAG content was determined in cytosolic (black columns) and membrane (gray columns) compartments in livers of 6 h-fasted mice on post-injection day 9. Total DAG content was calculated from the sum of cytosolic and membrane DAG content. B, relative mRNA levels of Pkrc. Gene expression was normalized using the 18s gene as a housekeeping gene. C, Western blotting analysis of PKC protein in whole liver homogenates. Tubulin was used as a loading control. D, PKC protein levels were determined in the cytosol (top panel) and membrane (bottom panel) compartments of livers of 6 h-fasted mice 9 days post-injection. GAPDH was used as a loading control for cytosolic lysate, whereas NaATPase served as a loading control for the membrane lysates. E, PKC protein expression was quantified using densitometric analysis of the autoradiographical signal. The membrane-to-cytosol (M/C) ratio as measure of PKC activity is expressed in the graph. Hepatic DAG species were analyzed in the cytosol (F) and membrane (G) compartments of livers of adLacZ- and adXBP1s-expressing mice. Error bars are represented as mean ± S.E.; p values were determined by Student's t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant.
In contrast to the chaperone expression profile, expression of lipogenic genes was reduced to the same extent in adXBP1s- and ΔDBD-expressing mice. This implies that the XBP1s-mediated reduction in lipogenic gene expression is independent of its DNA-binding capacity. We have shown previously that XBP1s, independent of its DNA-binding capacity, can directly interact with FoxO1 and promote its degradation in the 26S proteasome (16). We therefore postulate that the effect of XBP1s on lipogenic gene expression probably relies on direct interaction of XBP1s with lipogenic gene regulatory protein and its inhibition.

To further investigate the hepatic lipid phenotype in mice expressing adXBP1s, we next performed H&E staining to determine the histopathological changes in the livers. As shown in Fig. 3B, H&E staining documented that XBP1s reduced the number and size of the lipid droplets in the livers of XBP1s-expressing ob/ob mice compared with the controls. Quantitative analysis of hepatic TG content revealed a close to significant reduction in the livers of ob/ob mice expressing XBP1s (Fig. 3C).

Next we performed an in vivo lipogenesis assay to assess the hepatic fatty acid synthesis rate. For this, adXBP1s- and
adLacZ-injected mice were injected i.p. with tritiated water (3H2O) as a chaser on post-injection day 5. Livers were excised for analysis of newly synthesized fatty acids 1 h after the injection. The results indicated that the hepatic fatty acid synthesis rate in adXBP1s-injected mice was significantly (40%) lower compared with the controls (Fig. 3D). XBP1s expression also increased plasma TG levels in ob/ob mice (Fig. 3E).

Based on the suggestive reduction in lipid droplet size observed in H&E-stained livers of XBP1s-injected mice, we decided to further investigate the lipid droplet morphology. Using an electron microscopy approach, we observed increased double membrane structures in the lipid droplets that were suggestive of autophagosome formation in adXBP1s-injected ob/ob mice compared with those in the control group (Fig. 3F). Degradation of lipid droplets through autophagy (also termed macrolipophagy) has been suggested as an important process in regulation of hepatic lipid metabolism (34). To investigate whether macrolipophagy was indeed increased in the livers of XBP1s-expressing mice, we first determined hepatic protein levels of autophagy gene 5 (ATG5), a protein with a crucial role in early-stage autophagosome formation and thus in lipid droplet breakdown. ATG5 levels were markedly increased in the livers of ob/ob mice expressing XBP1s compared with LacZ controls, which indicates increased autophagosome formation (Fig. 3G). During autophagy, the cytosolic form of microtubule-associated protein 1A/1B light chain 3 (LC3BI) is conjugated to phosphatidylethanolamine to form LC3BII, which is subsequently recruited to autophagosomal membranes. LC3BII subsequently mediates the degradation of autophagosomal content. To determine the level of LC3BII associated with lipid droplets, we isolated hepatic lipid droplets and analyzed LC3BII levels in this compartment. Lipid droplet-associated LC3BII was significantly increased in the livers of adXBP1s-injected ob/ob mice (Fig. 3H), which indicates enhanced autophagosome formation. These results suggest that XBP1s, in addition to reducing the hepatic lipogenic rate, enhances breakdown of the lipid droplets by promoting autophagy.

**XBP1s Reduces Hepatic Lipogenic Gene Expression in Fructose-fed Mice**—Our results indicate that XBP1s lowers hepatic lipogenic gene expression in DIO and ob/ob mice and exerts anti-lipogenic effects. These results contradict the conclusions from a previous study (24), which concluded that XBP1s increases lipogenesis in mice fed a highly lipogenic fructose diet. To assess the effect of XBP1s expression in a setting with a high lipogenic rate without the confounding effects of obesity and insulin resistance, we fed mice a 60% fructose diet for 4 weeks. As expected, high-fructose feeding significantly induced expression of lipogenic genes (Fig. S4A). Nevertheless, we did not observe the previously reported increase in Xbp1s gene expression levels in fructose-fed mice compared with chow-fed mice (24). After 4 weeks of fructose feeding, mice were injected with adXBP1s or adLacZ. XBP1s expression significantly reduced expression of the key lipogenic genes Srebp1c, FasN, and Scd1 and led to a non-significant reduction in Acc1 and Acc2 (Fig. 4A). However, in contrast to DIO and ob/ob mouse models, XBP1s overexpression in this setting did not reduce hepatic TG content (Fig. 4B). Notably, we again observed increased plasma TG levels in XBP1s-expressing, fructose-fed
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mice compared with the controls (Fig. 4C). Additionally, XBP1s-expressing mice had a significant reduction in blood glucose levels in this setting too (Fig. 4D). These alterations were achieved without changes in food intake or body weight (Fig. 4, E and F). Liver and plasma hepatic cholesterol levels were unaffected by XBP1s expression in high fructose-fed mice (Fig. S4, B and C).

Discussion

NAFLD is one of the most serious pathologies of obesity, and it is currently the most common liver disease worldwide (35, 36). There is a strong association between obesity and NAFLD, and around 90% of obese, type 2 diabetic patients are diagnosed with NAFLD (37). NAFLD and type 2 diabetes increase morbidity and mortality rates in the obese population and create a serious economic burden on affected societies (38–40). NAFLD significantly increases the incidence of liver cirrhosis and hepatocellular carcinoma in the obese population (41). Despite continuing efforts in the field, the available treatment regimens can only minimally reduce NAFLD, and better treatment modalities have not been developed because of the fact that the underlying molecular pathologies are poorly understood. Identifying the molecular mechanisms that lead to NAFLD is therefore of great importance.

We (14–16) and others (42, 43) have previously identified XBP1s as a crucial regulator of glucose homeostasis in obesity. XBP1s action is severely impaired in mouse models of obesity (13), and this relative “XBP1s deficiency” contributes to the development of sustained ER stress, pathological activation of UPR signaling (13), and, ultimately, to disturbed glucose homeostasis. Reducing ER stress with chemical chaperones in vivo or by reinstating XBP1s action reduces ER stress and improves glucose intolerance and insulin resistance (14, 15, 19, 20).

Increased ER stress in obesity has also been implicated in the development of hepatosteatosis (21, 22). Despite several previous reports indicating that a homeostatic ER condition is critical for the maintenance of hepatic lipid homeostasis in obesity (19–23) and that reduction of ER stress is an anti-lipogenic modification, XBP1s, which probably has the strongest capacity to reduce ER stress, was suggested as a lipogenic factor (24). These postulates created a serious dilemma in the field and scrutinized the possibility of development of therapeutic approaches to reactivate XBP1s in the liver to treat obesity-associated insulin resistance and type 2 diabetes.

Here we document that, contrary to previous suggestion, XBP1s is an anti-lipogenic protein and that increasing XBP1s activity reduces hepatic TG content in two different and relevant mouse models of obesity and NAFLD. Interestingly, the anti-lipogenic activity of XBP1s is mediated independently of its transcriptional activity. These results indicate that XBP1s creates the anti-lipogenic effect through a protein-protein interaction. It will require further research to unravel the underlying molecular mechanism of the anti-lipogenic activity of XBP1s.

Fatty liver results from increased de novo synthesis and uptake of fatty acids, which are subsequently esterified in TG, and reduced TG catabolism because of reduced lipolysis and export of TG to the circulation (9). XBP1s consistently induced plasma TG levels in all mouse models described in this manuscript. This is in line with previous reports showing that depletion of hepatic XBP1s reduced TG secretion (24, 44). We propose that enhanced breakdown of hepatic lipid droplets and reduction in hepatic TG storage leads to redistribution of lipids from the liver to the periphery, thereby contributing to the rise in plasma TG levels in DIO and ob/ob mice.

Macrolipophagy has been suggested previously as an initiating step in lipolysis (34). Indeed, electron microscopy experiments performed in this study also indicate that macrolipophagy is increased in XBP1s-overexpressing mice and are suggestive of increased breakdown of lipid droplets. Biochemical experiments have strengthened this conclusion. Increased ATG5 levels in liver homogenates and increased LC3BII levels in isolated hepatic lipid droplets further confirm that autophagy of lipid droplets was indeed increased in adXBP1s-injected mice. In response to starvation, LC3BII association with lipid droplets and subsequent initiation of macrolipophagy is increased in healthy, lean mice, and this process is blunted in DIO mice (34). Excess nutrient supply, such as prolonged HFD feeding, has been reported to reduce autophagy efficiency, thereby contributing to the development of ER stress and insulin resistance (45–47). The vicious cycle in which increased cellular lipid content impairs macrolipophagy, which then further reduces autophagic function, eventually leads to more lipid retention. It is important to note that XBP1s activity is severely reduced in the liver of obese mice (13), where there is pronounced NAFLD, compared with lean mice. Our results indicate that increased XBP1s activity interrupts this defective macrolipophagy cycle by reinstating macrolipophagic activity and potentially increasing fatty acid availability for redistribution to the periphery, as shown by increased plasma TG levels in adXBP1s-injected mice. Taken together, the electron microscopy and biochemical results obtained from analysis of ATG5 and LC3BII levels strongly support that XBP1s promotes lipid droplet breakdown in the liver.

In addition to lowering hepatic TG levels, XBP1s reduced hepatic DAG content in DIO mice. DAGs are second messengers that activate members of the novel PKC family of proteins (26). Intracellular compartmentalization of DAGs has been indicated to play a critical role in activation of PKCe (27–29, 32, 33). We report that DAGs are specifically reduced in the cytoplasmic compartment of livers of XBP1s-expressing mice. The concomitant reduction in PKCe activation suggests that XBP1s-mediated reduction in cytoplasmic DAGs impairs PKCe activation. PKCe has been reported to be enhanced in fatty livers of insulin-resistant rodents (28) and humans (30). Reduction of PKCe activity following reactivation of XBP1s is a further support for the anti-lipogenic actions of this transcription factor.

In humans, increased levels of hepatic DAG and PKCe protein levels have been associated with development of insulin resistance (30, 31). PKCe directly interacts with and inhibits insulin receptor kinase activity, and depletion of hepatic PKCe improves hepatic insulin action in HFD-fed rats (28). Pkcre−/− mice are resistant to HFD-induced insulin resistance (48), which further provides evidence for a link between PKCe action and development of insulin resistance. Reducing
PKCe activation, as indicated by reduced cytoplasm-to-membrane translocation, is a novel mechanism for XBP1s to increase insulin sensitivity and thereby contribute to improved glucose homeostasis.

In fructose-fed mice, however, hepatic TG content remained unaffected by XBP1s expression despite significantly reduced lipogenic gene expression and enhanced plasma TG levels. It is interesting to note that we did not observe any alterations in Xbp1s levels during the high-fructose diet feeding, which indicates that isolated high-fructose diet feeding and development of fatty liver in this setting might not be associated with ER stress and XBP1s. Furthermore, the underlying core mechanism(s) for development of NAFLD under isolated high-fructose diet feeding could be overcoming the anti-lipogenic effects of XBP1s, even if XBP1s is exogenously activated in this setting. Thus, we conclude that lack of ER stress, obesity, and insulin resistance in fructose-fed mice versus DIO and ob/ob mice explains the lack of effect of XBP1s in this model.

Collectively, our results indicate that XBP1s is an anti-lipogenic protein and that hepatic reactivation of XBP1s in obesity does not create fatty liver but, on the contrary, reduces NAFLD. Therefore, it is interesting to note that we did not observe any alterations in lipogenic gene expression and enhanced plasma TG levels. It is unaffected by XBP1s expression despite significantly reduced lipogenic gene expression and enhanced plasma TG levels.

**Experimental Procedures**

**Reagents**—XBP1, PKCe, GAPDH, ADRP, and HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FoxO1, NUP98, FasN, LC3B, NaATPase, and α-tubulin antibodies were from Cell Signaling Technology (Beverley, MA). ATG5 antibody was from Novus Biologicals (Littleton, CO). Free glyceral reagent and the serum triglyceride determination kit were from Sigma-Aldrich (St. Louis, MO). Infinity total cholesterol reagent was from Fisher Scientific (Waltham, MA). The detergent-compatible protein assay kit, SYBR Green Supermix, and cDNA synthesis kit were from Bio-Rad. BM chemiluminescence blotting substrate was from Roche. The ultra-sensitive detergent-compatible protein assay kit, SYBR Green Supermix, and cDNA synthesis kit were from Bio-Rad. BM chemiluminescence blotting substrate was from Roche. The ultra-sensitive mouse insulin ELISA kit was from Crystal Chem.

**Adenovirus Production and Injection**—Adenovirus was generated using the ViraPower adenoviral expression system (Invitrogen) as described previously (13). Crude adenovirus was amplified, CsCl-purified, and titrated by Vector Biolabs (Malvern, PA). Prior to injection, the adenovirus was thawed at room temperature, and injection solutions were prepared in 100 μl of sterile NaCl (8*10E7 pfu/g). Mice were restrained in a restrainer, and adenovirus was injected through the tail vein using a 28-gauge needle. Mild pressure was applied on the site of injection to prevent backflow of the injected solution.

**Animals and Diets**—Wild-type C57BL6/J and leptin-deficient ob/ob mice (on a C57BL6/J background) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a temperature-controlled, air-conditioned, and specific pathogen-free animal facility at Boston Children’s Hospital. Mice were housed in groups (5 mice/cage) and maintained on a 12:12-h light/dark cycle (lights on at 7 a.m.). Diet-induced obesity was established by feeding wild-type mice a high-fat diet (HFD) (45% kcal, Research Diets Inc., New Brunswick, NJ) for 12 weeks prior to starting the experiments. A fructose diet (60% kcal, TD 00202, Teklad, Harlan Laboratories, Madison, WI) was provided for 4 weeks prior to starting the experiments. The HFD and especially the fructose diet are hypoglycemic and were therefore fully replaced every week. Ob/ob mice were fed standard laboratory chow. Mice had ad libitum access to food and water. All animal experiments were approved by the Animal Care and Use Committee of Boston Children’s Hospital.

**Glucose and Insulin Tolerance Test**—For glucose tolerance testing, mice were fasted overnight (7 p.m. to 8 a.m.). Blood glucose was measured from the tail vein prior to an i.p. injection of 1 g/kg of glucose in 200 μl of sterile NaCl. Glucose levels were measured from the tip of the tail using a glucometer (Bayer) at indicated time points. For insulin tolerance testing, food was removed for 6 h (8 a.m. to 2 p.m.). Blood glucose was measured from the tip of the tail prior to an i.p. injection of 0.75 IU/kg insulin (recombinant human insulin, Eli Lilly) in 200 μl of sterile NaCl. Glucose levels were measured from the tip of the tail at indicated time points.

**Blood and Tissue Collection**—Mice were terminated by cardiac puncture under isoflurane anesthesia after a 6-h fast (8 a.m. to 2 p.m.). Blood was collected in heparin-coated tubes and kept on ice until centrifugation (8000 × g, 4 °C, 20 min). Plasma was aliquoted and used for analysis immediately or stored at −80 °C. Tissues were quickly excised and snap-frozen in liquid nitrogen or fixed for microscopic analysis.

**Electron Microscopy**—Fresh liver was cut and immediately fixed in 2.5% glutaraldehyde-buffered solution at room temperature for 2 h and then at 4 °C overnight. After washing with PBS three times, tissues were post-fixed in 2% OsO4, dehydrated, and infiltrated. Samples were sectioned and photographed at the Electron Microscopy/Plastic Core at the Joslin Diabetes Center (Boston, MA).

**H&E Staining**—Fresh liver was cut and immediately fixed in 10% buffered formalin at 4 °C overnight. Tissues were paraffin-embedded, cut, and mounted onto glass slides prior to H&E staining according to the standard protocol of the Rodent Histopathology Core Facility of Harvard Medical School (Boston, MA).

**Tissue Lysis and Western Blotting**—Liver tissue (~75 mg) was homogenized in ice-cold lysis buffer (25 mM Tris–HCl (pH 7.4), 100 mM NaF, 50 mM Na2PO4, 10 mM Na3VO4, 10 mM EGTA, 10 mM EDTA, 1% Nonidet P-40, 2 mM PMSE, and protease inhibitor mixture) using a TissueLyserII (Qiagen). Homogenates were incubated on ice for 20 min and centrifuged at 16,100 × g at 4 °C for 1 h. The lipid layer was carefully removed, and supernatants were centrifuged for another hour (16,100 × g, 4 °C). Samples were denatured in 1:10 Laemmli buffer by boiling at 100 °C for 5 min. Nuclear proteins were extracted from 100 μg of liver tissue using a commercially available kit (NET-ER nuclear and cytoplasmic extraction kit, Fisher Scientific) according to the protocol of the manufacturer. Equivalent amounts of proteins from each sample were resolved by SDS-PAGE and then electrotransferred onto PVDF membranes.

The membranes were blocked in 10% blocking buffer provided with BM chemiluminescence bloting substrate (peroxidase) in TBS (pH 7.4) for 1 h at room temperature. The membranes were subsequently incubated with primary antibody in
for 1 h, pellets were washed with 10 volumes of acetone, 10 volumes of 1:1 acetone/ether, and 10 volumes of ether sequentially. Dried pellets were thoroughly resuspended in sample buffer (130 mM Tris-HCl (pH 6.8), 4% SDS, and 4% 2-mercaptoethanol) and incubated at 60 °C for 5 h in a sonication water bath. Dissolved samples were centrifuged at 18,500 × g at room temperature for 10 min, and supernatants were analyzed by Western blotting.

Reverse Transcription and Quantitative Real-time PCR—Total RNA was extracted using QIAzol reagent (Qiagen, Valencia, CA). 1 μg of RNA was reverse-transcribed to cDNA using a cDNA synthesis kit (Bio-Rad) under the following conditions: 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min. Transcripts were diluted 20 times, and QPCR analysis was performed using 4 μl of cDNA. Gene expression was analyzed using SYBR Green Supermix (Bio-Rad) on an iQ5 multicolor real-time PCR detection system (Bio-Rad) using the following primers: 18s rRNA forward, 5′-AGT CCC TGC CCT TTG TAC ACA-3′; 18s rRNA reverse, 5′-CGA TCC GAG GGC CTC ACT A-3′; Xbp1s forward, 5′-GGT CTG CTG AGT CCG CAG CAG CAG G-3′; Xbp1s reverse, 5′-AGG CTT GGT GTA TAC ATG Grp78 forward, 5′-GAG CTT GGT GTA TAC ATG Grp78 reverse, 5′-TCC CAG GAG TGT CTT TGG A-3′; Fasn forward, 5′-GGG TCT CCT GCA TTA CCA GCA GT-3′; Srebplc forward, 5′-CGG GTT GCC ACA ACG CTT-3′; Srebplc reverse, 5′-GGG CCT CCT GCC ATC AG-3′; Acc2 for- ward, 5′-ATC TCT GCA GTG GGA GCA-3′; Acc2 reverse, 5′-TTC CTG GCC ACT TGC CTT GAC GTG GT-3′; Xbp1s forward, 5′-ATG GCC CAC CCC AGA GCT A-3′; Xbp1s reverse, 5′-CCC CCT CCT TCA ATG TGC T-3′; Cac2 for- ward, 5′-GTC CCT CCT GCA TGA CAA C-3′; Cac2 reverse, 5′-TCC CAG GAG TGT CTT TGG A-3′; Srebplc forward, 5′-GGG CTT GGT GTA TAC ATG Grp78 forward, 5′-GAA ATG GGA GCA GGT TGT CTT GAC GT-3′; Srebplc reverse, 5′-TTC ATG GGA GCC CAC CCA GCA CAG C-3′; Scd1 forward, 5′-AGA TCT CCA GGT CTT ACC ACA CCA CCA C-3′; Scd1 reverse, 5′-GAG CTA GGT TTT CTT CCA GGT G-3′; Dgat2 forward, 5′-TTC CTG GCA TAA GGC CCT CTT AAT-3′; Dgat2 reverse, 5′-ATG CTA TGG TGT CTC TGC TGA TGT C-3′; Erdj4 for- ward, 5′-GCA ATG GGA GCA GGT CTT TGT AA-3′; Erdj4 reverse, 5′-TTC GGT GGT GCA CCT GTG GTG GT-3′; Grp58 for- ward, 5′-TAT GAT GGA CCTA GGA GGT CTT C-3′; Grp58 reverse, 5′-TGC TGG CTG CCT TTA GGA AT-3′; Grp78 for- ward, 5′-AGT GGT GGC CAC TAA TGG AG-3′; Grp78 reverse, 5′-CAA TCC CCTT GGT GCT GAT G-3′.

Statistical Analysis—Data are presented as mean ± S.E. Statistical significance was calculated using two-tailed Student’s t test or two-way analysis of variance testing with Bonferroni post-test for multiple-comparison analysis (for GTT and ITT) and was accepted at *p < 0.05; **p < 0.01; and ***p < 0.001.

Author Contributions—Conceptualization, H. H. and U. O.; Methodology, H. H., Y. Z., G. I. S., and U. O.; Investigation, H. H., Y. Z., D. Z., J. L., and M. A. S. H.; Writing—Original Draft, H. H. and U. O.; Writing—Review & Editing, H. H., G. I. S., and U. O.; Supervision, H. H. and U. O.; Funding Acquisition, U. O.

Acknowledgments—We thank Drs. Jonathon N. Winnay and C. Ronald Kahn (Joslin Diabetes Center, Harvard Medical School) for assistance with the in vivo fatty acid synthesis assay.
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