Function of inhibitor of Bruton’s tyrosine kinase isoform α (IBTKα) in nonalcoholic steatohepatitis links autophagy and the unfolded protein response

Nonalcoholic fatty liver disease (steatosis) is the most prevalent liver disease in the Western world. One of the advanced pathologies is nonalcoholic steatohepatitis (NASH), which is associated with induction of the unfolded protein response (UPR) and disruption of autophagic flux. However, the mechanisms by which these processes contribute to the pathogenesis of human diseases are unclear. Herein, we identify the α isoform of the inhibitor of Bruton’s tyrosine kinase (IBTKα) as a member of the UPR, whose expression is preferentially translated during endoplasmic reticulum (ER) stress. We found that IBTKα is located in the ER and associates with proteins LC3b, SEC16A, and SEC31A and plays a previously unrecognized role in phagophore initiation from ER exit sites. Depletion of IBTKα helps prevent accumulation of autophagosome intermediates stemming from exposure to saturated free fatty acids and rescues hepatocytes from death. Of note, induction of IBTKα and the UPR, along with inhibition of autophagic flux, was associated with progression from steatosis to NASH in liver biopsies. These results indicate a function for IBTKα in NASH that links autophagy with activation of the UPR.

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent liver disease in the Western world, and its associated pathologies range from simple steatosis to nonalcoholic steatohepatitis (NASH); hallmarks of NASH include hepatic inflammation, increased liver enzymes, and fibrosis that increases the risk of progression to cirrhosis, liver failure, and hepatocellular carcinoma (1–3). The liver plays a central role in metabolizing and processing fatty acids for export as complex lipids to the adipose tissue for storage; however, metabolic diseases such as obesity and insulin resistance trigger adipocytes to release excess levels of circulating free fatty acids (FFAs) into the serum. Elevated levels of saturated FFAs are suggested to cause lipotoxicity in peripheral tissues, including liver, activating cellular stress pathways, which can in turn lead to increased inflammation and hepatocyte death (4, 5).

Saturated FFAs can induce the unfolded protein response (UPR), allowing for changes in both transcriptional and translational modes of gene expression that can serve to alleviate stresses afflicting the endoplasmic reticulum (ER) (6, 7). The UPR is composed of sensor proteins for ER stress, including PERK (EIF2AK3/PEK) that phosphorylates the α subunit of eIF2 (eIF2α-P), which represses global translation initiation and lowers the influx of nascent polypeptides into the stressed ER (8). Additionally, eIF2α-P leads to preferential translation of key UPR genes that function in stress adaptation (9). Recently, we showed that eIF2α-P by PERK results in preferential translation of the transcription factor CHOP (GADD153/DDIT3), which plays a central role in saturated FFA-induced lipotoxicity and hepatic inflammation (10). Increased CHOP expression triggers a signaling pathway that induces NF-κB-directed transcriptional expression and consequent secretion of pro-inflammatory cytokines, such as IL-8 and TNFα, providing a linkage between the UPR and inflammation to disease progression (6, 7, 10). Although activation of CHOP by saturated FFAs is suggested to play a direct role in both the hepatotoxicity and inflammation observed during the progression of NASH, the underlying mechanisms of hepatocellular death remain unclear.

Recent literature has shown that autophagy is associated with the progression of NASH (11–14). Autophagy requires formation of double membrane vesicles that carry cytoplasmic cargo to the lysosome for degradation (15). Hepatic autophagy was reported to be impaired in NASH patients, as well as mice that displayed liver steatosis due to a high fat diet (11, 12). Of interest, although shorter term treatment of cultured human hepatocytes with the saturated FFA palmitate triggered autophagy, prolonged exposure led to a block in autophagic flux and cell death (11). It was reasoned that the block in autophagic flux contributes to the sensitivity of hepatocytes to prolonged...
exposure to saturated FFA, and it was reported that loss of CHOP function lowered the blockage of autophagic flux triggered by saturated FFA and protected hepatocytes from cell death (11). CHOP as well as its upstream effector—transcription factor ATF4 are suggested to contribute to the expression of select genes implicated in the formation and function of autophagosomes (16–18), which suggests that a blockage in autophagic flux rather than lowering of autophagy per se is a critical contributor to hepatotoxicity by saturated FFAs. A role for the UPR in the initiation of autophagy is also supported by the finding that substitutions in eIF2α that block phosphorylation by PERK result in defective lipiddation of LC3b and a consequent block in phagophore initiation (19). This finding suggests that there may be additional unknown direct translational target(s) of eIF2α-P that are essential for phagophore initiation during stress. The underlying mechanisms by which PERK and the UPR contribute to induction of autophagy will be a central focus of this study.

We recently identified the α isoform of inhibitor of Bruton’s tyrosine kinase (IBTKα) as being preferentially translated in response to eIF2α-P and ER stress (20). Although the biological functions of IBTKα are not yet understood, it is noted that IBTKα contains protein–protein interaction domains, including ankyrin repeats and the BTB/POZ domain, which is suggested to enable IBTKα to serve as a substrate adapter for the E3 ubiquitin ligase CUL3 (21, 22). In this study, we show that IBTKα expression is rapidly induced by PERK upon exposure to saturated FFA and that the ensuing activation of IBTKα plays an essential role in phagophore initiation by IBTKα assembly into a multisubunit complex with LC3b, SEC16A, and SEC31A at the endoplasmic reticulum exit site (ERES). We also show that depletion of IBTKα results in impaired autophagy, along with reduced protein secretion, resulting in suppression of FFA-induced hepatocyte cell death. Finally, we use human liver biopsy samples to demonstrate that the UPR-inflammation signaling axis and increased IBTKα expression are associated with progression from steatosis to NASH, thereby linking the UPR, inflammation, and inhibition of autophagic flux to disease pathogenesis in humans.

**Results**

**IBTKα is a novel UPR member induced by saturated FFAs**

To determine whether IBTKα is preferentially translated in human hepatocytes following metabolic stress, we treated human hepatoma HepG2 cells with palmitate or thapsigargin, a pharmacological agent that potently induces ER stress. Following 6 h of treatment, we performed polysome profiling (Fig. 1A). Both thapsigargin and palmitate resulted in a reduction of heavy polysomes coincident with accumulation of monosomes, indicative of lowered global translation initiation compared with vehicle treatment. IBTKα mRNA, as well as those encoding preferentially translated controls ATF4 and CHOP, was then measured by comparing the percent of each gene transcript in the gradient fractions (Fig. 1, B and C). After either stress treatment, there was a significant shift of IBTKα mRNA toward large polysomes compared with vehicle, similar to the expected increase in the ATF4 and CHOP transcripts. Interestingly, IBTKα was present in the heaviest polysomes fractions 6 and 7 after either thapsigargin or palmitate treatment, whereas ATF4 and CHOP were predominantly in polysome fractions 4–6. This shift of the IBTKα mRNA to the heaviest polysome fractions is consistent with the fact that IBTKα has a longer coding sequence that can accommodate more translating ribosomes compared with ATF4 and CHOP. HepG2 cells deleted for PERK (PERK-KO) by using CRISPR/Cas9 retained high levels of translation as viewed by heavy polysomes independent of stress (Fig. 1, D and E) and showed only modest changes in fraction distributions of IBTKα, ATF4, or CHOP mRNAs (Fig. 1, F and G). We conclude that PERK is required for repression of global protein synthesis, coincident with preferential translation of IBTKα and UPR members in human hepatocytes in response to ER stress triggered by lipotoxicity.

PERK and its downstream effector CHOP also trigger transcriptional expression of UPR target genes to alleviate stress or activate inflammation (8, 23). To determine whether IBTKα expression was also regulated at the transcriptional level during treatment with palmitate, we generated CHOP knock-out (CHOP-KO) HepG2 cells and exposed these cells along with their wild-type (WT) counterparts to saturated FFAs or vehicle (Fig. 1, H and I). IBTKα mRNA and protein were induced only in WT HepG2 cells treated with palmitate, whereas basal levels remained unchanged between WT and CHOP-KO cells. These results indicate that PERK activation and its downstream effector CHOP are also required for induced IBTKα mRNA expression in the UPR.

**Saturated FFAs induce cell death through inhibition of autophagic flux**

Although it was suggested that both apoptosis and autophagy are associated with lipotoxicity during NASH (5, 13, 24), it is unclear whether either of these pathways play a direct role in hepatocyte death. To determine whether apoptotic pathways are involved, we investigated the role of caspases in hepatocyte death during lipotoxicity by treating HepG2 cells with saturated and unsaturated FFAs, either alone or in the presence of pan-caspase inhibitor ZVAD/FMK (Fig. 2, A and B). In parallel, we also used controls, including staurosporine, that induce apoptosis and the potent ER stress agent tunicamycin. Treatment with saturated FFAs palmitate and stearate produced a modest increase in caspase activity, but the addition of ZVAD/FMK did not rescue cell death. By contrast, ZVAD/FMK blocked both staurosporine-induced cell death and caspase 3/7 activation. Neither the unsaturated FFA oleate nor the canonical UPR activator tunicamycin resulted in appreciable cell death after 24 h. During apoptosis, nuclear localization of cleaved caspase 3 is essential for breakdown of the nuclear lamina and DNA fragmentation (25). Although cleaved caspase 3 was localized predominantly to the nucleus after staurosporine treatment as judged by immunocytochemistry, total cleaved caspase 3 was reduced and retained in the cytoplasm with the addition of ZVAD/FMK (Fig. 2C). By contrast, the low levels of caspase 3 activation determined during treatment with saturated FFAs were coincident with caspase 3 being retained in the cytoplasm. These results indicate that apoptosis is not the predominant...
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Figure 1. PERK is required for preferential translation of IBTKα during palmitate exposure. A, polysome profiles of lysates prepared from HepG2 cells treated with palmitate, thapsigargin, or vehicle for 6 h. B and C, following polysome analysis, fractions 1–7 were collected, and the percentage of ATF4, CHOP, and IBTKα mRNA in each was quantified by qPCR and shown as a histogram. The percentage of the total gene transcripts in the heavy polysomes (fractions 5–7) for WT HepG2 treated with either palmitate or thapsigargin versus vehicle is indicated for each polysome profile. D and E, polysome profiles of lysates prepared from either WT or PERK-KO HepG2 cells treated for 6 h with palmitate, thapsigargin, or vehicle, as indicated. F and G, following polysome analysis of lysates prepared from PERK-KO HepG2 cells, the percentage of ATF4, CHOP, and IBTKα mRNA distributed in fractions 1–7 was quantified by qPCR and illustrated as histograms. Changes in the percentage of each gene transcript in the heavy polysomes (fractions 5–7) are indicated for the PERK-KO cells that were treated with either palmitate or thapsigargin versus vehicle. H, WT, PERK-KO, or CHOP-KO HepG2 cells were treated with either vehicle (−) or palmitate (+) for 12 h, followed by immunoblot analyses for the indicated proteins. I, WT, PERK-KO, or CHOP-KO HepG2 cells were treated with either vehicle (−) or palmitate (+) for 12 h, followed by qPCR measurements of IBTKα mRNA.

mode of hepatocyte death following exposure to saturated FFAs. Inhibition of autophagic flux is linked with NASH in human patients (11, 26). To determine whether autophagy was associated with activation of the UPR, induction of IBTKα, and cell death, we treated HepG2 cells with saturated or unsaturated FFAs for up to 24 h and measured key markers of UPR activation as well as LC3b and P62/SQSTM1 to assess changes in autophagy (Fig. 2, D and E). Increased levels of ATF4 and IBTKα proteins were observed by 3 h after treatment with palmitate and 6 h of tunicamycin, prior to induction of CHOP. Furthermore, palmitate and stearate triggered accumulation of lipidated LC3b-II and P62, suggesting that saturated FFAs either induced autophagy or, alternatively, blocked autophagic flux. Oleate had only a modest effect on the UPR and did not alter the autophagic markers, whereas treatment with tunicamycin...
mycin led to potent induction of the UPR and increased LC3b-II but produced only a transient increase in P62.

To confirm whether prolonged exposure to saturated FFAs interferes with autophagic flux as reported (11), we expressed N-terminal GFP-LC3b in HepG2 cells and assessed its co-localization with lysosomes by staining with LAMP2 (Fig. 2F). First, we treated the HepG2 cells with chloroquine, which inhibits proteolysis in the lysosome and hence stabilizes LC3b; 75% of GFP-LC3b was co-localized with the lysosomal marker LAMP2, indicating that autophagosomes were properly trafficked to the lysosomes. By contrast, treatment with palmitate disrupted autophagosome trafficking to the lysosome, with
only 10% of GFP-LC3b being co-localized with LAMP2 despite a robust induction of autophagy. These results are consistent with prior reports that prolonged treatment with palmitate leads to a block in autophagic flux, which would impair proper trafficking of damaged macromolecules and organelles to the lysosome for degradation.

Does disruption in the autophagic process play an active role in hepatocyte death upon exposure to saturated FFAs? We treated HepG2 cells with palmitate alone or in combination with agents that disrupt either autophagy initiation (3-methyladenine) or lysosomal function (chloroquine or bafilomycin A1) for 24 h and measured cell death (Fig. 2, G and H). To determine whether this mechanism was relevant to human hepatocytes, we also repeated the experiment with primary human hepatocytes. Addition of 3-methyladenine, which prevents induction of autophagy by inhibiting PI3K, increased the survival of both HepG2 cells and primary human hepatocytes, whereas the lysosome inhibitors resulted in increased death of HepG2 cells. Human primary hepatocytes were more sensitive to palmitate-induced cell death (>80% compared with 30% in HepG2 cells); therefore, adding chloroquine and bafilomycin A1 did not increase cell death further. These results suggest that inhibition of autophagic flux, rather than inhibition of phagophore formation per se, plays an important role in palmitate-induced hepatotoxicity.

**IBTKα is required for autophagosome formation and sensitizes hepatocytes to lipotoxicity**

To explore the hypothesis that IBTKα regulates cell survival by induction of autophagy, we generated IBTKα knockdown (shIBTKα) HepG2 cells using shRNA and measured key protein markers of stress and autophagy, in addition to cell death following palmitate treatment (Fig. 3A). As controls, we also knocked down ATG5, which is important for formation of the autophagosome (27), as well as CHOP that is suggested to contribute to expression of select autophagy genes. Following palmitate exposure, each of the three gene knockdowns resulted in decreased conversion of LC3b-I to LC3b-II and levels of P62 coincident with lowered cell death (Fig. 3, A and B). Of interest, either knockdown of IBTKα or overexpression of IBTKα similarly reduced LC3b lipidation and P62 levels, suggesting that the appropriate amount of IBTKα expressed in cells is critical for its function (Fig. 3C). Equivalent to depletion of IBTKα, overexpression of IBTKα also suppressed the toxicity of palmitate (Fig. 3D).

Earlier, we reported that knockdown of IBTKα in mouse embryonic fibroblasts in the absence of environmental stress and to a lesser extent in HepG2 cells can lead to increased apoptosis (9). In support of this idea, we determined that the small amounts of caspase 3/7 activity measured in HepG2 cells void of stress were modestly elevated by knockdown of IBTKα (supplemental Fig. 1). A similar result was determined for HepG2 cells treated with bafilomycin A1. By contrast, the modest amounts of caspase 3/7 activity measured in HepG2 cells exposed to palmitate (Fig. 2A) were significantly lowered by depletion of IBTKα (supplemental Fig. 1). These results combined with our lipotoxicity results indicate that IBTKα can have differential effects on cell viability depending on the nature and extent of the environmental stress and the affected cell types.

IBTKα mRNA levels and translation were increased by the PERK/CHOP pathway in response to palmitate (Fig. 1). Corresponding expression measurements of the IBTKα-depleted cells indicated that although the levels of IBTKα mRNA and protein were lowered as expected, CHOP and its known transcriptional target genes MAP1LC3B and SQSTM1 were fully induced after palmitate treatment, indicating that IBTKα functions as a unique downstream effector in the UPR (Fig. 3, A and C). Of note, mTORC1 remained repressed in all knockdowns following palmitate treatment, as measured by phosphorylation of S6 kinase, yet autophagy was not induced (Fig. 3A). These findings indicate that IBTKα is required for the induction of autophagy during activation of the UPR by lipotoxicity, and depletion of IBTKα or its upstream regulator CHOP rescues hepatocytes from exposure to saturated FFAs.

Both shIBTKα and control (shCTRL) cells were next assayed for accumulation of LC3b in the presence or absence of bafilomycin A1 or chloroquine (Fig. 3B). Depletion of IBTKα decreased conversion of LC3b-I to LC3b-II, as well as lowered accumulation of P62 upon treatment with chloroquine. Similar results were also observed upon overexpression of IBTKα (Fig. 3C). These results suggest that IBTKα is not only a downstream UPR target but is also an essential effector in a pathway leading to the induction of autophagy prior to LC3b lipidation.

To further test the proposed role of IBTKα in phagophore initiation, we expressed GFP-LC3b in HepG2 cells treated with chloroquine or vehicle and measured the accumulation of LC3b as judged by immunofluorescence microscopy (Fig. 3F). In the control HepG2 (shCTRL) cells, there was a distinct punctate pattern upon chloroquine treatment. However, knockdown of either ATG5 or IBTKα sharply lowered the detected punctate GFP-LC3b. These results support the idea that IBTKα is critical for phagophore formation.
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Port the idea that the UPR plays a direct role in phagophore formation through a signaling pathway involving CHOP and IBTKα.

IBTKα induces autophagy by binding to a multisubunit protein complex, including LC3b at the ERES

Phagophores have been suggested to form at the ER (28–32). Because both initiation of autophagy and ER morphology are dependent upon IBTKα (Fig. 3), we wanted to address the processes by IBTKα that can contribute to phagophore formation. We first utilized immunocytochemistry to determine the cellular location of IBTKα in WT HepG2 cells treated with chloroquine, palmitate, or vehicle by staining for endogenous IBTKα (Fig. 4A). IBTKα was primarily co-localized with the ER marker calnexin independent of stress. To assess whether IBTKα is also located at the site of autophagosome formation, we used GFP-LC3b HepG2 cells and followed a similar experimental design. IBTKα co-localized with only 5% of LC3b in both the vehicle and chloroquine-treated cells (Fig. 4B). However, upon treatment with palmitate, which would block autophagic flux, IBTKα co-localized with 90% of LC3b. These findings indicate that IBTKα co-localizes with phagophores initiating the ER, but not with mature autophagosomes at the lysosome.

To better understand the mechanisms by which IBTKα is involved in phagophore initiation at the ER membrane, we treated WT HepG2 cells with palmitate, thapsigargin, chloroquine, or vehicle and carried out immunoprecipitations using an antibody specific for endogenous IBTKα. Isolated proteins were then digested with trypsin, followed by mass spectrometry using multidimensional protein identification technology (MudPIT) (Fig. 4, C and D, and Supplemental Table 1). RAW

Figure 3. IBTKα is required for the induction of autophagy. A, shCTRL, shIBTKα, shCHOP, and shATG5 HepG2 cells were treated with either vehicle (−) or palmitate (+) for 12 h, and the indicated proteins were measured by immunoblot analyses. B, shCTRL, shIBTKα, shCHOP, and shATG5 HepG2 cells were treated with palmitate for 24 h, and cell viability was measured by LDH release. C, control (shCTRL) and shIBTKα HepG2 cells, or those overexpressing (OE) a FLAG-tagged IBTKα, were treated with vehicle (V), palmitate (PA), or chloroquine (CQ) for 24 h, and the indicated proteins were measured by immunoblot analyses. D, shCTRL, shIBTKα, and OE-IBTKα HepG2 cells were treated with palmitate for 24 h, and cell viability was determined by LDH release. E, shCTRL and shIBTKα HepG2 cells were treated with palmitate or vehicle for 12 h, and the indicated gene transcripts were measured by qPCR. F, HepG2 cells stably transduced with GFP-LC3b were treated with vehicle, chloroquine, or palmitate for 12 h, and co-localization with LAMP2 was visualized using immunofluorescence microscopy and quantified at the pixel level.

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The data files from each MudPIT step were used for FASTA database searching using SEQUEST HT, and the resulting dataset was filtered to allow for a false discovery rate of ≤1%. SAINT analysis was performed on the resulting peptide-spectrum matching counts from the database searches to identify proteins that displayed significant association with IBTK prepared from WT cells compared with affinity carried out using IgG control pulldowns. A total of 73 proteins were identified that met the SAINT probability score of ≥0.8. These IBTK-associated proteins include those involved in protein synthesis, translation, and vesicular transport.

**Figure 4.** IBTKα associates with protein complex at the ERES and induces formation of phagophores. A, HepG2 cells were treated with vehicle, chloroquine (CQ), or palmitate, and IBTKα and the ER marker calnexin were visualized using immunofluorescence microscopy. Co-localization of IBTKα and ER was quantified at the pixel level. B, HepG2 cells stably expressing GFP-LC3b were treated with vehicle, chloroquine, or palmitate, and co-localization with IBTKα was visualized using immunofluorescence microscopy and quantified. C, HepG2 cells were treated with vehicle, chloroquine, thapsigargin, or palmitate. Cell lysates were used in immunoprecipitation experiments with endogenous IBTKα as bait. The network represents proteins, and their functional classes, pulled down following LC/MS analysis of eluents. D, diagram showing SAINT score against fold change spectral abundance for proteins identified in LC/MS analysis of IBTKα immunoprecipitation experiment. E, shCTRL and shSEC16A HepG2 cells were treated with either vehicle (+) or palmitate (−) for 12 h and the indicated proteins were measured by immunoblot analyses. F, control (shCTRL) and shSEC16A HepG2 cells were treated for 24 h and viability was measured by LDH release. G, shCTRL and shSEC16A HepG2 cells were treated with palmitate or vehicle for 12 h and co-localization of IBTKα and calnexin was visualized using immunofluorescence microscopy.

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**IBTKα and the UPR activate NF-κB and secretion of cytokines triggering lipotoxicity**

Previously, we determined that treatment of hepatocytes with saturated FFAs induces the PERK/CHOP pathway in the UPR, leading to activation of NF-κB by a process involving phosphorylation of the subunit P65/RELA at serine 536 (10). Induced NF-κB contributes to increased expression and subsequent secretion of TNFα and IL-8; TNFα secreted from hepatocytes functions in a paracrine fashion, facilitating hepatocyte cell death and also further amplification of NF-κB activation, whereas both TNFα and IL-8 contribute to inflammation (10). The precise mechanisms by which CHOP contributes to activation of NF-κB in hepatocytes treated with saturated FFAs is uncertain. It is noteworthy that expression of the inhibitory IκBα was not significantly affected by loss of CHOP (10).

We reasoned that CHOP function in phagophore initiation and accumulation of suggested toxic autophagosomal intermediates may be contributors to CHOP activation of NF-κB and its downstream target genes. To test this idea, we determined whether depletion of IBTKα, or one of its interacting partners at the ERES-SEC31A, also prevented induction of NF-κB-directed gene expression. Similar to that observed for knockdown of CHOP, depletion of either IBTKα or SEC31A blocked P65 phosphorylation at serine 536, as well as induced secretion of TNFα and IL-8 and mRNA expression of these genes (Fig. 6, A–C). Finally, knockdown of SEC31A also thwarted death of HepG2 cells upon prolonged exposure to palmitate as observed for depletion of CHOP and IBTKα (Fig. 6D).

We next addressed whether SEC31A, along with SEC16A, has an important role in phagophore formation in HepG2 cells. As noted earlier, knockdown of SEC16A lowered the levels of LC3b-II and P62 in HepG2 cells treated with palmitate (Figs. 4E and 6E). Furthermore, depletion of SEC16A sharply lowered LC3b-II upon a block in autophagic flux during treatment with chloroquine (Fig. 6F). Similar results were observed upon depletion of SEC31A (Fig. 6G). These results support the idea that both SEC16A and SEC31A participate in initiation of autophagy.

SEC16A and SEC31A have well documented functions in the formation of COPII vesicles at the ERES (37), along with their suggested role in phagophores during assembly (32–34). Therefore, the role of these SEC proteins, and by inference CHOP and IBTKα, for activation of NF-κB and hepatotoxicity by saturated FFAs could result from the functions of these genes in facilitating secretion of TNFα and/or phagophore formation. ATG5 is central for phagophore formation, as visualized by lowered LC3b lipidation and P62 accumulation in ATG5-depleted HepG2 cells treated with palmitate (Figs. 3A and 6E). Of importance, knockdown of ATG5 blocked P65 phosphorylation upon treatment with the saturated FFA, strongly supporting the model that NF-κB activation during lipotoxicity is dependent upon the induction of autophagy (Fig. 6E). These findings indicate that lowered secretion of TNFα and IL-8 in the IBTKα-depleted cells is not merely a consequence of loss of COPII-directed secretion, which occurs with disruption of the protein complexes featuring SEC16A and SEC31A at the ERES (37).
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A

IP: Mock  IP: IBTKα

IBTKα  LC3b-I  SEC16A  GAPDH

ULK1  ULK2

B

IP: SEC16A

SEC16A  IBTKα  LC3b  ULK1  ULK2  IGG

C

Input  IP: Mock  IP: IBTKα

IBTKα  LC3b-I  SEC16A  GAPDH

ULK1  ULK2

D

Input  IP: Mock  IP: IBTKα

IBTKα  SEC31A  CUL3  LC3b-II  Actin

E

IP: Mock  IP: SEC31A

IBTKα  SEC31A  CUL3  LC3b  IGG

F

IP: Mock  IP: IBTKα

IBTKα  SEC16A  CUL3  LC3b-I  LC3b-II  GAPDH

GAPDH
To address whether general secretion also involves IBTKα and associated proteins at the ERES that facilitate initiation of autophagy, we transfected control and HepG2 cells depleted for IBTKα, CHOP, SEC16A, or SEC31A with a Gaussia luciferase reporter construct and measured secreted luciferase activity in the cell culture media following treatment with either vehicle of palmitate (Fig. 6H). Depletion of each of the four gene targets resulted in a reduction of Gaussia luciferase secretion. These findings suggest that the assembled multisubunit protein complexes containing IBTKα at the ERES are integral to secretion processes emanating from the ER, as well as the formation of autophagosomes.

We previously showed that secreted TNFα is central for hepatocellular death upon exposure to saturated FFAs (10). We addressed this idea by adding recombinant TNFα to WT, shCHOP, and shIBTKα HepG2 cells in the presence or absence of palmitate treatment. There was significant death of shCHOP and shIBTKα cells only after treatment with TNFα in combination with palmitate (Fig. 6I), which was accompanied by increased phosphorylation of P65 (Fig. 6J). These results indicate that IBTKα is critical for cytokine expression and secretion and consequent inhibition of autophagic flux during metabolic stress.

**Discussion**

Accumulation of saturated FFAs and activation of the UPR activation are common features associated with NAFLD; however, how the UPR contributes to the progression from simple steatosis to NASH is not clear (7). This report indicates that activation of the UPR and IBTKα, in combination with inhibition of autophagic flux during prolonged exposure to saturated FFAs, is as a key driver of hepatocellular death and the pathophysiology of NASH (Fig. 7A). IBTKα expression was induced as part of the UPR by both translational and transcriptional control mechanisms (Fig. 1), and loss of IBTKα in human hepatocytes thwarted induction of autophagy and enhanced survival of hepatocytes exposed to saturated FFAs (Fig. 2).

Translational control during ER stress allows for rapid enhanced expression of IBTKα and consequent assembly of a multisubunit complex at the ERES that included key factors, such as LC3b, ULK1/2, SEC16A, and SEC31A that served to promote phagophore initiation (Fig. 4). In response to saturated FFAs, the UPR and CHOP trigger a signaling pathway involving NF-κB, which induces transcriptional expression of key cytokines that contribute to inflammation and cell death (Figs. 6 and 8B). Among these cytokines, TNFα is suggested to function as an autocrine and paracrine factor that contributes to cell death and amplifies NF-κB-directed gene expression (Fig. 8B).

Enhanced levels of IBTKα are also suggested to promote formation of vesicles from the ER that facilitate secretion of these cytokines (Fig. 6). Lowered levels of secreted reporter Gaussia luciferase upon loss of IBTKα, as well as SEC16A and SEC31A, indicate that IBTKα facilitates at least a portion of the secretome. IBTKα may function as a multidomain adapter protein in the assembly of the protein complex at the ERES and/or help direct E3 ubiquitin ligase CUL3 ubiquitylation of proteins required for assembly or function of the complex. Importantly, loss of SEC31A did not disrupt IBTKα association with SEC16A or LC3b, suggesting that there are distinct complexes, including IBTKα, that are situated at the ERES (Fig. 8).

**Figure 5. IBTKα associates with SEC proteins.** A and B, HepG2 cells were treated for 12 h with the indicated stress compounds, and cell lysates were used for immunoprecipitation with IgG control (mock), IBTKα, or SEC16A followed by immunoblot analysis. On the left of A, input indicates the immunoblot analyses of total lysates. C, shCTRL and shSEC16A HepG2 cells were treated for 12 h with vehicle or palmitate, and cell lysates were used for immunoprecipitation with IgG control (mock), IBTKα, or SEC16A, followed by immunoblot analysis to measure the indicated proteins. On the left of D, input indicates immunoblot analyses carried out using total lysates. F, shCTRL and shSEC31A HepG2 cells were treated with vehicle or palmitate for 12 h, and cell lysates were used for immunoprecipitation with IgG control (mock) or IBTKα, followed by immunoblot analyses to measure the indicated proteins.
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**Figure 6. IBTKα and the UPR activate NF-κB and secretion of cytokines triggering lipotoxicity.** A, control (shCTRL), shCHOP, shIBTKα, and shSEC31A HepG2 cells were treated with vehicle (−) or palmitate (+) for 12 h, and cell lysates were used for immunoblot analyses to determine the levels of the indicated proteins. B, shCTRL, shCHOP, shIBTKα, and shSEC31A HepG2 cells were treated with vehicle or palmitate for 12 h, and IL-8 and TNFα release was measured by sandwich ELISA. C, shCTRL, shCHOP, and shIBTKα HepG2 cells were treated with vehicle or palmitate for 12 h, and the levels of CXCL8 (IL-8) and TNFα (TNFα) mRNAs were measured by qPCR. D, shCTRL and shSEC31A HepG2 cells were treated with palmitate for 24 h, and cell viability was measured by LDH release. E, shCTRL, shSEC16A, and shATG5 HepG2 cells were treated with either vehicle (−) or palmitate (+) for 12 h, and cell lysates were used for immunoblot analyses to determine the levels of the indicated proteins. F, shCTRL and shSEC16A HepG2 cells were treated with chloroquine (CQ) or vehicle (V) for 24 h, and the indicated proteins were measured by immunoblot. G, HepG2 cells depleted for SEC31A (shSEC31A) or control (shCTRL) were treated with chloroquine or vehicle for 24 h, and the indicated proteins were measured by immunoblot. H, shCTRL, shCHOP, shIBTKα, shSEC16A, and shSEC31A HepG2 cells were transfected with a Gaussia luciferase reporter and treated with vehicle or palmitate for 12 h. Luciferase activity was measured in the supernatant. I, cultured shCTRL, shCHOP, and shIBTKα HepG2 cells were treated for 24 h with recombinant TNFα and palmitate, alone or in combination, as indicated, and cell viability was measured by LDH release. J, shCTRL, shCHOP, and shIBTKα HepG2 cells were treated for 24 h with recombinant TNFα and/or palmitate, as indicated, and the indicated proteins were measured by immunoblot analyses.
Autophagy initiation and COP9-directed vesicle transport are suggested to be linked, as depletion of IBTKα prevents both the induction of autophagy and secretion of the secreted luciferase reporter and cytokines. The full complement of proteins functioning in conjunction with IBTKα in the ERES complexes is currently unknown. Furthermore, it is not known whether these distinct complexes are restricted to either formation of phagophores or to protein secretion or, alternatively, there are some common protein assembly steps at the ERES that contribute to both processes.
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**Figure 8. Model for the role of IBTKα in the regulation of autophagy and pathogenesis of NASH.** A, model for the UPR and IBTKα regulation of autophagy induction at the ERES during metabolic stress. Saturated FFAs induce the UPR, featuring induced transcripitional expression of IBTKα by CHOP and preferential translation by eIF2α-P. IBTKα assembles in a multisubunit complex with SEC16A, ULK1/2, and LC3b at the ERES, culminating in induction of phagophores. Saturated FFAs also block the autophagic flux, contributing to hepatocyte death. B, model for the UPR and IBTKα regulation of secretion through COPII vesicles during metabolic stress. Induced secretion of key cytokines, including TNFα and IL-8, play a key role in hepatocyte cell death and inflammation during lipotoxicity.

Autophagy is often thought of as a cell survival pathway that directs damaged organelles and misfolded proteins for degradation in lysosomes (39). However, autophagy can play a direct role in cell death, and recent studies have suggested that there are two main processes: 1) type II autophagic cell death, which consists of cell death accompanied by accumulation of large secondary vacuoles, and 2) autosis, which is characterized by a disappearance of the ER and general self-eating (40). Our results suggest that saturated FFAs induce neither type II cell death nor autosis, as autophagy is a key driver in hepatocellular death without the appearance of secondary lysosomes.

A key feature of our study is the localization of IBTKα to ERES through a complex that includes LC3b, SEC16A, and SEC31A (Fig. 4). When the hepatocytes were exposed to palmitate, LC3b-II was retained at the endoplasmic reticulum with IBTKα, suggesting either a defect in phagophore elongation or trafficking from the ERES to the lysosome. Further support that a block in autophagic flux rather than reduced autophagy per se is critical for the lipotoxicity of hepatocytes is that loss of ATG5, which facilitated phagophore formation, and CHOP that can direct expression of genes critical to formation of autophagosomes provides for resistance to saturated FFAs. These findings suggest that saturated FFAs induce a novel form of autophagic cell death, perhaps a subset of autosis, which allows for the lipidation of LC3b but interferes with LC3b trafficking from the ER to lysosomes. Importantly, this block in autophagic flux is coincident with increased inflammation and TNFα signaling thereby linking key features of NASH pathogenesis in a linked signaling cascade (Fig. 8B). Although it is possible that this form of cell death is unique to metabolic stress, it is also likely to be a driver in other diseases states or even drug-induced liver injury in which hepatocellular death and inflammation are prominent pathologies.

Induction of the UPR and a block in autophagic flux were also supported by our analysis of human liver biopsy samples, which indicated that levels of IBTKα, CHOP, as well as total and phosphorylated P65 were increased in both simple steatosis and in NASH (Fig. 7). Of importance, levels of LC3b-II and P62 were significantly elevated in NASH, which were strongly associated with hepatocellular injury markers, such as ballooning. Therefore, we have identified biomarkers suggested to be specific for NASH, as well as others associated with general NAFLD. Furthermore, analyses of the liver biopsy samples indicate that our in vitro model correlates well with the events occurring in NAFLD. Our principal component analysis combining protein quantification, histology, and serum analysis indicates that key factors and pathways described herein will provide new insight into the progression of simple steatosis to NASH.

In summary, we identified IBTKα as a novel preferentially translated member of the UPR during metabolic stress, which plays a role in both phagophore initiation and formation of transport vesicles at the ERES, linking autophagy and secretion to the pathogenesis of NASH. Furthermore, our study indicates that aberrant autophagy and the consequent inhibition of autophagic flux are key drivers in the pathogenesis of NASH at both the cellular level and as correlative biomarkers in patient samples. At present, no approved therapies exist to treat NASH, and there is a need for better mechanistic understanding of cellular targets that repress the associated pathologies and progression of NASH. The ability to target both hepatocellular death and inflammation through IBTKα in the treatment of simple steatosis and/or NASH could have therapeutic benefits, and further work in the validation of this target is warranted.
Experimental procedures

Stable gene knockdowns and knock-outs

Stable knockdown and control cells were generated by transducing HepG2 cells with lentivirus carrying shRNA from Sigma against shCHOP (TRCN0000364393 and TRCN0000007263), shIBTKα (TRCN0000082575 and TRCN000082577), shATG5 (TRCN0000151963 and TRCN0000151474), shSEC16A (TRCN0000246017), and shSEC31A (TRCN0000436177). Stable knockouts were constructed by using a plasmid from Sigma expressing the guide RNA, CAS9, long terminal repeats, and puromycin for CHOP (HS0000185403, GGAAATCGAGCG-CCTGACCCAGG), PERK (HS0000302986, AATTATCAG-CACTTTAGATGG), or IBTKα (HS0000392536, GCTTTGGATCTTGGTAATGAAGG). Stable N-terminal GFP-LC3b expressing the guide RNA, CAS9, long terminal repeats, and puromycin for CHOP (HS0000185403, GGAAATCGAGCG-CCTGACCCAGG), PERK (HS0000302986, AATTATCAG-CACTTTAGATGG), or IBTKα (HS0000392536, GCTTTGGATCTTGGTAATGAAGG). Stable N-terminal GFP-LC3b cells were constructed using a plasmid (EX-T0824-Lv103) from GeneCopoeia (Rockville, MD). Following transduction, cells taking up virus were selected using 10 μg/ml puromycin, and knock-out cells were sorted for single cells using flow cytometry. For the Gaussia luciferase assay, PSV40-g luc −gluc control plasmid from New England Biolabs (Ipswich, MA) was transfected to cells, and supernatant was used to measure secreted luciferase.

Cell culture and measurements of cell viability

Human hepatoma HepG2 cells were purchased from ATCC and were both cultured and treated with 600 μM FFAs as described previously (10). Furthermore, to invoke ER stress or to alter autophagy, cells were treated with 1 μM thapsigargin, 2 μM tunicamycin, 50 μM chloroquine, or 0.1 μM baflohimycin A1, as indicated. Cell viability was measured using lactate dehydrogenase (LDH) (41). Data were normalized to total LDH release by 10% Triton X-100. Recombinant TNFα (PHC03015L; Life Technologies, Inc.) was formulated in phosphate-buffered saline (PBS) with BSA and applied to cells as indicated in the figure legends. Caspase 3/7 activity was measured using the Apo-ONE homogeneous caspase 3/7 assay from Promega (Madison, WI).

Polysome profiling

HepG2 control and PERK-KO knock-out cells were cultured in the presence of vehicle, 600 μM palmitate, or 1 μM thapsigargin for 6 h. 50 μg/ml cycloheximide was added to each culture dish for 10 min prior to collection. Cell lysates were collected and subjected to centrifugation in a 10–50% sucrose gradient in a Beckman SW41Ti rotor for 2 h at 4 °C at 40,000 rpm. To measure RNA abundance, an absorbance of 254 nm was monitored as described (42). ATF4, CHOP, and IBTKα mRNA levels were measured by qPCR in each of the seven collected fractions as described (20). Firefly luciferase mRNA was added to each fraction to facilitate normalization in the cDNA and qPCR analysis for the transcript measurements as described. Data are represented as the percentage of transcript found in each fraction relative to the total for each mRNA, and a percentage change in large polysomes (fractions 5–7) with treatment relative to vehicle was measured.

Measurements of mRNA by qPCR

Following the indicated compound treatments, RNA was isolated using TRIzol reagent (Life Technologies, Inc.), and cDNA synthesis was performed using the TaqMan RT kit (Life Technologies, Inc.). Primers utilized for measuring mRNA levels are in supplemental Table 4, and transcripts were normalized to GAPDH.

Immunoblot analysis and ELISAs

Protein lysates were collected and quantified using Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Proteins were separated by electrophoresis using 4–12% BisTris gels via SDS-PAGE, transferred to nitrocellulose filters, and blocked for 1 h at room temperature. Membranes were incubated overnight with the following primary antibodies: from Cell Signaling Technology (Beverly, MA), ATF4 (11915S), GAPDH (2118S), p62/SQSTM1 (H00008878-M01), and ULK2 (NBP133136); from Thermo Fisher Scientific, calnexin (MA3–26795), and cleaved caspase 3 (9664L); from Santa Cruz Biotechnology (Dallas, TX), CHOP (sc-7351) and LAMP2 (sc-18822); from Sigma, β-actin (A5441); from Novus, LC3b (NB1002220), SEC16A (NB183016), IBTKα (NBP150533 and NBP188512), p62/SQSTM1 (H00008878-M01), and ULK2 (NBP133136); from Thermo Fisher Scientific, calnexin (MA3–027); and from Abcam (Cambridge, MA), SEC31A (AB8660). For human serum samples, the cytokine human magnetic 10-plex panel from Thermo Fisher Scientific was utilized on the Lumixi MAGPIX from EMD Millipore (Billerica, MA). Human IL-8 and TNFα in the conditioned media of HepG2 cells were measured using R&D Systems (Minneapolis, MN) Quantikine ELISA kits D8000C and DTA00C, respectively.

Immunoprecipitation

For immunoprecipitation analyses, cells were trypsinized, washed once with PBS, and flash-frozen following 5 min of incubation on ice in a hypotonic lysis buffer containing 20 mM Hapes, 2 mM MgCl2, and 10% glycerol. Following a rapid thaw in a pre-warmed water bath, NaCl (150 mM final concentration) was added to cell suspensions on ice for 5 min, followed by clarification by centrifugation using a microcentrifuge at 10,000 × g for 15 min. Supernatant was transferred to protein G Dynabeads coated with the following primary antibodies: from Abcam, SEC16A (ab70722) and SEC31A (AB8660); from Novus, IBTKα (NBP188512) and IgG control (3900S). After an overnight incubation in a cold room with continuous rotation to ensure mixing, beads were washed with a buffer containing 20 μM Hapes, 2 mM MgCl2, 10% glycerol, and 150 mM NaCl. Supernatant was removed, and proteins associated with beads were eluted with either urea for LC/MS analysis or with 1X SDS-PAGE buffer for immunoblot analysis.

Mass spectrometry and quantitative proteomics analysis

Mass spectrometry analysis of isolated protein complexes pulled down with antibodies against native IBTKα and peptide sequence matching via SEQUEST HT was performed using Proteome Discoverer™ 1.4 (Thermo Fisher Scientific), as described previously (43). Three biological replicates were car-
ried out for the IBTKα pulldown as compared with IgG control. For each biological replicate, four technical replicates were performed. Protein sequence matching was performed against a human FASTA database from UniProt (March 16, 2014) containing additional laboratory contaminant proteins, such as proteolytic enzymes. Nonspecific interactions were eliminated using the statistical model Significance Analysis of Interactome (SAINT) and a stringent SAINT threshold of \( p > 0.8 \) (44 – 46). This statistical tool assigns the number of peptide identifications for each target protein to a probability distribution, which is then used to measure the likelihood of biologically accurate protein interactions.

Cell imaging

For all cellular imaging, cultured HepG2 cells were treated with the indicated compounds, then fixed with 1× Prefer from Anatech (San Diego), and permeabilized with 0.1% Triton X-100 for 10 min. This was followed by overnight incubations with primary antibody in PBS containing 0.1% BSA, and then a 2-h incubation with the corresponding secondary Alexa Fluor conjugates and 10 μg/ml Hoechst (33342) from Life Technologies, Inc. Images were acquired by spinning disk confocal microscopy on the Opera (PerkinElmer Life Sciences) for three-dimensional rendering of the 0.5 μm z-stack sections and data quantification. Samples collected for electron microscopy were processed and imaged by DSimaging, LLC (West Lafayette, IN).

Human NAFLD samples

Samples were collected under a study protocol that was reviewed and approved by the Medical College of Wisconsin’s Institutional Review Board (previous institution for S. G.). Each participating subject gave a written Informed Consent for participation. Subjects were morbidly obese (BMI \( \geq 40 \) kg/m² or >35 kg/m² with significant co-morbidities) with prior unsuccessful attempts to lose and maintain weight, and who underwent bariatric surgery. A protocol intraoperative liver biopsy was performed on all patients for histological phenotyping. Patients with alcohol intake \( >20 \) g/day and those with other liver diseases based on positive disease-specific serological tests and suggestive liver histology were excluded. Patients using drugs associated with NAFLD prior to liver biopsy were excluded. An experienced pathologist read the liver biopsy according to the NASH Clinical Research Network working group system (National Institutes of Health) (38).

Statistical analysis

Data are depicted as \( \pm \) S.D. unless otherwise noted. Differences between multiple groups were analyzed using analysis of variance and a post hoc Tukey’s honest significant difference. For all figures, \( p < 0.05 \) was considered statistically significant and is depicted using an asterisk, whereas treatment groups in knockdown/knock-out experiments considered statistically significant relative to the respective shCTRL treatment are depicted by a number sign (#).

**IBTKα facilitates autophagy during NASH**

**Author contributions**—J. A. W. conceived the study, designed, performed, and analyzed experiments and wrote the manuscript. S. K. Y. designed and performed translational control experiments in Fig. 1. A. L. M. performed and analyzed proteomics data. S. G. provided human liver biopsy and serum samples and consulted with the principal component analysis. J. L. S. and H. C. M. designed and analyzed experiments and contributed to the preparation of the manuscript. R. C. W. conceived and coordinated the study, designed and analyzed experiments, and wrote the manuscript. All authors approved the final manuscript version.

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