Inhibition of Fatty Acid Synthase Induces Endoplasmic Reticulum Stress in Tumor Cells

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Abstract

Fatty acid synthase (FAS), the cellular enzyme that synthesizes palmitate, is expressed at high levels in tumor cells and is vital for their survival. Through the synthesis of palmitate, FAS primarily drives the synthesis of phospholipids in tumor cells. In this study, we tested the hypothesis that the FAS inhibitors induce endoplasmic reticulum (ER) stress in tumor cells. Treatment of tumor cells with FAS inhibitors induces robust PERK-dependent phosphorylation of the translation initiation factor eIF2α and concomitant inhibition of protein synthesis. PERK-deficient transformed mouse embryonic fibroblasts and HT-29 colon carcinoma cells that express a dominant negative PERK (ΔC-PERK) are hypersensitive to FAS inhibitor–induced cell death. Pharmacologic inhibition of FAS also induces the processing of X-box binding protein-1, indicating that the IRE1 arm of the ER stress response is activated when FAS is inhibited. Induction of ER stress is further confirmed by the increased expression of the ER stress–regulated genes *CHOP, ATF4*, and *GRP78*. FAS inhibitor–induced ER stress is activated prior to the detection of caspase 3 and PARP cleavage, primary indicators of cell death, whereas orlistat-induced cell death is rescued by coincubation with the global translation inhibitor cycloheximide. Lastly, FAS inhibitors cooperate with the ER stress inducer thapsigargin to enhance tumor cell killing. These results provide the first evidence that FAS inhibitors induce ER stress and establish an important mechanistic link between FAS activity and ER function. [Cancer Res 2007;67(3):1262–9]

Introduction

Fatty acid synthase (FAS) is a multifunctional enzyme that catalyzes the terminal steps in the synthesis of the 16-carbon fatty acid palmitate in cells (1, 2). In normal tissue, the FAS expression levels are relatively low because fatty acid is generally supplied by dietary fatty acids. On the other hand, FAS is expressed at significantly higher levels in many tumors including those of the prostate, breast, colon, ovary, and others (3–5). This expression profile suggests that tumors require higher levels of fatty acids than can be supplied from the circulation. Several reports have shown that FAS expression levels correlate with tumor progression, aggressiveness, and metastasis (5–7). In fact, FAS expression levels are predictive of the progression from organ-confined prostate cancer to metastatic prostate cancer (6), indicating that FAS provides a metabolic advantage to tumor cells. Because of the strong link between FAS expression and cancer, FAS has become an attractive target for therapeutic intervention.

The functional connection between FAS and tumor progression has been provided by the discovery and design of small molecule drugs that inhibit the catalytic activity of FAS (8, 9). Cerulenin and C75, which target the keto-acyl synthase domain of FAS, were the first small molecules to be described as inhibitors of FAS activity in human tumor cells. These pharmacologic agents inhibit FAS activity and induce cell death in many tumor cell lines *in vitro* (5, 7). The compounds are also effective at inhibiting the growth of human tumor xenografts *in vivo* and have chemopreventive abilities (10–12). We were the first to describe orlistat as an inhibitor of the thioesterase domain of FAS (13). Orlistat inhibits FAS activity and induces cell death in a variety of tumor cell lines and is able to effectively inhibit the growth of prostate tumor xenografts in mice (13–15). The data linking FAS function and tumor cell survival emphasizes the relevance of FAS as an attractive antitumor target. The importance of fatty acid synthesis in tumor cells is further underscored by data demonstrating that pharmacologic and genetic inhibition of two upstream enzymes in the fatty acid synthesis pathway, ATP citrate lyase and acetyl CoA carboxylase, also induces cell death in tumor cell lines (16–18).

Because FAS is a target for therapeutic intervention, it is important to fully understand the role of FAS in tumor cells as well as the antitumor effects of FAS inhibitors. Given that the endoplasmic reticulum (ER) is the major site for phospholipid synthesis in cells, it is not surprising that previous studies have identified a link between pathways that regulate lipid synthesis and the ER stress response (19–21). Fatty acid synthesis in general, and FAS activity in particular, drives phospholipid synthesis which primarily occurs in the ER (22). Because of the direct connection between FAS activity and phospholipid synthesis, we tested the hypothesis that pharmacologic blockade of FAS activity might induce ER stress in tumor cells (22). The data presented herein shows for the first time that inhibition of FAS induces ER stress specifically in a variety of tumor cells and not in normal cells. Importantly, we also show that FAS inhibitors cooperate with a known ER stress inducer, thapsigargin, to induce cell death. The data also provide evidence that FAS inhibitors might be combined with PERK inhibitors to more effectively treat cancer. The evidence suggests that increased FAS expression in tumor cells is important for ER function to maintain membrane biogenesis and suggests a role for ER stress in the antitumor effects of FAS inhibitors.

Materials and Methods

Materials. The PC-3, DU145, HT-29, HeLa, and FS-4 cell lines were obtained from American Type Culture Collection (Manassas, VA). Cell culture medium and supplements were from Invitrogen (Carlsbad, CA).

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Antibodies against eIF2α, phospho-eIF2α, cleaved caspase 3, and cleaved PARP were from Cell Signaling Technologies (Beverly, MA). Antibody against FAS was from BD Transduction Labs (San Diego, CA). Antibody against β-tubulin was from NeoMarkers (Fremont, CA). TRIzol was from Invitrogen. Avian myeloblastosis virus–reverse transcriptase and Taq Polymerase were from Promega (Madison, WI). ^{35}S-Methionine and ^{14}C-acetate were purchased from GE Healthcare (formerly Amersham Biosciences, Piscataway, NJ). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA), except for those designed for short interfering RNA (siRNA), which were synthesized by Dharmacon (Lafayette, CO). All other reagents were purchased from Sigma (St. Louis, MO), Calbiochem (San Diego, CA), or Bio-Rad (Hercules, CA).

Cell culture and drug treatments. Prostate tumor cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Wild-type and *PERK*$^{-/-}$ mouse embryonic fibroblasts (MEF), obtained from David Ron (Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY), HeLa cervical cancer cells, and FS-4 human foreskin fibroblasts were maintained in DMEM-high glucose supplemented with 10% fetal bovine serum. HT-29 colon carcinoma cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum. HT-29 cells expressing the pBabe-puro empty vector or the pBabe-puro-ΔC-PERK construct were maintained with 1 μg/mL of puromycin and supplemented with 20% fetal bovine serum, nonessential amino acids, and 2-mercaptoethanol. Cells were treated for the indicated times and drug concentrations as indicated. Orlistat was extracted from capsules in ethanol as described previously and stored at −80°C (13). Further dilutions were made in DMSO.

Generation of ΔC-PERK–expressing cells. To generate human tumor cells with deficient PERK signaling, HT-29 cells seeded in six-well plates were transfected with 1 μg of pBabe-puro or pBabe-puro-ΔC-PERK using LipofectAMINE (Invitrogen). These plasmids have been described previously (23). Stable populations of each construct were selected by incubating transfected cells with 3 μg/mL of puromycin for 48 h. The transfected cell populations were then maintained in 1 μg/mL of puromycin for subsequent experiments in the medium described above.

Immunoblot analysis. Cells were harvested after the indicated treatments, washed with ice-cold PBS, and lysed in buffer containing 1% Triton X-100 and a complete protease, kinase, and phosphatase inhibitor cocktail. Protein samples were electrophoresed through 7.5%, 10%, or 12% SDS-polyacrylamide gels and transferred to nitrocellulose, except for blots to detect phospho-eIF2α and eIF2α, which were transferred to Immobilon-P membrane (polyvinylidene difluoride). Immunoreactive bands were detected by enhanced chemiluminescence (Perkin Elmer Life Sciences, Inc., Boston, MA).

Metabolic labeling of protein and fatty acid synthesis. To measure fatty acid synthesis, 1×10^5 cells per well were seeded in 24-well plates. Cells were treated with C75 (10 μg/mL), orlistat (25, 50 μmol/L), or cerulenin (5, 10 μg/mL) for 2 h. ^{14}C-Acetate (1 μCi) was added to each well for 2 h. Cells were collected, washed, and lipids were extracted and quantified as previously described (13). To measure new protein synthesis, PC-3 cells were seeded in six-well plates. Orlistat (50 μmol/L) and thapsigargin (1 μmol/L) were added for the indicated times. After incubation with orlistat or thapsigargin, the cells were switched to methionine-deficient medium although maintaining the drug concentrations. Methionine-deficient medium supplemented with 100 μCi/mL of ^{35}S-methionine was added to the cells for 30 min to label newly synthesized proteins. After the labeling period, cells were washed, lysed, and samples were resolved by electrophoresis through a 10% SDS-polyacrylamide gel. The gel was then stained with Coomassie, dried, and the relative protein synthesis of each sample was quantified after scanning with a Typhoon 9210 (Amersham) using ImageQuant software.

Clonogenic survival assays. Cells were plated in six-well plates at a low density depending on the individual cell type. PC-3 cells were plated at a density of 800 cells per well, except for the experiment combining C75 with thapsigargin, for which PC-3 cells were plated at 3,000 cells per well. HT-29 and MEF cells were plated at a density of 400 cells per well. Human tumor cells were plated 48 h prior to each experiment, whereas MEFs were plated 24 h prior to treatment. Fresh medium containing the indicated drugs was added at the indicated concentrations for 12 to 20 h as indicated. The medium was then removed, the wells were washed and fresh medium was added. Plates were incubated until macroscopic colonies formed. To visualize colonies, the wells were washed twice with ice-cold PBS and fixed for 10 min with a 10% methanol/10% acetic acid solution. Colonies were stained with a 0.4% crystal violet/20% methanol solution for 10 min. The crystal violet solution was removed, the wells were washed with water to remove excess dye, and dried at room temperature overnight. Colonies were quantified by counting and by solubilization in 33% acetic acid followed by spectrophotometric analysis at 540 nm. The survival of treated cells was normalized relative to vehicle-treated cells and statistical significance was determined by two-tailed Student's *t* tests.

Detection of X-box binding protein-1 splicing and ATF4, GRP78, CHOP, and GADD34 expression. Cells were exposed to the various drug treatments or transfected with siRNA for the indicated times. Total RNA was isolated from cells using TRIzol according to the manufacturer's directions. cDNA was generated from 2 μg of total RNA using Avian myeloblastosis virus–reverse transcriptase. X-box binding protein-1 (XBP-1) was amplified by PCR with Taq polymerase using the oligonucleotides AAACAGAGTAGCAGCTCAGACTGC (sense) and TCCTTCTGGGTAGACCTCTGGGAG (antisense). The XBP-1 products were resolved on 2% Tris acetate-EDTA agarose gels and imaged on the Typhoon 9210 at 610 nm. The expression of CHOP, ATF4, GRP78, and GADD34 was determined by semiquantitative PCR using RNA collected as described above. Multiple cycles were tested for each gene to determine the optimum cycles in the linear range. The oligonucleotide sequences used were: CHOP, CAGAACCAGCAGAGGTCACA and AGCTGTGCCACTTTCCTTTC; GRP78, CTGGGTACATTTGATCTGACTGG and GCATCCTGGTGGCTTTCCAGCCATTC; ATF4, CTTACGTTGCCATGATCCCT and CTTCTGGCGGTACCTAGTGG; and GADD34, GTGGAAGCAGTAAAAGGAGCAG and CAGCAACTCCCTCTTCCTCG. The CHOP, GRP78, and ATF4 products were resolved on 1% Tris acetate-EDTA agarose gels and imaged on the Typhoon 9210 at 610 nm.

Suppression of FAS expression with siRNA. A paired siRNA oligonucleotide against the *FAS* gene (FAS1 sense, GUAGGCCUUCCACUCCUAUU) and one siRNA against luciferase as a negative control (Luc sense, CUUACGUGAUACUUCGAUU) were designed and synthesized by Dharmacon. The individual siRNAs (30 nmol/L) were transfected into cells upon plating with siPORT *NeoFX* transfection reagent (Ambion, Austin, TX) according to the instructions of the manufacturer. Cells were collected at the indicated times after transfection and then harvested for RNA to perform reverse transcription-PCR (RT-PCR) or protein for immunoblot analysis.

Results

Pharmacologic inhibition of FAS induces phosphorylation of eIF2α in tumor cells. Several studies have shown that lipid composition is important for maintaining ER function (19–21, 24, 25). Other studies have shown that FAS drives phospholipid synthesis in tumor cells (22). Because of this fact, we hypothesized that FAS inhibitors might induce ER stress. One hallmark of ER stress is the PERK-dependent phosphorylation of the translation initiation factor eIF2α. We first examined the phosphorylation status of eIF2α in cells treated with three different pharmacologic inhibitors of FAS (Fig. 1*A*). PC-3 cells were treated with orlistat (12.5–50 μmol/L, *left*) or cerulenin (5 or 10 μg/mL, *middle*) for 16 h, or C75 (10 μg/mL) for 8 to 24 h (*right*). Each FAS inhibitor induced robust phosphorylation of eIF2α at each concentration after 16 h of treatment, as did thapsigargin (data not shown). Similarly, all three inhibitors induced eIF2α phosphorylation regardless of tumor cell type tested (data not shown). Likewise, fatty acid synthesis was inhibited to similar degrees by each treatment, as measured by ^{14}C-acetate incorporation into total cellular lipids (Fig. 1*B, left*). Because the phosphorylation of eIF2α leads to the inhibition of

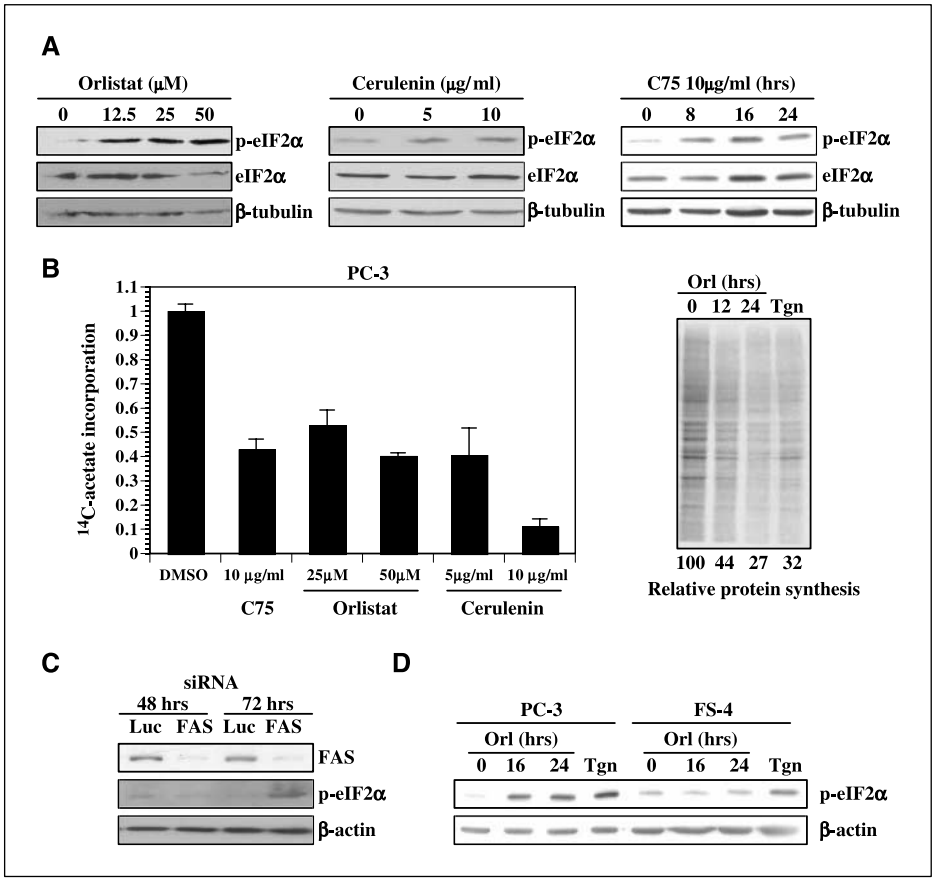

Figure 1. FAS inhibitors induce phosphorylation of eIF2α in tumor cells. *A,* PC-3 cells were treated with the indicated concentrations of orlistat (*left*) or cerulenin (*middle*) for 16 h, or C75 (10 μg/mL) for 8 to 24 h (*right*). Samples were resolved by SDS-PAGE, transferred to polyvinylidene difluoride and the membrane was probed with antibodies specific for phospho-eIF2α, total eIF2α, and β-tubulin. *B,* PC-3 cells were treated for 2 h with C75 (10 μg/mL), orlistat (25 and 50 μmol/L), or cerulenin (5 and 10 μg/mL), then incubated with ^{14}C-acetate (1 μCi) for 2 h. Cells were collected, washed, and lipids were extracted and quantified relative to vehicle-treated control (*left*). PC-3 cells were treated with orlistat (50 μmol/L) for the indicated times or thapsigargin (*Tgn*, 1 μmol/L) for 1 h and then pulsed with 10 μCi of ^{35}S-methionine for 30 min. Protein aliquots were then resolved by SDS-PAGE and new protein synthesis was quantified by scanning on a Typhoon 9210. Quantification was relative to vehicle-treated controls (*right*). *C,* PC-3 cells were transfected with siRNA against FAS or luciferase (*Luc*) for the indicated times and analyzed by immunoblot. *D,* FS-4 normal foreskin fibroblasts were treated with orlistat (25 μmol/L) for 24 h or Tgn (1 μmol/L) for 1 h side by side with PC3 cells and prepared for immunoblot analysis.

protein synthesis, we did a ^{35}S-methionine labeling experiment to measure the levels of newly synthesized proteins in cells treated with orlistat. PC-3 cells were treated with orlistat (50 μmol/L) for 12 and 24 h or thapsigargin (1 μmol/L) as a positive control for 1 h (Fig. 1*B, right*). Orlistat treatment reduced protein synthesis by 56% after 12 h and by 73% at 24 h, similar to treatment with thapsigargin. Therefore, orlistat treatment is sufficient to induce the phosphorylation of eIF2α and, subsequently, inhibit protein synthesis. To further confirm our findings, a genetic approach was also used to inhibit FAS expression. PC-3 cells were transfected with FAS-specific siRNA or siRNA against luciferase as a negative control. Immunoblot analysis showed a nearly 70% reduction of FAS protein in the samples 48 h after transfection, which continued through 72 h (Fig. 1*C*). Consistent with our findings using pharmacologic inhibitors, the reduction of FAS expression levels resulted in the detection of significant levels of phosphorylated eIF2α at 72 h (Fig. 1*C*). Conversely, treatment of normal human foreskin FS-4 fibroblasts with the FAS inhibitor orlistat did not result in the phosphorylation of eIF2a (Fig. 1*D*). Collectively, these data indicate that eIF2α phosphorylation induced by FAS inhibition is, indeed, specific to both FAS and tumor cells.

PERK mediates eIF2α phosphorylation in response to orlistat treatment. There are four known eIF2α kinases: PERK, GCN2, PKR, and HRI; however, PERK is the kinase that phosphorylates eIF2α during the ER stress response (26, 27). To determine whether PERK is the kinase responsible for the phosphorylation eIF2α in response to FAS inhibition, wild-type and *PERK*$^{-/-}$ MEFs transformed with *Ki-Ras*^{V12} were obtained and tested for their sensitivity to orlistat (27). The wild-type and *PERK*$^{-/-}$ MEFs were treated with orlistat (12.5 μmol/L) for 8, 16, and 24 h (Fig. 2*A*) or treated with thapsigargin (1 μmol/L) for 1 h (data not shown). In the wild-type MEFs, orlistat induced the phosphorylation of eIF2α within 8 h (Fig. 2*A*), consistent with our findings in prostate tumor cell lines (Fig. 1*A*). On the other hand, no significant phosphorylation of eIF2α was evident during the same time course of orlistat treatment in the PERK-deficient cells (Fig. 2*A*). As expected, thapsigargin only induced phosphorylation of eIF2α in the wild-type and not the *PERK*$^{-/-}$ MEFs (data not shown). These data indicate that FAS inhibition results in PERK-dependent phosphorylation of eIF2α.

It has been shown that PERK-deficient cells are hypersensitive to ER stress–induced apoptosis (28). Because of this, we tested whether *PERK*$^{-/-}$ MEFs were hypersensitive to orlistat-induced cell death using clonogenic survival assays. Wild-type and *PERK*$^{-/-}$ MEFs were treated with vehicle, orlistat (25 μmol/L), or thapsigargin (100 nmol/L) for 16 h (Fig. 2*B*). As expected, the *PERK*$^{-/-}$ MEFs were hypersensitive to thapsigargin-induced cell death as shown by a 3-fold decrease in clonogenic survival ($P < 0.005$). Similarly, the *PERK*$^{-/-}$ MEFs showed hypersensitivity to orlistat treatment, showing reduced clonogenic survival of wild-type–transformed MEFs to 70% of vehicle-treated cells. On the other hand, clonogenic survival was decreased nearly 4-fold to <20% ($P < 0.005$) in the *PERK*$^{-/-}$ MEFs following orlistat treatment. These data indicate that the inhibition of FAS activity induces ER stress which is exacerbated by the loss of PERK.

To further support the results obtained in MEFs, we generated stable populations of HT-29 colon carcinoma cells transfected with a dominant negative PERK construct that lacks the kinase domain

(ΔC-PERK) or the corresponding empty vector (23). These cells were seeded at a low density and treated with C75 (9 μg/mL), orlistat (25 μmol/L), or thapsigargin (10 nmol/L) to assess clonogenic survival. As expected, the HT-29 ΔC-PERK cells were hypersensitive to thapsigargin as shown by a nearly 3-fold reduction in clonogenic survival (Fig. 2*C*, $P < 0.005$). Similarly, the HT-29 ΔC-PERK cells were also hypersensitive to both orlistat and C75 compared with the empty vector–transfected cells. Clonogenic survival was reduced >2-fold in C75-treated cells and nearly 4-fold in orlistat-treated cells (Fig. 2*C*, $P < 0.005$). These results confirm that PERK function is important for an adaptive response in tumor cells when FAS activity is inhibited.

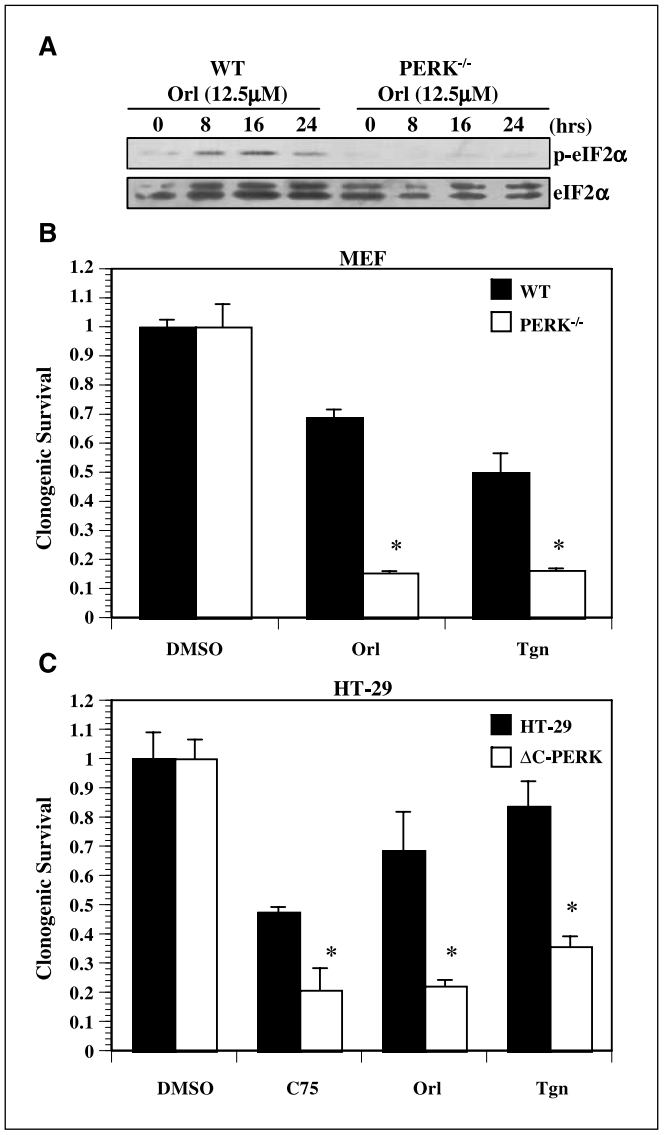

Figure 2. PERK phosphorylates eIF2α in response to, and protects cells against, orlistat treatment. *A,* wild-type (*WT*) and *PERK*$^{-/-}$ MEFs transformed with with *Ki-Ras*^{V12} were treated with orlistat (12.5 μmol/L) for the indicated times. Samples were analyzed with antibodies specific for phospho-eIF2α and total eIF2α. *B,* clonogenic survival assays were done in WT and *PERK*$^{-/-}$ MEFs, treated with vehicle, orlistat (25 μmol/L), or Tgn (100 nmol/L) for 16 h, in triplicate. The surviving fraction was normalized relative to vehicle-treated controls. *C,* HT-29 cells and HT-29/ΔC-PERK cells were treated with vehicle, orlistat (25 μmol/L), C75 (9 μg/mL), or Tgn (10 nmol/L) for 16 h, in triplicate. Clonogenic survival was normalized relative to vehicle-treated controls and statistical significance was determined by two-tailed Student's *t* tests.

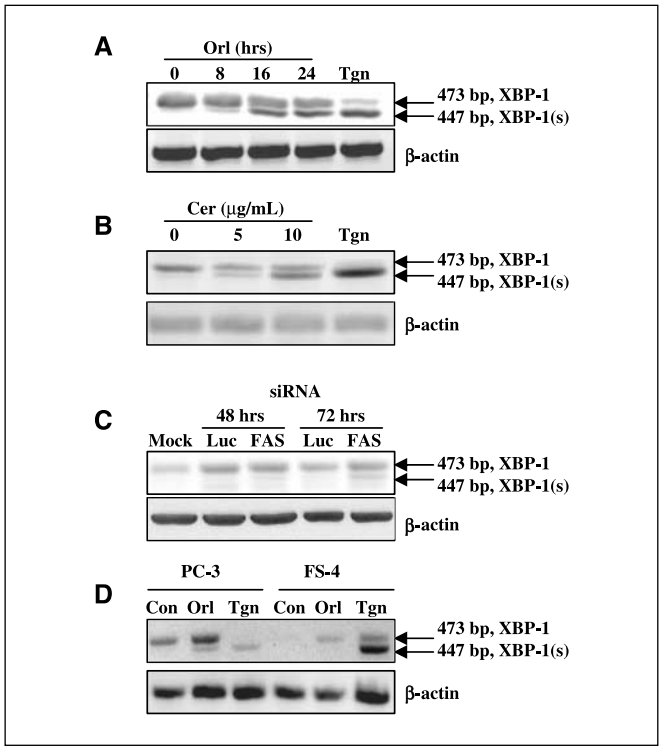

Figure 3. FAS inhibitor treatment activates processing of XBP-1. *A,* PC-3 cells were treated with orlistat (50 μmol/L) for the indicated times or Tgn (500 nmol/L) for 8 h. *B,* PC-3 cells were treated with the indicated concentrations of cerulenin for 16 h or Tgn (500 nmol/L) for 8 h. *C,* HeLa cells were transfected with siRNA against FAS or luciferase (*Luc*) for the indicated times. *D,* PC-3 and FS-4 cells were treated with orlistat (50 μmol/L) for 16 h or Tgn (1 μmol/L) for 1 h. Total RNA was collected and RT-PCR was done as described in experimental procedures. XBP-1 is indicated by the 473 bp product and XBP-1(s) is indicated by the 447 bp fragment.

Treatment with orlistat induces processing of XBP-1. In addition to the PERK-regulated arm, the ER stress response can also be mediated by IRE1, a kinase with endonuclease activity that facilitates the splicing of XBP-1 mRNA to yield the splice variant XBP-1(s) during ER stress (29). To determine whether pharmacologic inhibition of FAS also activates the IRE1 pathway, the status of XBP-1 mRNA was assessed by RT-PCR using oligonucleotides which flank the splice site in the XBP-1 mRNA. Total RNA was collected from PC-3 cells treated with orlistat (50 μmol/L) for 8 to 24 h (Fig. 3*A*) or with cerulenin (5 or 10 μg/mL) for 16 h (Fig. 3*B*). Thapsigargin treatment resulted in a loss of the 473 bp product associated with unspliced XBP-1 and the appearance of the processed 447 bp form associated with XBP-1(s) that is produced only in response to ER stress (Fig. 3*A*). Similarly, orlistat treatment resulted in the appearance of the 447 bp XBP-1(s) product in as few as 8 h with maximum processing by 16 h (Fig. 3*A*). Cerulenin also induced the processing of XBP-1 (Fig. 3*B*). To further confirm the role of FAS inhibition in the processing of XBP-1 to XBP-1(s), HeLa cells were transfected with FAS-specific siRNA or siRNA against luciferase as a negative control and collected for RT-PCR (Fig. 3*C*). The 447 bp fragment that corresponds to the ER stress–specific XBP-1(s) product was detected at 72 h in the FAS-specific siRNA samples. Although PC-3 cells exhibit XBP-1 splicing after both orlistat and thapsigargin treatment, FS-4 cells exhibited only minor induction of XBP-1 and no splicing in response to orlistat (Fig. 3*D*). These results suggest that the IRE1 pathway of the ER stress

response is activated in parallel to the PERK pathway when FAS is inhibited in tumor cells.

Inhibition of FAS activity induces expression of ER stress–regulated genes. Activation of the ER stress response induces the expression of a number of genes associated with adaptation and cell death, including *CHOP, GRP78,* and *ATF4*. CHOP has been implicated in ER stress–dependent apoptosis and $CHOP^{-/-}$ cells are mildly resistant to apoptosis following treatment with ER-stressing agents (30). The mRNA expression of CHOP is induced in DU145 cells treated with orlistat (50 μmol/L) or C75 (9 μg/mL; Fig. 4*A*). Similar effects were seen in PC-3 cells (data not shown). CHOP mRNA expression was also induced following siRNA-mediated knockdown of FAS expression in PC-3 cells (Fig. 4*B*). Increased mRNA expression of CHOP may indicate that ER stress could play a role in cell death induced by FAS inhibition (30). The mRNA expression of GRP78, an ER stress–regulated chaperone, is also induced in orlistat-treated cells (Fig. 4*C*; ref. 31). Furthermore, mRNA expression of the transcription factor ATF4, which is dependent on eIF2α phosphorylation, was also induced by orlistat treatment (Fig. 4*C*). Collectively, the data in Figs. 1–4 show that FAS inhibitors induce the ER stress response.

ER stress is an early event in cells treated with FAS inhibitors. To determine whether phosphorylation of eIF2α and subsequent indications of the ER stress response events coincide or precede cell death induced by FAS inhibition, we analyzed the temporal phosphorylation of eIF2α and markers of caspase activity and cell death. In PC-3 cells treated with orlistat, phosphorylation of eIF2α was evident at 16 h of treatment, whereas significant cleavage of caspase 3 and PARP are not detectable until 24 and 48 h, respectively, consistent with previous reports (13). These data

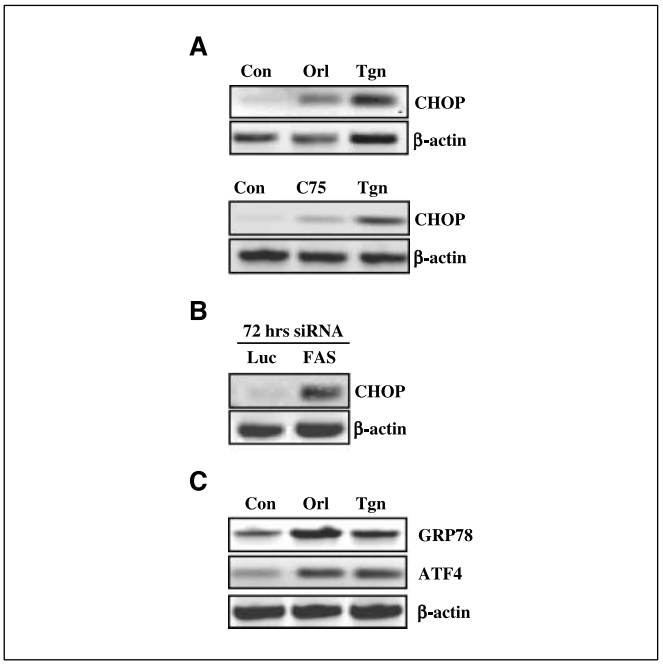

Figure 4. Inhibition of FAS activity induces mRNA expression of ER stress–regulated genes. *A,* DU145 cells were treated with vehicle control, orlistat (50 μmol/L, *top*), C75 (9 μg/mL, *bottom*), or Tgn (500 nmol/L) for 16 h. *B,* PC-3 cells were transfected with siRNA against FAS or luciferase (*Luc*) for 72 h. *C,* PC-3 cells were treated with vehicle control or orlistat (25 μmol/L) for 24 h or Tgn (1 μmol/L) for 1 h. Total RNA was collected with TRIzol and semiquantitative RT-PCR was done with oligonucleotides specific for CHOP (*A* and *B*), GRP78 and ATF4 (*C*), or β-actin (*A–C*).

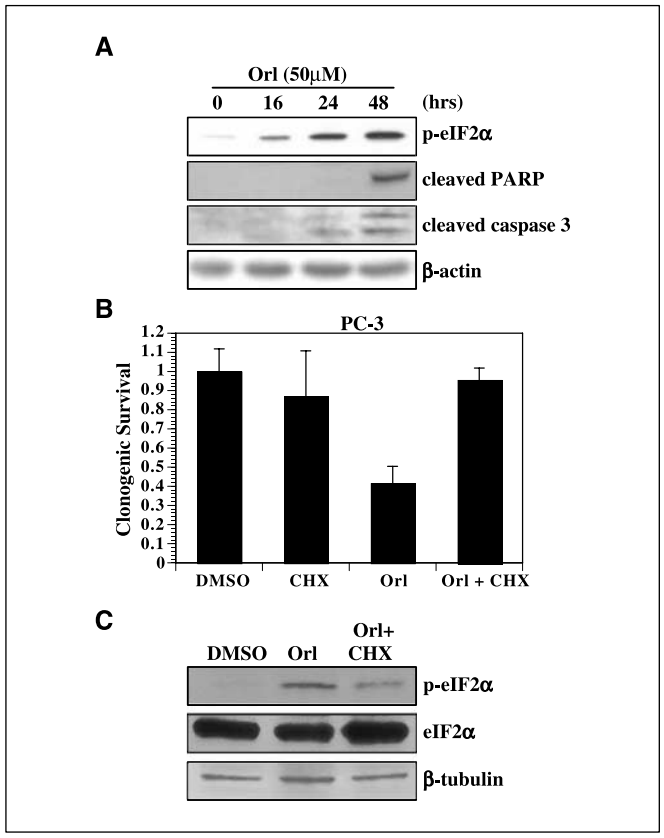

Figure 5. Phosphorylation of eIF2α is an early event in cells treated with FAS inhibitors. *A,* PC-3 cells were treated with 50 μmol/L of orlistat for the indicated times and samples were collected for immunoblot analysis and probed with antibodies specific for phospho-eIF2α, cleaved PARP, cleaved caspase 3, and β-actin. *B,* PC-3 cells were treated with DMSO, cycloheximide (CHX, 1 μg/mL), orlistat (25 μmol/L), or orlistat with cycloheximide for 16 h. Clonogenic survival was normalized relative to vehicle-treated controls. *C,* PC-3 cells were treated with DMSO, cycloheximide (1 μg/mL), orlistat (25 μmol/L), or the combination of orlistat and cycloheximide for 16 h. Samples were collected for immunoblot analysis and probed with antibodies specific for phospho-eIF2α, total eIF2α, and β-tubulin.

indicate that the ER stress response is induced prior to caspase 3 activation and PARP cleavage. These data are further supported by the timeline of XBP-1 processing following orlistat treatment (Fig. 3*A*). Previous studies have shown that inhibition of protein translation with cycloheximide can ameliorate the effects of ER stress, likely by decreasing protein burden on the ER (28). Clonogenic survival assays and immunoblot analysis were done in PC-3 cells treated with vehicle control, cycloheximide (1 μg/mL), orlistat (25 μmol/L), or orlistat with cycloheximide (Fig. 5*B* and *C*). Cycloheximide treatment rescued clonogenic survival of PC-3 cells treated with orlistat (Fig. 5*B*), and also inhibited orlistat-induced phosphorylation of eIF2α (Fig. 5*C*). These data are consistent with previous reports on the protective effect of cycloheximide on cells under ER stress and suggest that reduced protein burden on the ER or reduced expression of a specific proapoptotic factor could be responsible.

Cooperation between FAS inhibitors and thapsigargin. At least one study has shown that ER stress can have a negative effect on the activity of chemotherapeutic drugs (32). To determine the effect of ER stress on FAS inhibitor–induced cell death, clonogenic survival assays, immunoblot, and RT-PCR analysis were done on tumor cells treated with orlistat or C75 in combination with the ER

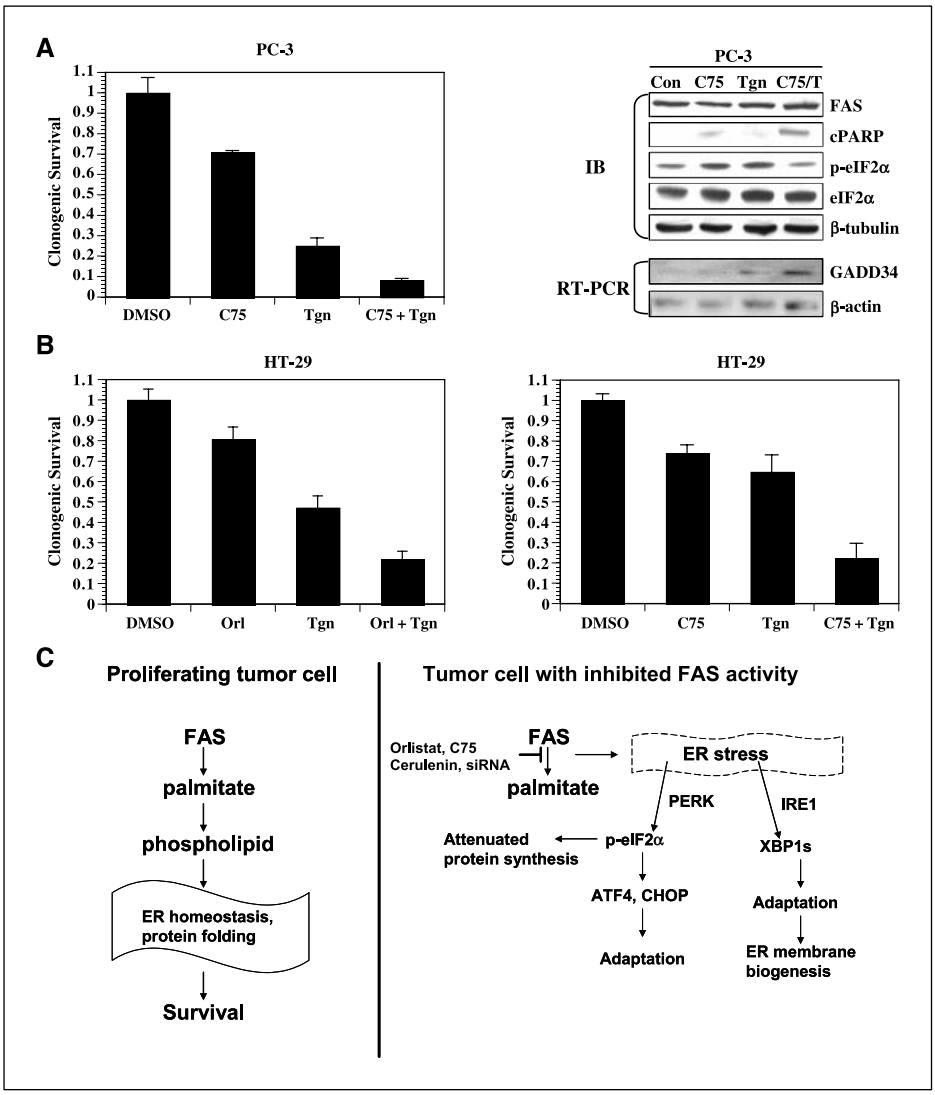

Figure 6. Pharmacologic FAS inhibitors cooperate with thapsigargin. *A,* PC-3 cells were treated with DMSO, C75 (9 μg/mL), Tgn (25 nmol/L), or the combination of each for 12 h and clonogenic survival was normalized relative to vehicle-treated controls (*left*). Times and doses of these various experiments were selected to achieve minimal cell death from single agents, so that the effect of the combination would be most clear. PC-3 cells were treated with DMSO, C75 (9 μg/mL), Tgn (25 nmol/L), or the combination of both for 20 h and samples were subjected to immunoblot analysis (*top right*), or RNA was collected and semiquantitative RT-PCR was done using primers specific for GADD34 or β-actin (*bottom right*). *B,* HT-29 were treated with DMSO, orlistat (25 μmol/L), Tgn (25 nmol/L), or the combination of each for 12 h (*left*). Cells were treated with DMSO, C75 (9 μg/mL), Tgn (25 nmol/L), or the combination of each for 12 h (*right*). Clonogenic survival was normalized relative to vehicle-treated controls. *C,* model demonstrating that in a proliferating tumor cell, FAS contributes to ER function by driving phospholipid synthesis (*left*). When FAS is inhibited (*right*), ER stress is induced, which activates a series of downstream events that mediate adaptation and promote ER membrane biogenesis.

stressing agent, thapsigargin (Fig. 6*A* and *B*). Lower doses of FAS inhibitors and thapsigargin were used to achieve reduced cell killing by either single agent in order to maximize the effect of the combination of the two drugs. PC-3 cells were seeded at a low density and treated with C75 (9 μg/mL), thapsigargin (25 nmol/L), or the combination of both for 12 h and assayed for clonogenic survival. The clonogenic survival of cells treated with the combination of drugs was significantly reduced compared with either agent alone (Fig. 6*A, left*). This coincides with immunoblot data demonstrating that levels of cleaved PARP are highest in lysates from cells treated with both drugs (Fig. 6*A, top right*). Interestingly, whereas both C75 and thapsigargin induced the phosphorylation of eIF2α separately, the level of phosphorylated eIF2α was significantly reduced in cells treated with the two agents combined, with no change in total eIF2α levels (Fig. 6*A, top right*). This data suggests that the combined agents facilitated a more rapid progression of the ER stress response and induction of the GADD34 feedback loop (33). In support of this, mRNA expression of GADD34 was increased in PC-3 cells treated with both agents, as compared with either agent alone (Fig. 6*A, bottom right*). Confirming that the effects of this combination of drugs are not cell type–specific, clonogenic survival was assessed in HT-29 cells treated with one of two combinations: either (*a*) orlistat (25 μmol/L), thapsigargin (25 nmol/L), or the combination of both for 12 h (Fig. 6*B, left*); or (*b*) C75 (9 μg/mL), thapsigargin (25 nmol/L), or the combination of both for 12 h (Fig. 6*B, right*). Although clonogenic survival only indicates an additive interaction, these data, importantly, show that ER stress does not inhibit the actions of FAS inhibitors and may actually enhance their efficacy.

Discussion

The ER stress response is a choreographed series of cellular events activated by specific insults that result in altered ER function (26, 34). The combined effect of this response is the activation of genes that are specifically expressed to engage an adaptation protocol. Upon prolonged stress, the adaptation mechanism of the ER stress response is saturated, thus, activating cell death. Several studies have developed important connections between lipid synthesis pathways and the ER stress response (19–21, 35). Inhibition of phospholipid synthesis, especially that of phosphatidylcholine, induces ER stress–related pathways (20). Similarly, altering phospholipid metabolism by manipulation of phospholipase activity amplifies the ER stress response in β-cells

(36). In addition, the accumulation of the G_{M1}-ganglioside activates the ER stress response in neurons, indicating that sphingolipid levels are also important for regulating ER function (35). Recent studies have shown that cholesterol-induced apoptosis in macrophages is triggered by ER stress induction, and small molecule inhibitors of cholesterol synthesis activate the integrated stress response (24, 25). There are also direct links between the individual ER stress components and lipid synthesis pathways. For instance, overexpression of the ER stress–specific XBP-1(s) expands ER volume by increasing the activity of enzymes responsible for phosphatidylcholine synthesis (19). Moreover, mice that are null for *XBP-1* have underdeveloped ER (37, 38). Collectively, these data show that lipid and sterol levels are important for maintaining ER function.

Because FAS inhibitors are being developed as antitumor agents, it is important to understand the effects these drugs have on both normal and tumor cells. The evidence here shows that FAS inhibitors induce the ER stress response in a variety of tumor cells, but not in normal cells. A model summarizing these data is presented in Fig. 6*C*. Our results suggest that in a proliferating tumor cell, FAS activity drives phospholipid synthesis, which facilitates ER homeostasis and function. When FAS activity is inhibited in tumor cells, the result is PERK-dependent phosphorylation of eIF2α, a concomitant attenuation of protein synthesis and the IRE1-mediated processing of XBP-1. Interestingly, phosphorylation of eIF2α persists for as long as 48 h with no attenuation (Fig. 5*A*), suggesting that protein phosphatase 1 activity is not activated or that GADD34 is not induced as is evidenced by the lack of expression in Fig. 6*A*. Downstream of PERK and IRE1 activation, inhibiting FAS activity also induces mRNA expression of canonical markers of the ER stress response pathway including CHOP, GRP78, and ATF4. The precise role of these players has not been determined in cells that have been treated with FAS inhibitors. However, it has previously been shown that ATF4 acts to protect cells from ER-generated reactive oxygen species (40). Another report showed that siRNA-mediated knockdown of FAS or acetyl CoA carboxylase in breast cancer cells results in cell death that is mediated by reactive oxygen species and is attenuated by supplementation with the antioxidant vitamin E, which suggests that the ER stress response in general, and ATF4 expression specifically, may be a response to changes in the redox status of FAS inhibitor–treated cells (18). These data are consistent with the hypothesis that the ER stress response initially acts to protect cells from FAS inhibitors, but do not rule out that ER stress could also facilitate cell death after prolonged stress.

It is interesting to note that several indicators of ER stress, including phosphorylation of eIF2α, inhibition of protein synthesis, and XBP-1 processing are detected well before the canonical hallmarks of apoptosis, cleaved caspase 3 and cleaved PARP. This indicates that the ER may be an early sensor of fatty acid and phospholipid levels in tumor cells. When the ER stress response is unable to fully restore ER function perturbed by FAS inhibition, it is possible that this could lead to the initiation of a cell death program. Consistent with this notion, cycloheximide is able to inhibit the orlistat-induced cell death and phosphorylation of eIF2α. These data do not conflict with previous reports demonstrating that FAS inhibitors activate the intrinsic cell death pathway and that ceramide accumulation contributes to FAS inhibitor–induced cell death (34, 41). In fact, a previous study showed that ceramide accumulation was also associated with thapsigargin-induced ER stress and apoptosis (36). It is possible that FAS inhibitor–induced ER stress may result in ceramide accumulation which is important for cell death; however, the relationship between FAS inhibition, ER stress, and subsequent downstream events remain to be determined. Given the importance of phospholipid synthesis during S phase of the cell cycle, showing that FAS inhibitors induce ER stress in tumor cells also compliments a previous study which established that FAS inhibitors induce apoptosis during S phase (42, 43). Collectively, the data presented herein fill a critical gap in our understanding of how endogenous fatty acid synthesis is required to maintain proper ER integrity and function.

We have shown that inhibiting FAS activity in tumor cells induces an ER stress response. Based on these findings, we propose that one teleologic explanation for high FAS levels in tumors is to provide support for a dynamic ER in rapidly proliferating cells. Furthermore, we hypothesize that the ER acts as a sensor of FAS activity and resulting phospholipid levels. In addition, we show that orlistat and C75 cooperate with the ER stressing agent thapsigargin to enhance cell death *in vitro*. Although thapsigargin is highly toxic and not a likely candidate for tumor therapy, these data imply that tumor microenvironment–induced ER stress will not hinder the efficacy of FAS inhibitors (44). The data also suggests that FAS inhibitors might be combined with PERK inhibitors to enhance tumor cell cytotoxicity. In summary, these data provide the first evidence that FAS inhibitors induce ER stress, which may explain some antitumor effects of FAS inhibitors, and they also establish an important mechanistic link between FAS and ER function in tumor cells.

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