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Endoplasmic reticulum stress causes the activation of sterol regulatory element binding protein-2

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Abstract

Background: Sterol regulatory element binding protein-2 (SREBP-2) is a membrane-bound transcription factor that upon proteolytic processing can activate the expression of genes involved in cholesterol biosynthesis and uptake. We as well as others have demonstrated that the accumulation of misfolded proteins within the endoplasmic reticulum (ER), a condition known as ER stress, can dysregulate lipid metabolism by activating the SREBPs. The purpose of this study was to determine the mechanism by which ER stress induces SREBP-2 activation.
Methods and results: HeLa and MCF7 cells were treated with ER stress-inducing agents to determine the effect of ER stress on SREBP-2 cleavage and subsequent cholesterol accumulation. Cells treated with thapsigargin (Tg) exhibit proteolytic cleavage of SREBP-2. Proteolytic cleavage of SREBP-2 induced by Tg occurred independently of caspase activation and was inhibited by the site-1 protease inhibitor AEBSF, suggesting that Tg-induced SREBP-2 cleavage occurs through the conventional site-1/-2 pathway. Treatment of HeLa cells with Tg also led to the accumulation of free cholesterol as measured by Filipin staining.
Conclusions: These results imply that ER stress-induced SREBP-2 activation occurs through the conventional pathway that normally regulates SREBP in accordance with intracellular sterol concentration.

Keywords: SREBP; ER stress; UPR; Cholesterol; Caspases

1. Introduction

Sterol regulatory element binding proteins (SREBPs) are transcription factors synthesized as inactive precursors bound to the endoplasmic reticulum (ER) membrane responsible for the upregulation of genes involved in cholesterol synthesis. SREBP-1a and -2 are the predominant isoforms in most cultured cell lines, whereas SREBP-1c and -2 are found in the majority of tissues (Shimomura, Shimano, Horton, Goldstein, & Brown, 1997). SREBP-1a and -2 are responsible for the upregulation of genes involved in cholesterol synthesis. SREBP-1c is preferentially responsible for the upregulation of genes involved in the fatty acid biosynthesis pathway (Horton, Goldstein, & Brown, 2002). The SREBPs are localized in the ER through their interaction with SREBP cleavage activating protein (SCAP) (Hua, Nohturfft, Goldstein, & Brown, 1996) and Insig (Yabe, Brown, & Goldstein, 2002; Yang et al., 2002). SCAP forms a complex with the SREBPs and is essential for their activation. Cells that lack SCAP require the addition

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of exogenous free cholesterol for survival (Rawson, DeBose-Boyd, Goldstein, & Brown, 1999). Insig is an ER-resident protein anchor that binds SCAP and maintains the ER localization of the SCAP–SREBP complex. Upon cellular sterol depletion, cholesterol dissociates from the sterol-sensing domain of SCAP, releasing Insig and allowing SCAP–SREBP to exit the ER through COPII vesicle transport (Sun, Li, Goldstein, & Brown, 2005). SCAP transports SREBP to the Golgi where the site-1-serine protease (S1P) and site-2-zinc metalloproteinase (S2P) release the active SREBP transcription factor. Upon sterol depletion and the initiation of the SREBP proteolytic pathway, the active transcription factor translocates to the nucleus causing SREBP-associated gene expression that encodes for enzymes in the cholesterol/triglyceride biosynthesis and uptake pathways (Goldstein, DeBose-Boyd, & Brown, 2006).

In addition to the location of newly synthesized SREBP, the ER is the principal site for folding and maturation of transmembrane, secretory and ER-resident proteins (Lee, 2001). The ER contains a high level of protein chaperones such as GRP78, GRP94, and calreticulin to assist in the correct folding of synthesized proteins and to prevent the accumulation of misfolded proteins. Disruption in ER function that interferes with proper folding and maturation of proteins causes ER stress and initiates the unfolded protein response (UPR), an integrated intracellular signalling pathway that induces temporary translational inhibition followed by upregulation of ER chaperones. The UPR is mediated via three ER-resident sensors: a type-I ER transmembrane protein kinase (IRE-1), activating transcription factor 6 (ATF-6) and the PKR-like ER kinase (PERK). Activation of these three sensors is mediated by the dissociation of GRP78 following ER stress (Lawrence de Koning, Werstuck, Zhou, & Austin, 2003; Rutkowski & Kaufman, 2004). As a result, the UPR enhances cell survival by ensuring that the adverse effects of ER stress are dealt with in a timely and efficient manner. Although the UPR may provide a protective advantage for the cell, prolonged or severe ER stress can result in caspase activation and apoptosis (Morishima, Nakanishi, Takenouchi, Shibata, & Yasuhiko, 2002; Nakagawa et al., 2000).

We as well as others have reported that conditions that cause ER stress or apoptosis, induce SREBP activation, independent of intracellular cholesterol content (Higgins & Ioannou, 2001; Lee & Ye, 2004; Pai, Brown, & Goldstein, 1996; Wang et al., 1995, 1996; Werstuck et al., 2001). Previous studies also found that caspase-3 and -7 cleave the SREBPs following apoptotic stimuli in a sterol-independent manner (Pai et al., 1996; Wang et al., 1995, 1996). The suggested caspase cleavage site is located on the N-terminal, cytoplasmic side of SREBP (Pai et al., 1996; Wang et al., 1995, 1996). These studies revealed that caspase-3/-7 cleaves SREBP at a site other then S1P/S2P. In support of these findings, Higgins and Ioannou (2001) found that the caspase-induced SREBP cleavage was transcriptionally active, caused expression of reporter constructs, and occurred very early in the apoptotic process (Higgins & Ioannou, 2001). Since conditions that cause ER stress can lead to caspase activation and SREBP cleavage, studies were initiated to determine whether ER stress may cause SREBP activation and subsequent cholesterol accumulation through a caspase-dependent mechanism. In the present study, we demonstrate that ER stress induces SREBP activation and lipid accumulation independent of caspase activation. Our findings also suggest that ER stress-mediated SREBP activation occurs through the conventional S1P/S2P proteolytic pathway.

2. Materials and methods

2.1. Cell culture conditions

The human breast adenocarcinoma cell line MCF7, stably transfected to express caspase-3 (MCF7/cas3) or vector control (MCF7/pbabe), was kindly provided by Dr. Damu Tang (Department of Medicine, McMaster University). The human cervical carcinoma cell line HeLa was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cell lines were cultured in DMEM (ATCC; Manassas, VA) containing 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin. Cell lines were cultured in a humidified incubator at 37 °C with 5% CO_2.

2.2. Cell lysis

Cell lysates were prepared by the addition of 4× SDS-PAGE buffer containing 25 μg/ml of the proteasome inhibitor, *N*-acetyl-leucinal-leucinal-norleucinal (ALLN) (Calbiochem) and a protease inhibitor cocktail (Roche). Prior to Western Blot analysis, lysates were subjected to the Bio-Rad Dc Protein Assay to determine the protein concentration and stored at −20 °C.

2.3. Western blot analysis

The anti-SREBP-2 (IgG-1C6) monoclonal antibody was purchased from BD Pharmingen (Mississauga, Ont.). The anti-KDEL monoclonal antibody, which

recognizes GRP78, GRP94 and HSP47, was purchased from Stressgen Biotechnologies (Victoria, BC). The anti-caspase-3 monoclonal antibody, which recognizes full-length caspase-3, was purchased from Transduction Laboratories (BD Pharmingen, Mississauga, Ont.). The anti-β-actin monoclonal antibody used as a loading control for Western blots was purchased from Sigma (Oakville, Ont.). Following protein lysate preparation, 50 μg of the protein lysate was separated on SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes. Following incubation with the appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (DakoCytomation Inc., Mississauga, Ont.), the membranes were developed using the Renaissance Chemiluminescence Reagent kit (Perkin-Elmer, Boston, MA). Nitrocellulose membranes were exposed to film (Kodak, X-Omat Blue Film) for 10 min (SREBP-2, Caspase-3) or 10 s (β-actin).

2.4. Transient transfection of HeLa cells with pSRE-GFP

HeLa cells were transiently transfected with a green fluorescent protein (GFP) reporter plasmid under the control of the sterol regulatory element (SRE) (kindly provided by Dr. Yiannis A. Ioannou, Mount Sinai School of Medicine). HeLa cells were transfected with 1 μg of pSRE-GFP using Effectene transfection reagent (Qiagen, Mississauga, Ont.) for 24 h. Fol-

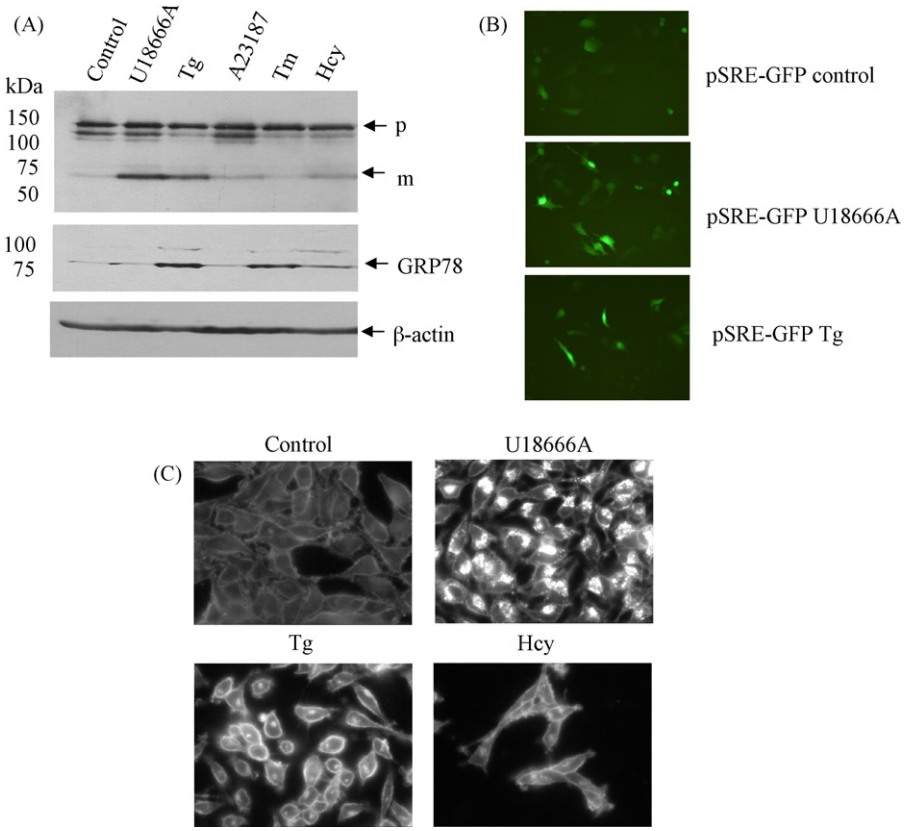

Fig. 1. ER stress agents activate SREBP-2, induce SRE-controlled gene expression, and increase free cholesterol accumulation in HeLa cells. (Panel A) Treatment of HeLa cells with ER stress agents cause SREBP cleavage and activation. HeLa cells were treated with various ER stress agents, including thapsigargin (Tg; 0.01 μM), A23187 (1 μM), tunicamycin (Tm; 5 μg/ml), and homocysteine (Hcy; 5 mM). As a positive control for SREBP activation, cells were treated with U18666A (2 μg/ml). Following incubation for 18 h, protein lysates were subjected to immunoblot analysis using antibodies against SREBP-2 (to assess SREBP activation) or GRP78 (to assess ER stress). p, ER membrane-bound precursor form of SREBP-2. m, mature active form of SREBP-2. (Panel B) HeLa cells were transiently transfected with the reporter plasmid pSRE-GFP using Effectene transfection reagent. Transiently transfected HeLa/pSRE-GFP cells were treated with U18666A (2 μg/ml) or Tg (0.01 μM) for 18 h and examined for SRE-controlled GFP expression. (Panel C) HeLa cells were treated with U18666A (2 μg/ml) as a positive control for free cholesterol accumulation or the ER stress-inducing agents, Tg (0.01 μM) or Hcy (5 mM), for 24 h. Following treatment, cells were stained with Filipin to examine intracellular free cholesterol content.

lowing transient transfection, HeLa/pSRE-GFP cells were treated with 2 μg/ml 3β-(2-diethylaminoethoxy)-androstenone•HCl (U18666A) (BIOMOL International, Plymouth Meeting, PA) as a positive control for free cholesterol accumulation or the ER stress-inducing agent thapsigargin (Tg; 0.01 μM) for 18 h. Following treatment, GFP expression was visualized by fluorescence microscopy.

2.5. Filipin staining

HeLa cells were cultured on coverslips and incubated for 24 h with either 2 μg/ml U18666A, 0.01 μM Tg, 5 μg/ml tunicamycin (Tm), or 5 mM homocysteine (Hcy). Cells were washed three times with phosphate buffer saline (PBS) and fixed with 3% paraformaldehyde for 1 h at room temperature (RT). Following fixation, cells were washed with PBS and incubated with Filipin (Sigma) for 2 h at RT. Stained cells were washed three times with PBS and Filipin-cholesterol complexes were visualized by fluorescence microscopy. Relative fluorescence was quantified using Sigma Scan Pro 5 software, and results were normalized to total cell count.

3. Results

3.1. ER stress agents activate SREBP-2, induce SRE-controlled gene expression and increase intracellular free cholesterol

To determine whether various agents known to induce ER stress could activate SREBP-2, HeLa cells were treated with ER stress-inducing agents, including thapsigargin (Tg), A23187, tunicamycin (Tm) and homocysteine (Hcy). As a positive control for SREBP-2 activation, the lysosomal cholesterol efflux inhibitor U18666A was used to block cholesterol transport to the ER. Although the various ER stress-inducing agents activated SREBP-2 to different degrees, Tg-induced SREBP-2 cleavage was similar to that observed for U18666A (Fig. 1A). To determine whether the Tg-dependent cleaved SREBP-2 was transcriptionally active, HeLa cells were transfected with the pSRE-GFP reporter plasmid and treated with either Tg or U18666A. Both U18666A and Tg increased GFP expression compared to untreated cells (Fig. 1B). Since the downstream effect of SREBP-2 activation is cellular lipid accumulation, HeLa cells were treated with Tg

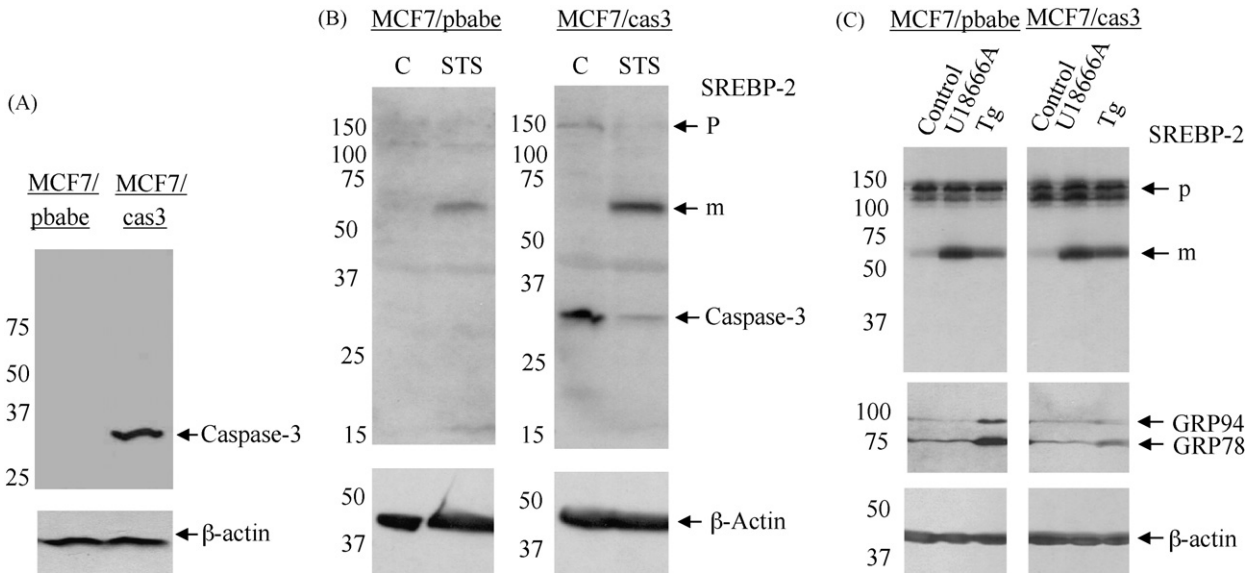

Fig. 2. Thapsigargin and staurosporine increases SREBP-2 cleavage in MCF7 cells. (Panel A) MCF7 cells that do not express caspase-3 (MCF7/pBabe) were stably transfected to overexpress caspase-3 (MCF7/cas3). (Panel B) Treatment of MCF7 cells with staurosporine (STS) induces SREBP cleavage. MCF7/pBabe or MCF7/cas3, which overexpress functional caspase-3, were treated with staurosporine (1 μM) for 4 h. Total cell lysates were subjected to immunoblot analysis using antibodies against SREBP-2 (to assess SREBP activation) or caspase-3 (to assess caspase-3 activation). p, ER membrane-bound precursor form of SREBP-2. m, mature active form of SREBP-2. (Panel C) Treatment of MCF7 cells with Tg increases SREBP cleavage. MCF7/pbabe or MCF7/cas3, which overexpress functional caspase-3, were treated with U18666A (2 μg/ml) or Tg (0.01 μM) for 18 h. Protein lysates were subjected to immunoblot analysis using antibodies against SREBP-2 (to assess SREBP activation) or GRP78 (to assess ER stress).

and stained with Filipin to assess whether ER stress-induced SREBP-2 cleavage is functional. HeLa cells treated with Tg or Hcy had increased free cholesterol associated with membranes compared to the characteristic lysosomal accumulation seen with U18666A treatment (Fig. 1C). The results in Fig. 1B and C are consistent with the results seen in panel 1A and provide evidence that ER stress-induced SREBP-2 activation is functional and leads to lipid accumulation.

3.2. ER stress-induced SREBP-2 cleavage is not dependant on caspase-3

It was previously shown that caspase-3 could activate SREBP-2 in a sterol-independent process (Higgins & Ioannou, 2001; Pai et al., 1996; Wang et al., 1995, 1996). MCF7 cells contains a mutation in the caspase-3 gene causing complete loss of caspase-3 expression (Janicke, Sprengart, Wati, & Porter, 1998). Since caspase-3 has been shown to induce SREBP-2 cleavage in response to apoptotic stimuli, MCF7 cells stably transfected to express functional caspase-3 (Fig. 2A) were used to examine the effects of caspase-3 on ER stress-induced SREBP-2 cleavage. MCF7/pBabe and MCF7/cas3 cells were treated with staurosporine to examine SREBP-2 cleavage. Although staurosporine caused SREBP-2 cleavage in MCF7/pbabe cells, it activated caspase-3 and induced SREBP-2 cleavage to a much greater degree in MCF7/cas3 cells (Fig. 2B). MCF7/pbabe and MCF7/cas3 cells were treated with U18666A or Tg to induce SREBP-2 cleavage and it was observed that the absence of caspase-3 in MCF7 cells had no effect on Tg-induced SREBP-2 cleavage (Fig. 2C).

3.3. Thapsigargin-induced SREBP-2 cleavage is not mediated by caspases

To determine whether other caspases are involved in SREBP-2 activation during ER stress, HeLa cells were pretreated with the pan-caspase inhibitor ZVAD-fmk and exposed to either staurosporine or Tg to induce caspase activation (staurosporine) or ER stress (Tg), respectively. Although ZVAD-fmk decreased staurosporine-induced caspase-3 and SREBP-2 cleavage, it had no effect on Tg-induced SREBP-2 processing (Fig. 3). In addition to ZVAD-fmk, inhibitors specific for caspase-1, caspase-7 or calpain did not inhibit Tg-induced SREBP-2 activation (data not shown).

3.4. S1P inhibitor blocks thapsigargin-induced SREBP-2 cleavage

The effects of ER stress agents on SREBP-2 cleavage occur independent of caspase activation and can be blocked by the serine protease inhibitor Pefabloc (AEBSF), a known inhibitor of S1P (Okada et al., 2003). MCF7 cells were treated with AEBSF in the presence of U18666A or Tg to induce SREBP-2 cleavage. AEBSF inhibited U18666A and Tg-induced SREBP-2 cleavage (Fig. 4A). In contrast, staurosporine-induced SREBP activation in HeLa cells was not blocked by AEBSF (Fig. 4B). Based on these findings, we have hypothesized that ER stress induces SREBP-2 activation through a mechanism independent of caspase activation and likely involves the expression and/or activity of the known cellular components that regulate SREBP-2 activation.

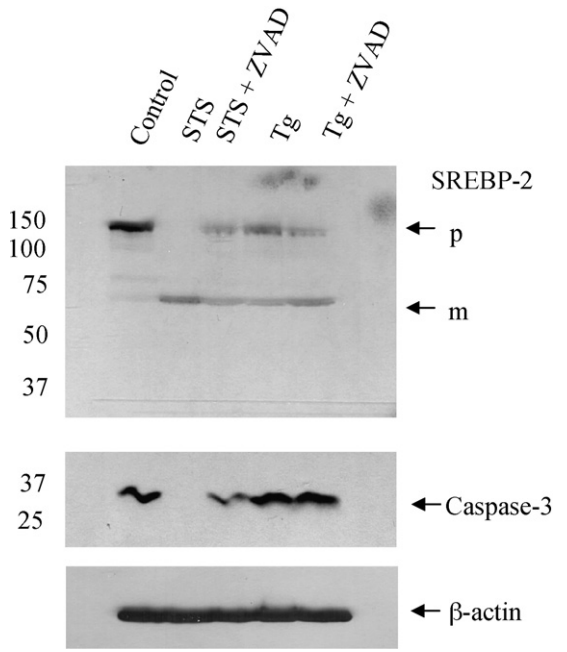

Fig. 3. Thapsigargin-induced SREBP cleavage is not mediated by caspases. Thapsigargin induces SREBP activation in the presence of a pan-caspase inhibitor, ZVAD-fmk. Although ZVAD decreased staurosporine (STS)-induced SREBP and caspase-3 activation, it had no effect on Tg-induced SREBP processing. HeLa cells pretreated for 2 h with the pan-caspase inhibitor ZVAD-fmk (100 μM) were treated with 1 μM (STS) or 0.5 μM Tg for 4 h to examine SREBP processing. Protein lysates were subjected to immunoblot analysis using antibodies against SREBP-2 (to assess SREBP activation) or caspase-3 (to assess caspase activation). p, ER membrane-bound precursor form of SREBP-2. m, mature active form of SREBP-2.

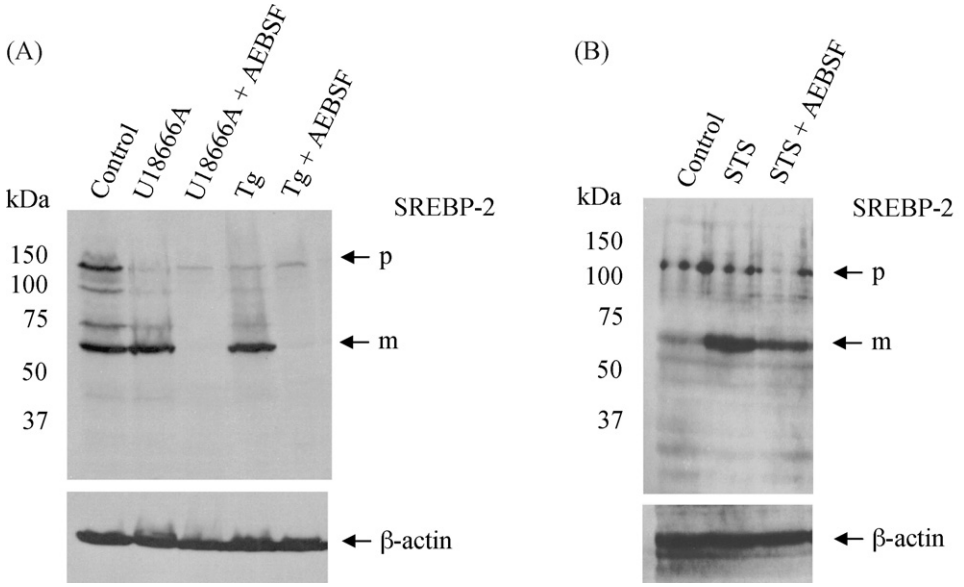

Fig. 4. The serine protease inhibitor, AEBSF, blocks thapsigargin but not staurosporine-induced SREBP-2 activation. Thapsigargin-induced SREBP-2 activation is inhibited by the site-1, serine protease inhibitor AEBSF. (Panel A) MCF7 cells were pretreated with or without the serine protease inhibitor AEBSF (0.3 mM) for 2 h. MCF7 cells were then treated with U18666A (2 μg/ml), or thapsigargin (0.1 μM) for 18 h. (Panel B) HeLa cells were pretreated with or without AEBSF (0.3 mM) for 2 h. HeLa cells were then treated with staurosporine (STS; 1 μM) for 4 h. Protein lysates were subjected to immunoblot analysis using antibodies against SREBP-2. p, ER membrane-bound precursor form of SREBP-2. m, mature active form of SREBP-2.

3.5. AEBSF inhibits ER stress-induced cholesterol accumulation

To provide further evidence that ER stress induces SREBP-2 activation through the conventional S1P/S2P proteolytic pathway, HeLa cells were treated with the S1P serine protease inhibitor (AEBSF) in the presence of ER stress-inducing agents. When HeLa cells were treated with U18666A, free cholesterol accumulated in a punctate pattern compared to the membrane-like incorporation seen with Tg or Tm (Fig. 5). The degree of cholesterol accumulation is consistent with the increase in SREBP cleavage observed in Fig. 1. HeLa cells incubated in the presence of AEBSF had decreased free cholesterol accumulation as measured by Filipin staining (Fig. 5). This experiment provides additional evidence that ER stress-induced SREBP-2 activation and cholesterol accumulation occurs through the conventional S1P/S2P proteolytic pathway.

4. Discussion

Activation of the SREBP-2 pathway leading to cholesterol biosynthesis and uptake is a well-defined response to decreased membrane cholesterol content. How SREBP-2 is activated under apoptotic (Higgins & Ioannou, 2001; Pai et al., 1996; Wang et al., 1995, 1996) or ER stress conditions (Lee & Ye, 2004; Werstuck et al., 2001) is not well understood. Our findings provide evidence that apoptosis and ER stress activate SREBP-2 by distinctly different mechanisms. We provide evidence that ER stress-induced SREBP-2 activation and downstream cholesterol accumulation does not require caspase activation and can be inhibited by disrupting the conventional SREBP-2 proteolytic pathway involving S1P and S2P.

Disruption in ER function that interferes with proper folding and maturation of proteins causes ER stress and initiates the UPR, an intracellular signalling pathway that induces temporary translational inhibition followed by upregulation of ER chaperones. Recent studies (Lee & Ye, 2004) report that cellular stress from hypotonic medium or Tg, induces translation inhibition and Insig degradation. In the absence of cholesterol, Insig degradation allows the SCAP–SREBP-2 complex to exit the ER and be activated through the conventional SREBP-2 proteolytic pathway (Gong et al., 2006). Others have observed that increased cell volume caused by hypotonic medium blocks ER to Golgi transport but retrograde transport remains active, resulting in the collapse of the ER intermediate Golgi compartment (ERIGC) and the Golgi into the ER (Lee & Linstedt, 1999). It has also

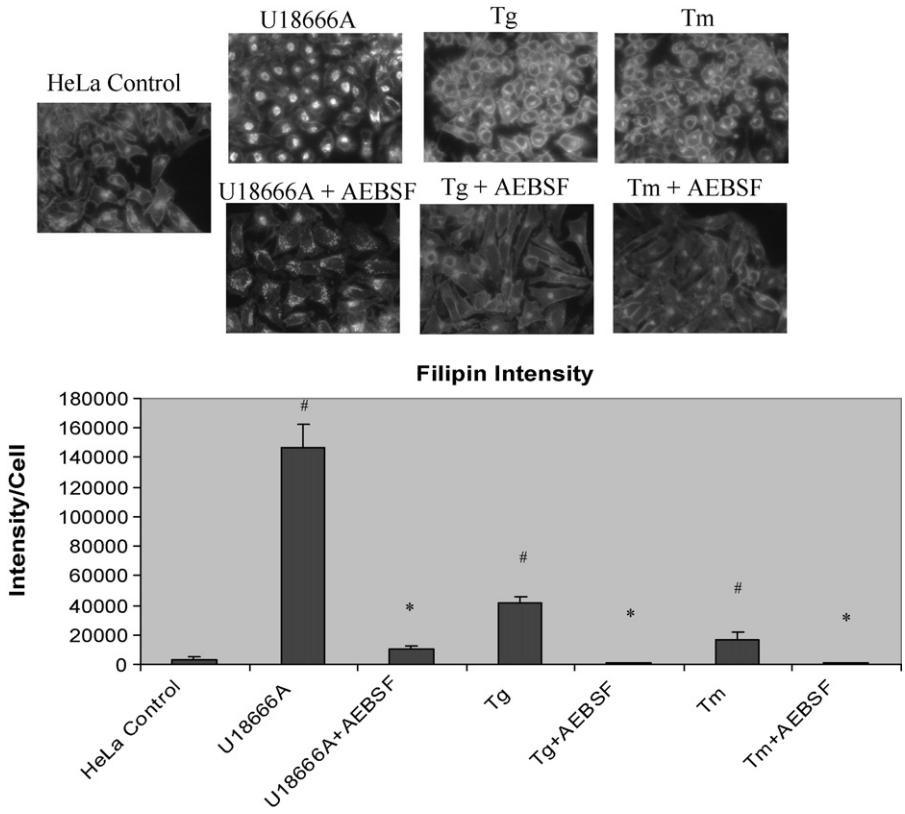

Fig. 5. The serine protease inhibitor, AEBSF, blocks cholesterol depleted and ER stress-induced lipid accumulation. HeLa cells were incubated for 18 h with U18666A (2 μg/ml), thapsigargin (0.3 μM), or tunicamycin (2 μg/ml) in the presence or absence of AEBSF (0.3 mM). Following treatment, cells were washed, fixed, and stained with Filipin to examine free cholesterol accumulation. Cellular free cholesterol content was measure using Sigma Scan Pro 5 software. Filipin intensity was determined by intensity per number of cells in measured field (# $p < 0.05$, treatment compared to control; * $p < 0.05$, treatment plus AEBSF compared to treatment alone).

been observed that SREBP-2 activation can be induced by brefeldin A, which induces a collapse of the Golgi into the ER allowing the S1P/S2P proteases to cleave SREBP (DeBose-Boyd et al., 1999). This phenomenon was also achieved by transfecting cells with a mutant S1P containing the ER retention signal KDEL (DeBose-Boyd et al., 1999). Based on these previous findings, it is not surprising that hypotonic stress-induced SREBP-2 cleavage occurs, possibly through the retrograde transport of S1P/S2P proteases from the Golgi to the ER. Nonetheless, these findings (Lee & Ye, 2004) suggest that Tg-induced SREBP-2 cleavage is caused by Insig depletion.

While examining the conventional process of decreased cholesterol-induced Insig–SCAP dissociation, Gong et al. (2006) found that Insig is rapidly degraded by the proteasome system following sterol depleted dissociation from SCAP. They determined that the interaction of Insig with SCAP was essential for elevated Insig protein levels, which maintains the SCAP–SREBP-2 complex in the ER (Gong et al., 2006). It is possible that the observed Tg/hypotonic medium-induced Insig degradation and SREBP-2 cleavage described by Lee and Ye (2004) occurs following Insig–SCAP dissociation. Future studies will be necessary to determine whether ER stress and other forms of cellular stress alter ER lumen homeostasis releasing Insig from SCAP in a cholesterol-independent manner, thereby allowing SCAP to transport SREBP-2 to the Golgi for activation. Experiments are currently being conducted to determine how ER stress affects the Insig–SCAP interaction.

In contrast to the results described by Lee and Ye (2004), Ron and co-workers (Harding et al., 2005) reported that compounds that induce eIF2α phosphorylation cause decreased SREBP-2 activation and SRE-gene expression. They examined the effect of the PERK pathway on SREBP-2 activation and found that under their experimental conditions, eIF2α phosphorylation caused a decrease in SREBP-2 activation that correlated with a decrease in SREBP-2 controlled gene expression (Harding et al., 2005). Although our find-

ings (Outinen et al., 1998, 1999; Werstuck et al., 2001) as well as others (Ji & Kaplowitz, 2003; Lee & Ye, 2004; Wang, Kouri, & Wollheim, 2005) suggest that ER stress induces SREBP-2 activation, these seemingly contrasting results (Harding et al., 2005) could represent a negative feedback loop to dampen cholesterol biosynthesis following ER stress. Indeed, ER stress rapidly induces SREBP-2 activation prior to Insig degradation (Colgan and Austin, unpublished results). Therefore, the subsequent translation inhibition induced by PERK-mediated eIF2α phosphorylation would represent an important regulatory mechanism to prevent uncontrolled lipid biosynthesis from being continuously activated following ER stress. Once GADD34-mediated eIF2α dephosphorylation occurs, SREBP-2-induced gene expression may cause a shift to promote a positive balance between lipid synthesis and uptake. Future experiments will be designed to determine the mechanism, timing, and physiological relevance of ER stress-induced SREBP-2 activation.

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