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Airway mesenchymal cell death by mevalonate cascade inhibition: Integration of autophagy, unfolded protein response and apoptosis focusing on Bcl2 family proteins

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ABSTRACT

HMG-CoA reductase, the proximal rate-limiting enzyme in the mevalonate pathway, is inhibited by statins. Beyond their cholesterol lowering impact, statins have pleiotropic effects and their use is linked to improved lung health. We have shown that mevalonate cascade inhibition induces apoptosis and autophagy in cultured human airway mesenchymal cells. Here, we show that simvastatin also induces endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) in these cells. We tested whether coordination of ER stress, autophagy and apoptosis determines survival or demise of human lung mesenchymal cells exposed to statin. We observed that simvastatin exposure activates UPR (activated transcription factor 4, activated transcription factor 6 and IRE1α) and caspase-4 in primary human airway fibroblasts and smooth muscle cells. Exogenous mevalonate inhibited apoptosis, autophagy and UPR, but exogenous cholesterol was without impact, indicating that sterol intermediates are involved with mechanisms mediating statin effects. Caspase-4 inhibition decreased simvastatin-induced apoptosis, whereas inhibition of autophagy by *ATG7* or *ATG3* knockdown significantly increased cell death. In $BAX^{-/-}/BAK^{-/-}$ murine embryonic fibroblasts, simvastatin-triggered apoptotic and UPR events were abrogated, but autophagy flux was increased leading to cell death via necrosis. Our data indicate that mevalonate cascade inhibition, likely associated with depletion of sterol intermediates, can lead to cell death via coordinated apoptosis, autophagy, and ER stress. The interplay between these pathways appears to be principally regulated by autophagy and Bcl-2-family pro-apoptotic proteins. These findings uncover multiple mechanisms of action of statins that could contribute to refining the use of such agent in treatment of lung disease.

1. Introduction

Statins are inhibitors of HMG-CoA reductase, the proximal effector in the multistep mevalonate cascade that leads to synthesis of cholesterol and other sterols [1,2]. HMG-CoA reductase inhibition depletes cells of lipid intermediates, an effect that limits the availability of isoprenoid, substrates needed for posttranslational protein lipidation. This modification controls activation state, stability and sub-cellular distribution of many proteins in signaling pathways that regulate cell growth, survival, differentiation and synthetic function [3].

Statins have emerged as compounds with pleiotropic inhibitory effects on inflammation, oxidative stress, and tissue repair; impact that is linked with positive health outcomes that go beyond serum lipid-lowering in several conditions, including lung function in the aged and in smokers as well as in patients with respiratory tract

Abbreviations: ATF6, Activating transcription factor 6; ATG5, Autophagy protein 5; BAK, BCL-2-antagonist/killer; BAX, The Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; BIP, Binding immunoglobulin protein; Caspase, Cysteine-dependent aspartate-directed protease; CHOP, C/EBP homologous protein; DMSO, Dimethyl sulfoxide; ER, Endoplasmic reticulum; ERK, Extracellular signal-regulated kinase; Ero1α, ER oxidase1α; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HAF, Human airway fibroblast cell; HASM, Human airway smooth muscle cell; HMGB1, High Mobility Group Box 1; HMG-CoA reductase, 3-hydroxy-3-methyl-glutaryl-CoA reductase; IRE1α and β, Inositol requiring kinase 1α and β; JNK, Jun N-terminal Kinase; LC-3, Microtubule-associated protein light chain 3; MTT Assay, Methylthiazol Tetrazolium Assay; NF-κB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; PARP, Poly (ADP-ribose) polymerase; PERK, Protein kinase-like endoplasmic reticulum kinase; PKR, Double-stranded RNA-activated protein kinase; TEM, Transmission electron microscopy; UPR, Unfolded protein response; XBP1, X-box binding protein

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infection and pneumonia [4,5]. Conversely positive impact of statin use on asthma symptoms is equivocal [6]. At a cellular level, statins have broad effects, for instance, we and others reported the suppression of human airway mesenchymal cell proliferation and synthesis of extracellular matrix proteins, as well as promoting autophagy and apoptosis at some concentrations [7–9]. Given the diverse effects of statins both on lung health and on the function of lung mesenchymal cells that modulate tissue repair, better understanding of their impact at a cellular level is needed to appreciate potential for treating lung disorders.

The ability of cells to respond to perturbations in endoplasmic reticulum (ER) function, or ER stress, is critical for cell survival. The ER is the primary intracellular site for folding and bio-assembly of membrane-associated and secreted proteins. Within the cell, ER stress is sensed by three upstream signaling proteins, PERK, IRE1 and ATF6, that upon release from the luminal ER binding protein, BIP, initiate intracellular transcriptional and translational activity known as the unfolded protein response (UPR) [10,11]. The UPR is aimed at reestablishing ER homeostasis; however, if UPR mechanisms of adaptation are insufficient to decrease the unfolded protein load, UPR can initiate apoptosis leading to cell death [12–14]. In addition to apoptosis, autophagy may also be triggered by ER stress via the UPR stress sensors IRE1α and PERK [15,16].

In addition to evidence in different cell types that mevalonate cascade inhibition can induce apoptosis and autophagy [7, 17–21], the induction of ER stress has been documented in macrophages [22]; whether mevalonate cascade inhibition with statins leads to a concomitant ER stress response in human airway mesenchymal cells in not known. In the present study, we investigate the effect of simvastatin exposure on ER stress and the UPR in primary cultured human airway fibroblasts and smooth muscle cells. Furthermore, we investigate the degree of interplay that exists between mevalonate cascade inhibition-induced apoptosis, autophagy and ER stress and how this impacts cell survival or demise. These studies extend our previous work and shed needed understanding of the complex cellular response to statins that may be at the root of the impact of these compounds on lung health.

2. Material and methods

2.1. Reagents

Cell culture plastic ware was obtained from Corning Costar Co. (Canada). Cell culture media, simvastatin, mevalonate, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (MTT), rabbit anti-human/mouse/rat LC3β, and cholesterol were obtained from Sigma (Sigma-Aldrich, Oakville, CA). Z-LEVD-FMK is purchased from Calbiochem (Canada). Rabbit anti-human cleaved caspase-7, -3, rabbit anti Bak, Bax, GRP78 (BIP), IRE1α, and Atg5 were purchased from Cell Signaling (Canada). Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Santa Cruz Biotechnologies (USA). Rabbit anti-ATF6, anti-ATF4, anti-XBP1, anti-Caspase-4, and anti-Caspase-12, were obtained from Abcam (USA). ATP luminescence assay kit (Abcam, Canada). Caspase Glo Kit (Caspase-9, -3/-7) (Promega, USA).

2.2. Primary HASM and HAF cell culture preparation

For all experiments we used primary cultured human airway smooth muscle (HASM) cells and airway fibroblasts (HAF) that were prepared from 2nd to 4th generation of bronchi in macroscopically healthy segments of resected lung specimens. After microdissection to separate submucosal compartments from airway smooth muscle, HAF and HASM cells respectively, were isolated by enzymatic dissociation as described [7,20,23,24]. All procedures were approved by the Human Research Ethics Board (University of Manitoba). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum with antibiotic. For all experiments cells were starved in 0.5% fetal bovine serum for 48 h prior to all treatments. Medium was changed every 48 h. For all experiments passages 3–7 of HASM and HAF were used.

2.3. HASM immortalisation

HASM immortalisation has been done according to our previous established method [25]. Briefly HASM cells were infected with a retrovirus vector encoding the hTERT gene. Later hTERT expression cassette was cloned into pLXIN (Clontech), and replication-incompetent Moloney murine leukemia virus retrovirus was generated in HEK293 retroviral packaging cells. Primary and first-passage cultures of human airway smooth muscle cells were infected with the hTERT retrovirus and selected with 100 mg/ml G418 for 1 wk. Infection was verified in immortalised cells by RT-PCR using telomerase-specific primers. There is no evidence of senescence up to 60 passages. For all experiments, passages 10–20 were used.

2.4. MTT assay

Cytotoxicity of simvastatin towards the HASM and HAF was determined by MTT-assays as previously described [7]. In experiments investigating the effect of mevalonate (0–2 mM), cholesterol (0–50 μM), and Z-LEVD-FMK, HASM were pre-treated with indicated concentrations of the inhibitors or chemicals (4 h) and then co-treated with indicated concentrations of simvastatin for the experimental time points.

2.5. Apoptosis measurement

Apoptosis was measured using the Nicoletti method which has been previously used by our group [20,26]. Briefly, HASM grown in 12 well plates were treated with simvastatin (10 μM) for 72 h, after craping, cells were harvested by centrifugation at 1500 *g*, 4 °C, for 5 min. The cells were washed once with PBS, and then resuspended in a hypotonic PI lysis buffer (1% sodium citrate, 0.1% Triton X-100, 0.5 mg/ml RNase A, 40 μg/ml propidium iodide). Cell nuclei were then incubated for 30 min at 30 °C and the nuclei were subsequently analyzed by flow cytometry. Nuclei to the left of the G1 peak containing hypodiploid DNA were considered to be apoptotic.

2.6. Immunoblotting

HASM, HAF, immortalised HASM, and different MEF knock out models were treated with indicated dose of simvastatin, in each time point attached and floating cells were collected in control and simvastatin treated cells. Western blotting was used to detect cleaved caspase-3, -7, ATF6, caspase-12, caspase-4, ATF4, IRE1α, Atg5–12, LC3β, XBP1, HMGB1, beta actin, and GAPDH. Briefly, cells were washed and protein extracts prepared in lysis buffer (20 mM Tris–HCl (pH 7.5), 0.5% Nonidet P-40, 0.5 mM PMSF, 100 μM β-glycerol 3-phosphate and 0.5% protease inhibitor cocktail). After centrifugation at 13,000 ×*g* for 10 min, protein content in the supernatant was determined by Lowry protein assay, proteins were then size-fractionated by SDS-PAGE and transferred onto nylon membranes under reducing conditions. After blocking membranes with non-fat dried milk and Tween 20, blots were incubated overnight with the primary antibodies at 4 °C. HRP-conjugated secondary antibody incubation was for 1 h at room temperature (RT), then blots were developed by enhanced chemiluminescence (ECL) detection (Amersham-Pharmacia Biotech) [7].

2.7. Caspase luminometric assay

Caspase-9, and caspase-3/-7 activity was measured using a luminescence based assay (Promega, CA) based on previously established method [27]. Briefly MEF WT, *ATG3* KO, and *Bax*/*Bak* KO were cultured in 96 well plates (5000 cells per well). After being 65 confluent, they were treated with simvastatin (10 μM) and in the proper time point

the reagent were added to the wells according to manufacturer protocol and in 30 min the luminescence emission was detected.

2.8. Electron microscopy

HASM, HAF, immortalised HASM, and different MEF knock out models were treated with indicated dose of simvastatin, in each time point attached and floating cells were collected in control and simvastatin treated cells. For transmission electron microscopy (TEM), cells were fixed (2.5% glutaraldehyde in PBS (pH 7.4) for 1 h at 4 °C) and post-fixed (1% osmium tetroxide) before embedding in Epon. TEM was performed with a Philips CM10, at 80 kV, on ultra-thin sections (100 nm on 200 mesh grids) stained with uranyl acetate and counterstained with lead citrate [7].

2.9. Stable gene silencing by lentiviral delivery of shRNA

ATG7-shRNA lentiviral particles (sc-41447-V) and control shRNA lentiviral particles (sc-108080) were purchased from Santa Cruz Biotechnology (US). For infection, immortalised HASM cells were grown to 70% confluence and transduced at an MOI of 10 in the presence of 30 μg/mL polybrene (final concentration) overnight. Excess viral vectors were removed and the transduced cells were cultured in fresh medium for 2 days before selection of stable expression of shRNA by growing in culture media containing puromycin (4 μg/mL) for at least 3 weeks. For control cells, a GIPZ vector harboring "scrambled" non-coding shRNA was prepared and used to generate lentivirus for transduction of the same primary HASM cell lines that were used to generate ATG7-deficient stable cultures.

2.10. Sub-cellular fractionation

Following induction of cell death using simvastatin (10 μM) cytosolic, mitochondrial and nuclear fractions were generated using a digitonin-based subcellular fractionation technique as described previously [20]. Briefly, 10^7 cells were harvested by centrifugation at 800 ×*g*, washed in phosphate buffered saline (PBS) pH 7.2, and re-pelleted. Cells were digitonin-permeabilised for 5 min on ice at a density of 3×10^7/ml in cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na2HPO4, 1.4 mM KH_2PO_4 pH 7.2, 100 μM PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin, containing 200 μg/ml digitonin). Plasma membrane permeabilisation of cells was confirmed by staining in a 0.2% trypan blue solution. Cells were then centrifuged at 1000 ×*g* for 5 min at 4 °C. The supernatants (cytosolic and mitochondria fractions) were saved and pellets solubilised in the same volume of nuclear lysis buffer, followed by pelleting at 12,500 ×*g* for 10 min at 4 °C.

2.11. HMGB1 release

HMGB1 release was assessed by immunoblot analysis. Cells were seeded in 100 mm dishes. After 24 h (80% confluence), cells were treated with simvastatin (10 μM) and at the indicated time point (72 h) the supernatant was collected, centrifuged in 10,000 ×*g* for 20 min, transferred to 50 ml filter tubes (10 kD mesh, Amicon), and centrifuged in 3000 ×*g* for 35 min. The remaining supernatant on top of the filter was collected and Western blot analysis was performed using a rabbit anti-HMGB1 antibody (Abcam, Canada).

2.12. ATP assay

Abcam's ATP assay kit was used to measure ATP. Briefly the Assay Kit (Bioluminescent) utilises bioluminescent detection of ATP levels for a rapid screening of apoptosis, necrosis, growth arrest, and cell proliferation simultaneously in mammalian cells. The assay utilises the enzyme luciferase to catalyze the formation of light from ATP and luciferin, and the light can be measured using a luminometer [28].

2.13. Statistical analysis

Results were expressed as means ± SDE and statistical differences were evaluated by one-way or two-way ANOVA followed by Tukey's or Bonferroni's post hoc test, using Graph Pad Prism 5.0. $P < 0.05$ was considered significant. For all experiments data were collected in triplicates from at least three cell lines unless otherwise indicated.

3. Results

3.1. Loss of cholesterol intermediates triggers simvastatin-induced cell death

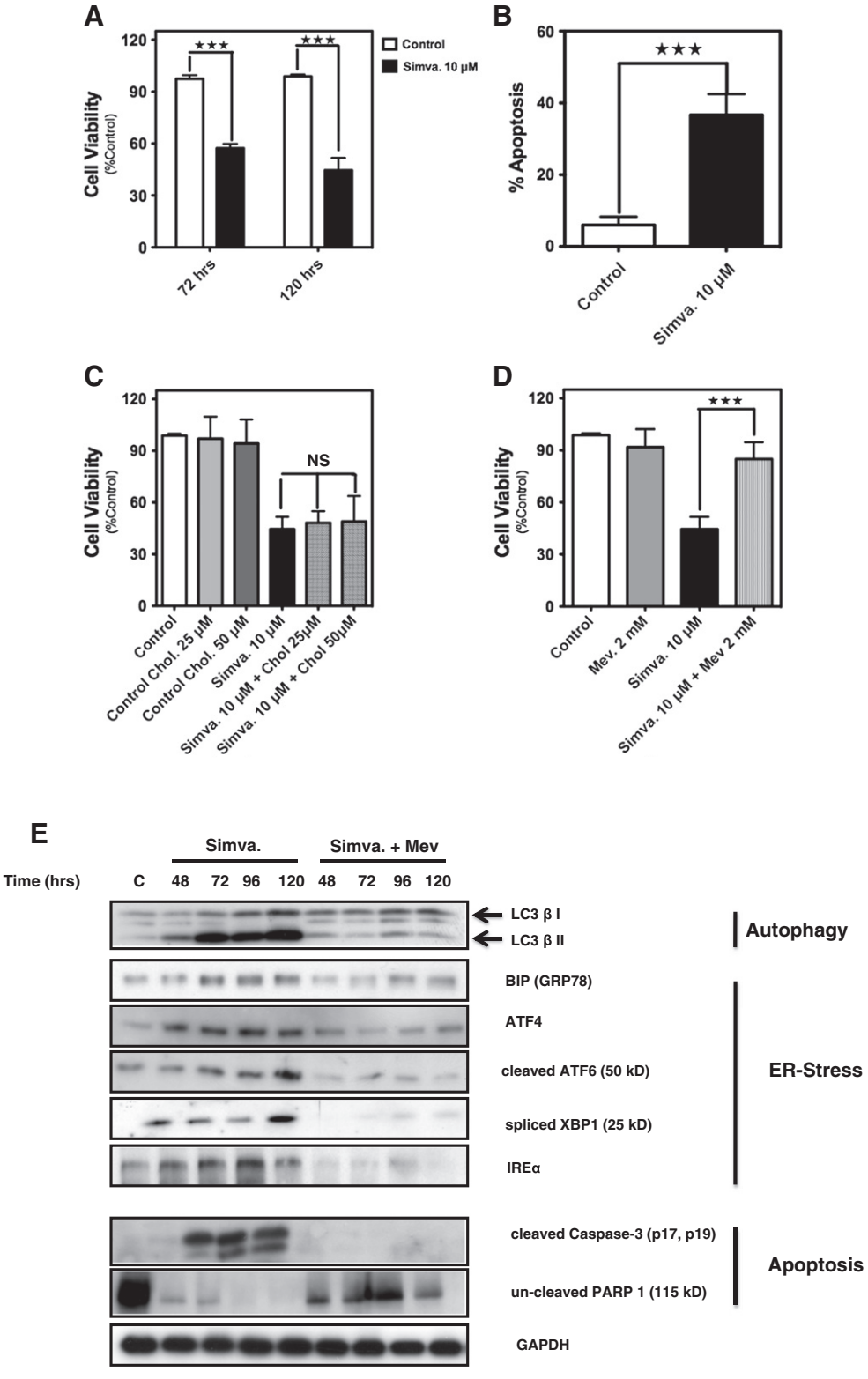
We and others have shown that inhibition of mevalonate synthesis by statins can activate autophagy, apoptosis, and UPR in different cells [7,20,22,26,29]. We have also recently showed that simvastatin induces cell death in both HASM and HAF [7,20]. Here we assessed whether cell death induced by mevalonate cascade inhibition in HASM could be prevented by the addition of exogenous mevalonate and confirmed that depletion of intermediate cascade lipid moieties is the likely cause of cytotoxicity (Fig. 1A & D). Simvastatin induced apoptosis was also confirmed using PI based FACS assay (Fig. 1B). Of note, addition of exogenous cholesterol (0–50 μM) to cells treated with simvastatin had no impact on cell viability, indicating that depletion of cellular cholesterol per se did not invoke cell death (Fig. 1C). To decipher intracellular responses associated with mevalonate cascade inhibition, we used immunoblotting to track abundance of: lipidated LC3β II, a marker for autophagy [30], cleaved caspase-3 and PARP-1, markers for apoptosis [27], as well as, UPR markers BIP, activating transcription factor 4 (ATF4), cleaved activating transcription factor 6 (ATF6), and spliced X box-binding protein1 (sXBP1) [26,31]. In simvastatin-treated HASM cells, mevalonate (2 mM) significantly blunted hallmarks of autophagy and UPR, as well as protecting again loss of PARP-1 whilst inhibiting cleaved caspase-3 accumulation (apoptosis markers). These observations indicate that lipid intermediate depletion is a critical determinant of a multi-faceted cell response to simvastatin (Fig. 1E–K). As the response of primary HASM and HAF to simvastatin is following the same trends in apoptosis, UPR, and autophagy we used primary HASM cells and senescence-resistant cell lines generated from them in the subsequent experiments. On the other hand in some experiments we used MEFs in our work exclusively as a tool for proof-of-principle related to mechanisms that are suggested from our experiments using HASM cells.

3.2. Mevalonate cascade inhibition activates UPR in airway mesenchymal cells

As our initial experiments indicated that markers for UPR are concomitantly activated with those for apoptosis and autophagy, we more rigorously assessed simvastatin-induced UPR in human airway mesenchymal cells. UPR is driven by the activation of ER resident proteins including IRE1α (inositol-requiring protein1 α), the protein chaperone BiP (immunoglobulinbinding protein; Grp78), CHOP, ER oxidase1α (Ero1α) and calnexin [10,31–33]. Under conditions where ER stress mediates cell death and apoptosis, ER-located caspase-12 also activates UPR signaling [34,35]. Our experiments assessed the degree to which the abundance of UPR effectors are impacted in HAF and HASM cells by simvastatin (10 μM, 0–120 h) (Fig. 2A–G). Western blotting and densitometry revealed that mevalonate cascade inhibition induced a rapid increase of Ero1α and calnexin as well as caspase-4 activation, with subsequent accumulation of CHOP, BIP and IRE1α. These data confirm that simvastatin induces UPR, and its individual effectors are activated with different kinetics.

We next examined the impact of mevalonate cascade inhibition on several transcription factors that promote UPR, including spliced XBP1, ATF6 and ATF4. These factors regulate genes encoding CHOP, $p58^{IPK}$, and proteins involved in amino acid transport and synthesis, stress responses, redox reactions and protein degradation [31,36,37].

Activation (splicing or cleavage) and nuclear translocation of XBP1 is induced by IRE1α, whereas during ER stress ATF6 is cleaved prior to nuclear localisation, and ATF4 is activated by PERK [36,37]. Consistent with these mechanisms, using cell fractionation and immunoblot analysis we observed that mevalonate cascade inhibition (simvastatin 10 μM) induced accumulation of nuclear spliced XBP1, ATF4, and cleaved ATF6 concomitant with PERK phosphorylation (Fig. 2H). These findings confirm that the three main signaling arms of UPR (IRE1α, PERK, and ATF6) are activated in response to simvastatin treatment in primary cultured human airway mesenchymal cells.



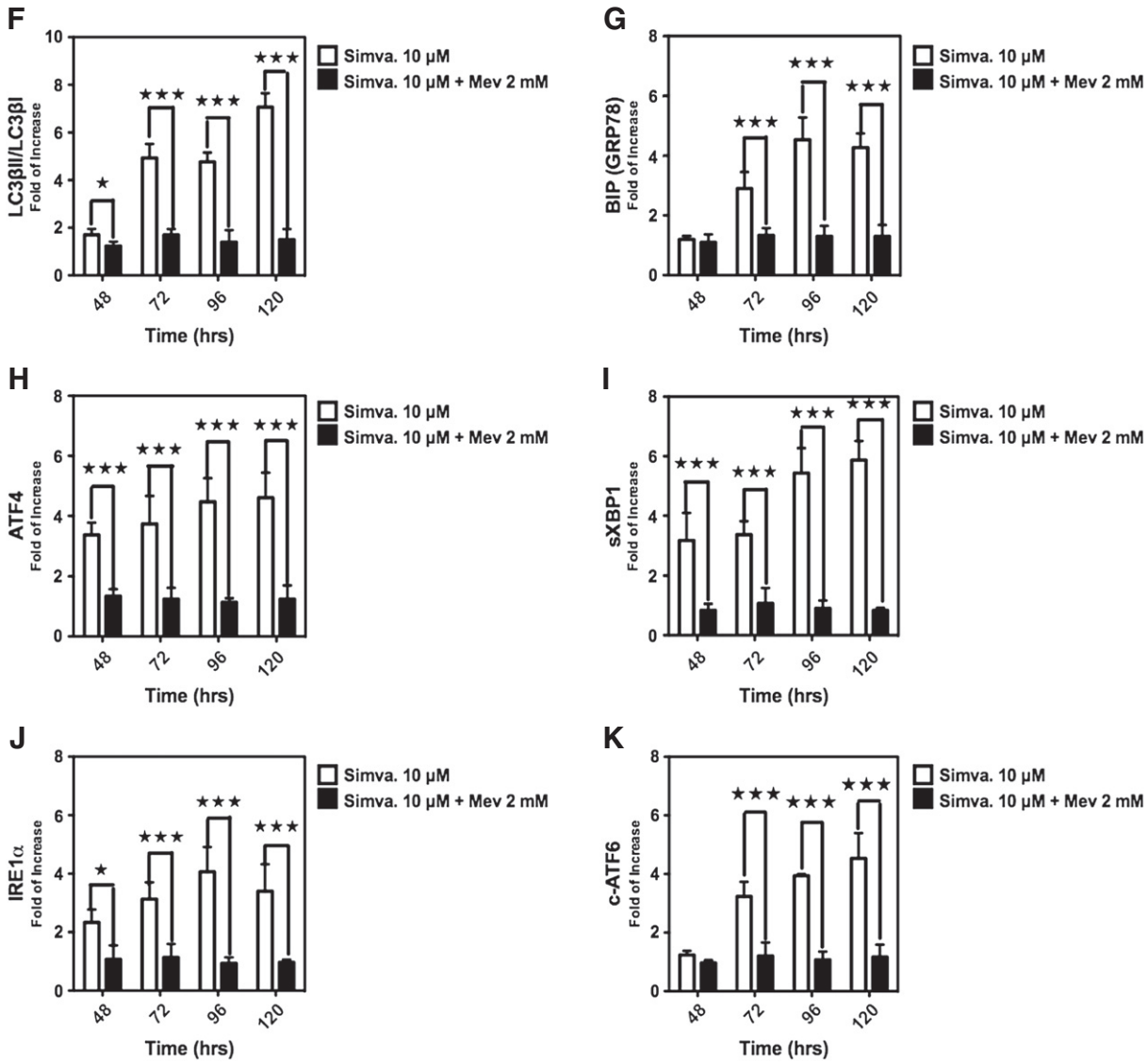


Fig. 1. Mevalonate inhibits simvastatin-induced cell death response including ER-stress, autophagy, and apoptosis. (A) HASM cells were treated with simvastatin (10 μM) and cell viability was assessed 72 and 120 h thereafter by MTT assay. Control cells for each time point were treated with the solvent control (DMSO) at the same concentration. Results are expressed as percentage of corresponding time point control and represent the means ± SD of 6 independent experiments in three different HASM (★★★ $P < 0.001$). (B) HASM cells were treated with simvastatin (10 μM), floating and attached cells were collected and apoptosis was assessed 72 h thereafter by propidium iodide FACS assay (Nicolleti). (C) Effects of supplementation with cholesterol (0–50 μM) on HASM cell death, 4 h prior to treatment with simvastatin (10 μM, 96 h) on simvastatin-induced cell death. For each experiment control cells were treated with simvastatin solvent (DMSO) alone (control) or with both DMSO and the appropriate solvent (ethanol). Results are expressed as mean ± SD of 6 independent experiments using three different HASM primary cells. (**C**) Effects of supplementation with 2 mM mevalonate evaluate on cell death, 4 h prior to treatment with simvastatin (10 μM, 96 h) on simvastatin-induced cell death. For each experiment control cells were treated with simvastatin solvent (DMSO) alone (control) or with both DMSO and the appropriate solvent (ethanol). Results are expressed as mean ± SD of 6 independent experiments using three different HASM primary cells (★★★ $P < 0.001$). The results also indicated that there was not any significant difference between mevaloante control and simvastatin and mevalonate cotreatment ($P > 0.05$). (E) Mevalonate inhibited simvastatin-induced (10 μM) autophagy, UPR response, and apoptosis. Immunoblotting of autophagy (LC3β), UPR (BIP, ATF, cleaved ATF6, spliced XBP1, and IRE1α) and apoptosis (cleaved PARP1 and caspase-3) confirmed the inhibitory effect of mevalonate on simvastatin-induced autophagy, UPR, and apoptotic response. Results are representative of three different primary HASM. Protein loading was checked using GAPDH protein. (F–K) Densitometry analysis confirms that mevalonate significantly (★★★ $P < 0.001$) decrease simvastatin (10 μM) induced autophagy (LC3β II/LC3β I, F), and endoplasmic reticulum stress (BIP, ATF4, sXBP, IRE1α, c-ATF6 G, H, I, J, K respectively).

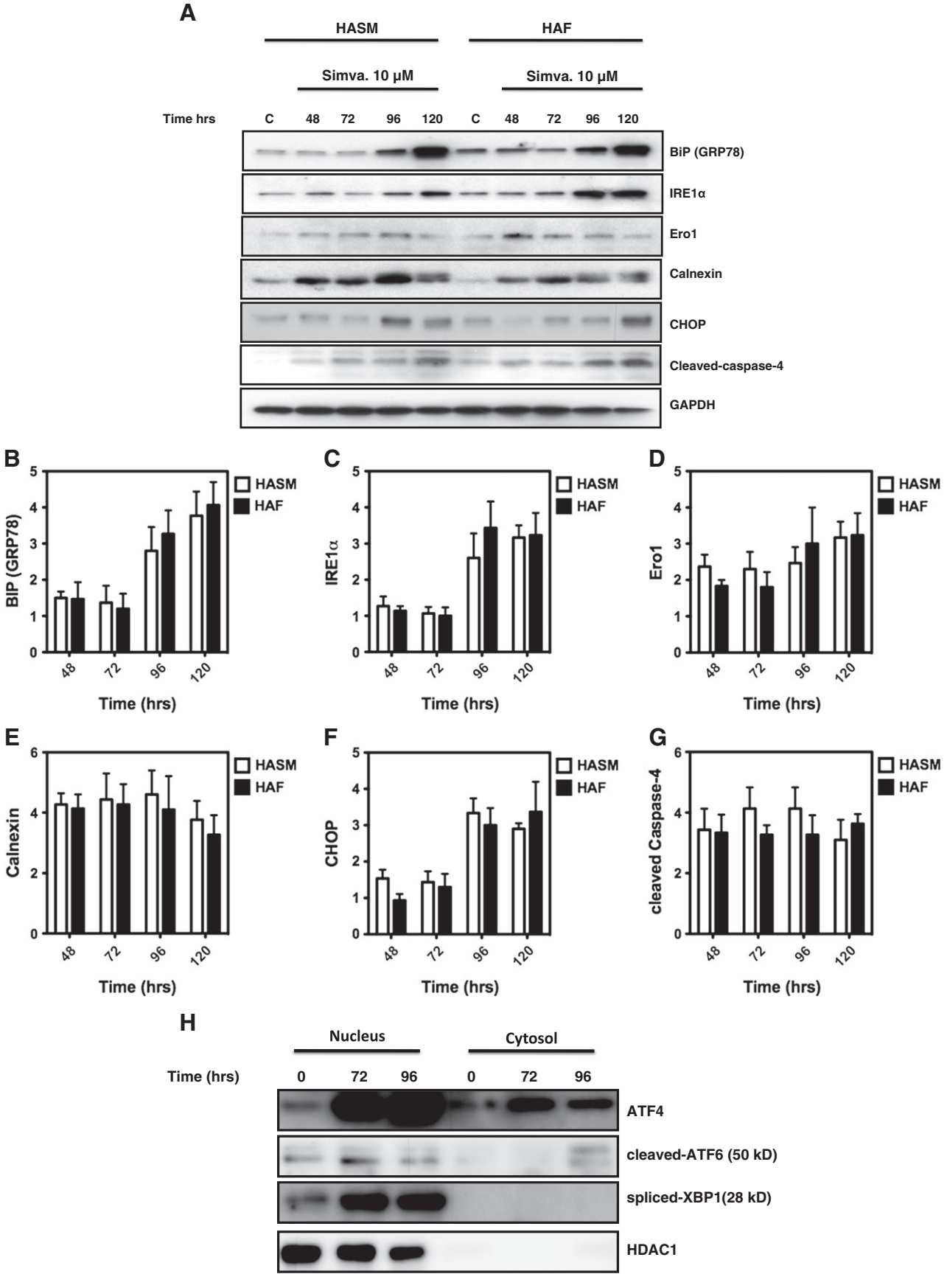
3.3. Caspase-4 mediates simvastatin-induced apoptosis and cell death in human airway smooth muscle cells

As we observed parallel activation of UPR and apoptosis during simvastatin exposure, we investigated whether these responses were linked or mutually exclusive. Several reports indicate that UPR-induced apoptosis is, in part, dependent on the activation of caspase-4/-12 [38–41]. Since caspase-12 is only present in about 20% of individuals of African descent, we assessed the impact of inhibiting caspase-4 activity using Z-LEVD-FMK (25 μM) (Fig. 3). We found that both mevalonate cascade inhibition-induced cell death and activation of caspase-3/-7 were markedly blunted by caspase-4 inhibition. This observation suggests that simvastatin-induced apoptosis may occur as the result of UPR that leads to caspase-4 activation.

3.4. Autophagy limits simvastatin-induced apoptosis and UPR

In addition to interplay between apoptosis and UPR, a functional relationship exists between autophagy and ER stress [42]. Indeed, autophagy may serve as a mechanism to eliminate portions of damaged

ER and control the rate of ER expansion under stress conditions [15]. Our group has recently shown that coordination of autophagy and apoptosis in various cell models, including simvastatin exposed human lung mesenchymal cells, can determine cell survival and demise [7,30]. In this context, we used two different approaches to investigate the impact of inhibiting autophagy during mevalonate cascade



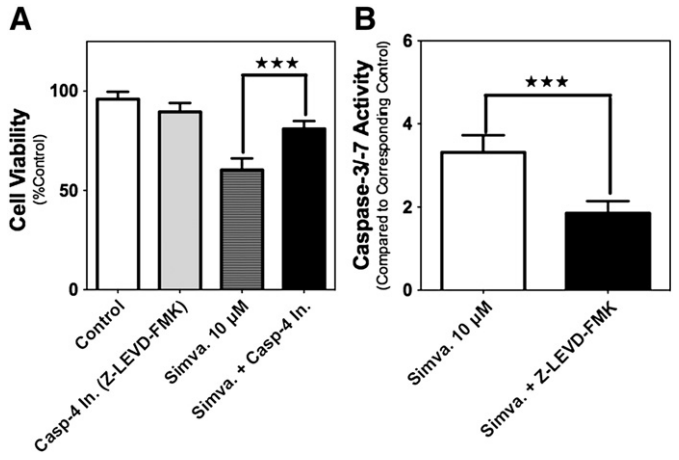


Fig. 3. Specific ER-dependent caspase inhibition reduced mevalonate cascade inhibition-induced cell death and caspase-3/-7 activation. Primary HASM were pre-treated with caspase-4 (Z-LEVD-FMK) inhibitor (25 μM) for 4 h and then co-treated with simvastatin for 96 h. Caspase-4 inhibition not only decreased simvastatin-induced cell death (A) but also decreased simvastatin-induced caspase-3/-7 activation (B). Results are expressed as mean ± SD of 4 independent experiments using three different primary HASM cells (★★★ $P < 0.001$).

inhibition-induced apoptosis and UPR. As knockout (KO) or knockdown (KD) of Atg3 and Atg7 inhibits autophagy [7,43,44], we used *ATG3* KO mouse embryonic fibroblasts (MEF) as well as employing shRNA mediated Atg7 KD in immortalised human airway smooth muscle cells. We found that cell death triggered by mevalonate cascade inhibition (simvastatin 10 μM, 0–96 h) was significantly *increased* in *ATG3* KO MEFs (Fig. 4A). Similarly, simvastatin more profoundly increased both the apoptotic response (caspase-7 cleavage, western blot and caspase-3/-7 luminometric assay) and UPR (IRE1α expression) in *ATG3* KO MEFs (Fig. 4B&C). In Atg7 KD human airway smooth muscle cells, mevalonate cascade inhibition augmented cell death (Fig. 4D) as well as increasing caspase-3 cleavage in concert with significantly augmented IRE1α expression when compared to control cells (Fig. 4E) (as Atg7 KD cells was produced from immortalised HASM (hTERT) cell viability in Fig. 4D showed slightly difference from HASM cells which was previously showed in Fig. 1A). These data indicate that autophagy modulates both apoptosis and UPR induced by mevalonate cascade inhibition. Moreover, inhibiting autophagy increases cytotoxic effects of simvastatin in human airway smooth muscle cells.

3.5. Pro-apoptotic BH3-only proteins coordinate cell death pathways in response to simvastatin

Our prior work shows that apoptosis induced by mevalonate cascade inhibition in primary human airway mesenchymal cells is mediated, in part, by the BH3 only pro-apoptotic proteins, Bax and Bak [20]. Of note, Bax and Bak, which are partially localised on the ER membrane, undergo changes in conformation and oligomerise to commit a cell to apoptosis; during ER stress these proteins can be further impacted, leading to greater damage to ER membranes [45,46]. On this basis we aimed to determine whether Bax and Bak play a coordinating role in the multifaceted cellular response that we observe upon simvastatin exposure. We used MEFs from wild type (WT) and *BAX*/*BAK* double knockout mice and assessed cytotoxic effects 24–72 h after simvastatin (10 μM) exposure (Fig. 5A–C). Using phase contrast microscopy we observed excessive cell rounding and detachment in $BAX^{-/-}$/$BAK^{-/-}$ MEFs (24 h), a response that was consistent with statistically significant greater loss of cell viability (48 and 72 h), as determined by MTT assay. Paradoxically, these data suggest that cell viability is compromised in the absence of a functional Bax/Bak pathway that we have shown promotes apoptosis upon simvastatin exposure [20].

To more fully understand this response we used immunoblotting to track hallmark markers of apoptosis, UPR and autophagy after mevalonate cascade inhibition. We observed that a $BAX^{-/-}/BAK^{-/-}$ background inhibits both apoptosis (reduced PARP-1 cleavage) and UPR (reduced expression of BIP and IRE1α). In contrast, absence of Bax and Bak was associated with excess autophagy, as evident by increased LC3βII and Atg5–12 conjugation compared to WT cells (Fig. 5D). We also showed that simvastatin (10 μM, 24 h) induced significant activation of caspase-9 in MEF WT cells while in $BAX^{-/-}$/$BAK^{-/-}$ MEF cells caspase-9 was not activated (Fig. 5E). These observations indicate that in the absence of a functional mitochondrial-apoptosome pathway, exaggerated autophagy, but reduced apoptosis and UPR, correlates with compromised cell viability in the presence of simvastatin.

We next investigated the nature of simvastatin-induced cell death in $BAX^{-/-}/BAK^{-/-}$ cells. First we used TEM to assess cellular ultrastructure and observed, particularly in $BAX^{-/-}/BAK^{-/-}$ MEFs, evidence for cell swelling and rupture, features associated with cells undergoing necrosis (Fig. 5F–G). To better determine whether simvastatin induced necrosis, we measured the level of cellular ATP, which is depleted during necrosis [47]. We found that mevalonate cascade inhibition induced a significant decrease in ATP content exclusively in $BAX^{-/-}/BAK^{-/-}$ MEFs (Fig. 5H). The release of the alarmin, High Mobility Group Box 1 (HMGB1), is another marker of necrosis [48], thus we also used immunoblotting of concentrated cell culture media to monitor HMGB1 release after simvastatin treatment (10 μM, 72 h). We determined that HMGB1 was released exclusively from $BAX^{-/-}/BAK^{-/-}$ cells (Fig. 5I). Taken together, our experiments demonstrate that though mevalonate cascade inhibition in the absence of pro-apoptotic Bax and Bak is associated with limited ER stress, cells undergo exaggerated autophagy that is associated with cell death via necrosis. These observations also emphasise a role for BH3 only pro-apoptotic proteins in mitochondria and the ER for simvastatin-induced ER-stress and apoptosis.

4. Discussion

Statin use has been linked with improved lung health, particularly in COPD patients, but allergen challenge murine models suggest this may also be the case in asthma, despite small clinical trials in asthmatics that have been equivocal with respect to patient benefit [5,6]. The mechanisms for these effects, or lack thereof, are not fully appreciated. Though statin use is linked with a significant anti-inflammatory impact at the systemic level, emerging in vitro data strongly indicate that they have broad effects on cell growth, survival and synthetic function [3,4,20]. The present study reveals the degree of complexity in cell response that can result from mevalonate cascade inhibition and suggests this may be a reason that the impact of statins at a (patho)physiological and patient symptom level can be variable. We demonstrate for the first time that human airway fibroblasts and smooth muscle cells exhibit ER stress and UPR upon statin exposure. Moreover, these processes are integrated with pathways that regulate apoptosis and autophagy. We further demonstrate that BH3-only pro-apoptosis proteins (Bax and Bak) that associate with the ER and mitochondria are pivotal

Fig. 2. Mevalonate cascade inhibition induces UPR expression and translocation of UPR-induced transcription factors to nucleous. (A) Western blot analysis of cell lysates from matched pairs of HASM and HAF. Cells were treated with 10 μM simvastatin for the indicated time periods, and then immunoblotted using the indicated specific antibodies. Simvastatin provoked BIP, IRE1α, Ero1, calnexin, and CHOP expression and activated caspase-4. Protein loading was checked using GAPDH protein. (C–G) Densitometry analysis of UPR proteins has been done on the results of western blot of matched pairs of HASM and HAF which were treated with 10 μM simvastatin for the indicated time periods. The results show the fold of increase of BIP, IRE1α, Ero1, calnexin, and CHOP expression and activated caspase-4 compared (C to G respectively) to time matched control after normalizing against GAPDH loading control. (H) Simvastatin (10 μM, 0–72 h) induced ATF4, cleaved ATF6, and spliced XBP1, translocation to nucleus. Primary HASM were treated with indicated simvastatin concentration and nuclear/cytosolic expression of each protein was evaluated using immunoblotting. Loading and fraction purity were confirmed using HDAC protein.

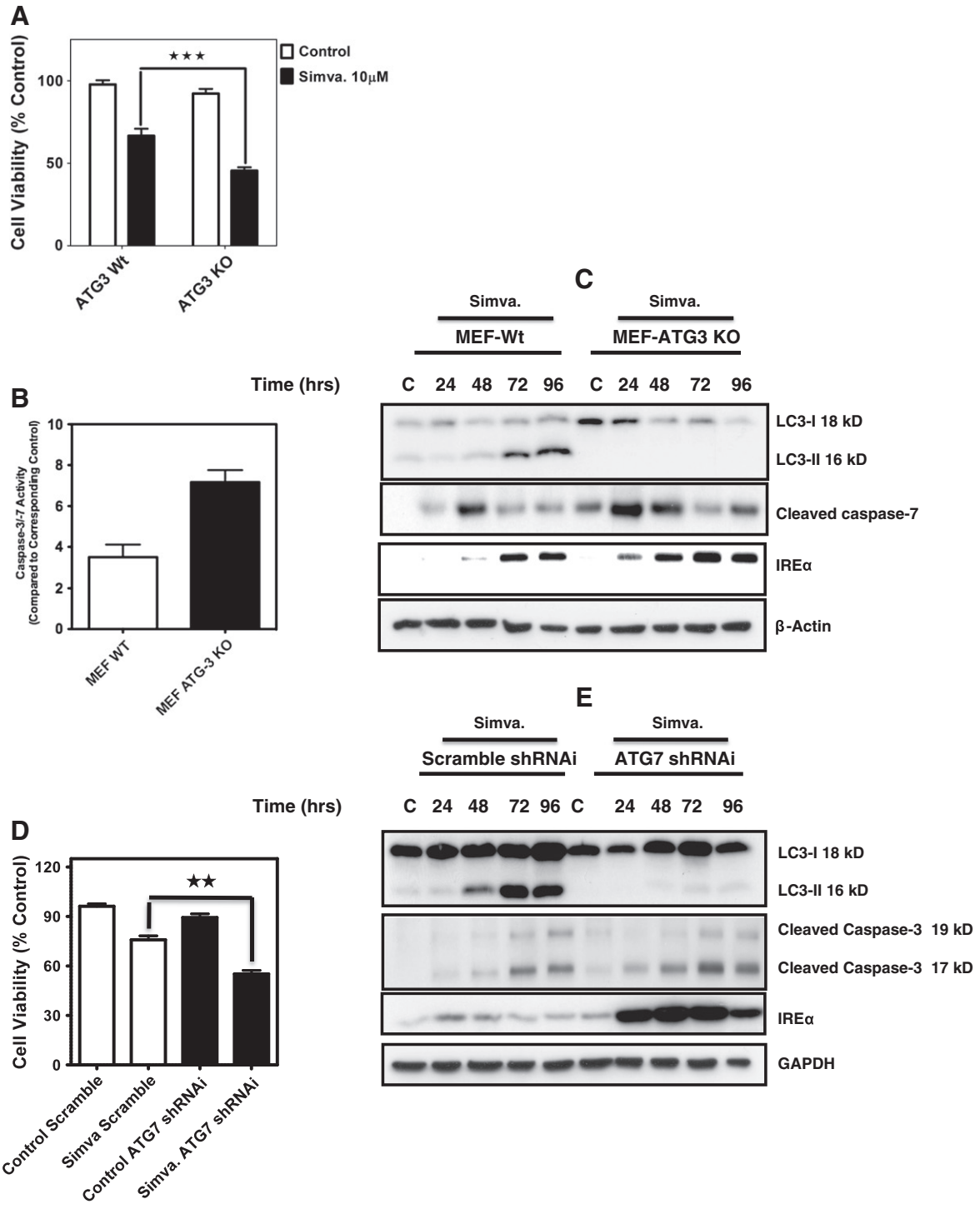


Fig. 4. Autophagy inhibition increased mevalonate cascade-induced cell death—study in knockdown and knockout models. (A) Wt MEF and *ATG3* KO were treated with simvastatin (10 μM, 96 h). We observed significant differences between Wt and *ATG3* KO cell in simvastatin-induced cell death (★★★ $P < 0.001$). For each experiment control cells were treated with simvastatin solvent (DMSO) at the same concentration. Results are expressed as mean ± SD of 6 independent experiments. (B) Wt MEF and *ATG3* KO were treated with simvastatin (10 μM, 48 h). Caspase-3/-7 activity was measured in time-matched control and simvastatin treated cells. We observed significant differences between Wt and *ATG3* KO cell in simvastatin-induced caspase-3/-7 activation (★★★ $P < 0.001$) (C) Immunoblot analysis showed that mevalonate cascade inhibition in ATG3 KO MEF cells inhibited LC3 lipidation and increased caspase-7 cleavage and IRE1α expression. Equal protein loading was confirmed using β-actin. (D) Immortalised human airway smooth muscle (Wt) and *ATG7* KO were treated with simvastatin (10 μM, 96 h). We observed significant differences between Wt and ATG3 KO cell in simvastatin-induced cell death (★★ $P < 0.01$). For each experiment control cells were treated with simvastatin solvent (DMSO). Results were expressed as mean ± SD of 6 independent experiments. (E) Immunoblot analysis showed that mevalonate cascade inhibition in human immortalised smooth muscle *ATG7* KO cells inhibited LC3 lipidation and increased caspase-3 cleavage and IRE1α expression. Protein loading was checked using GAPDH protein.

determinants in the coordination of the final cell response. This understanding is important in light of the untapped potential of statins and other therapeutics that can impact protein lipidation or effectors downstream from signaling proteins that are regulated by post translational lipidation.

Though our study is the first to describe the activating effects of mevalonate cascade inhibition on ER stress and UPR in human airway mesenchymal cells, studies using different cell models have generated diverse data [22,49–51]. For instance, while some studies reveal statins to have inhibitory impact on ER stress in the heart of C57BL/6J male

mice, and mice macrophages [50,51], but other studies using animal models of abdominal aortic aneurysm indicate no impact on ER stress [52]. Notably, as we observed in our current studies with human lung mesenchymal cells, UPR is activated by statin exposure in RAW264.7 macrophages and K562 cells [22,53]. Apart from subtle differences in the experimental design, these reports and our work suggest that cell type-specific effects may exist with respect to regulatory mechanisms involving intermediates of the mevalonate cascade on signaling pathways that can modulate ER stress and UPR. On this basis the data from our study using human lung airway mesenchymal cells may be of importance in understanding the impact of statins on lung health and disease.

The UPR is a highly conserved, multi-pathway survival mechanism designed to restore cellular homeostasis in cells subjected to unfavorable environmental conditions that perturb ER function and the production of properly folded proteins leading to ER stress [31,54]. ER stress triggers pathways that attenuate protein synthesis rate and up regulate activity of genes that encode proteins involved in folding, retrotranslocation and proteasomal degradation of ER-localised proteins [55,56]. There are three main ER trans-membrane proteins that act as stress sensors to initiate different UPR signaling cascades: double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) alpha and beta, and inositol requiring kinase 1 (IRE1) alpha and beta [14]. Activation of PERK is mediated by its oligomerisation and autophosphorylation, leading to ATF4 translation [57], while IRE1α activation leads to splicing of XPB1 to generate sXBP1 [31,58]. Notably, HMG-CoA reductase is anchored in the ER membrane [59], thus it is intriguing to speculate that this subcellular localisation may be permissive to statin induction of UPR sensor proteins in airway fibroblasts and smooth muscle cells. Our results show that mevalonate cascade inhibition induces different arms of the UPR with different kinetics, with early activation of ER

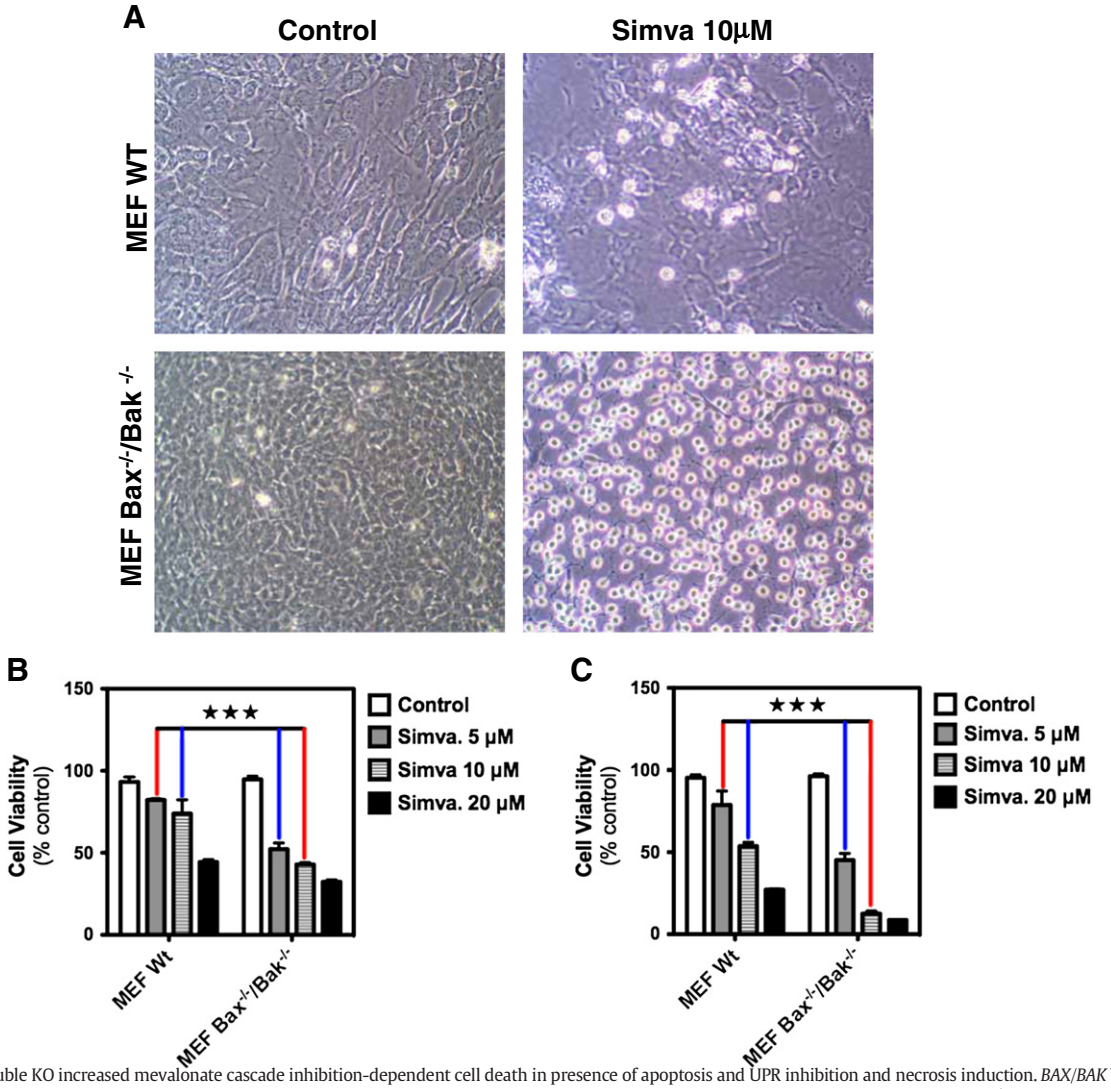


Fig. 5. *BAX/BAK* double KO increased mevalonate cascade inhibition-dependent cell death in presence of apoptosis and UPR inhibition and necrosis induction. *BAX/BAK* Wt MEF cells and *BAX/BAK*$^{-/-}$ KO MEF cells treated with simvastatin (10 μM). (A) Cell morphology confirmed that simvastatin caused increased cell death in *BAX/BAK*$^{-/-}$ KO MEF cells as early as 24 h. (B, C) Simvastatin (10 μM, 24 and 72 h) induced significantly higher cell death in Bax/Bak$^{-/-}$ MEF than in Wt MEF cells (★★★ $P < 0.001$) at 24 and 72 h. For each experiment, control cells were treated with simvastatin solvent (DMSO) at the same concentration as used in the simvastatin experiments. Results are expressed as mean ± SD of 6 independent experiments. (D) Immunoblot analysis show that mevalonate cascade inhibition in *BAX/BAK*$^{-/-}$ KO MEF cells increased LC3 lipidation and Atg5–12 conjugation, while decreasing un-cleaved PARP-1, BIP and IRE1α expression when compared to corresponding Wt cells. Equal protein loading was assessed using β-actin. (E) Wt MEF and *Bax/Bak* KO were treated with simvastatin (10 μM, 24 h). Caspase-9 activity was measured in time-matched control and simvastatin treated cells. We observed significant differences between Wt and *Bax/Bak* KO cell in simvastatin-induced caspase-9 activation (★★★ $P < 0.001$) (F, G) Ultrastructural (TEM) studies of Wt and *BAX/BAK*$^{-/-}$ MEF cells confirmed that simvastatin (10 μM, 72 h) treatment induced autophagosome formation in Wt MEF. By contrast, simvastatin treated Bax/Bak$^{-/-}$ MEF cells show hallmark of both, necrosis (cell swelling and cell membrane disintegration), and autophagy. (H) Simvastatin induced a significant (★★★ $P < 0.001$) decrease in total cellular ATP in *BAX/BAK*$^{-/-}$ MEF as compared to Wt MEF at 24 and 72 h. For each experiment, control cells were treated with simvastatin solvent (DMSO) as a control. Results are expressed as mean ± SD of 6 independent experiments. (I) Wt MEF and *BAX/BAK*$^{-/-}$ KO MEF cells were treated with simvastatin (10 μM, 72 h) and concentrated supernatant of these cells were immunoblotted for HMGB1 detection. Simvastatin induced HMGB1 release exclusively from *BAX/BAK*$^{-/-}$ MEF cells.

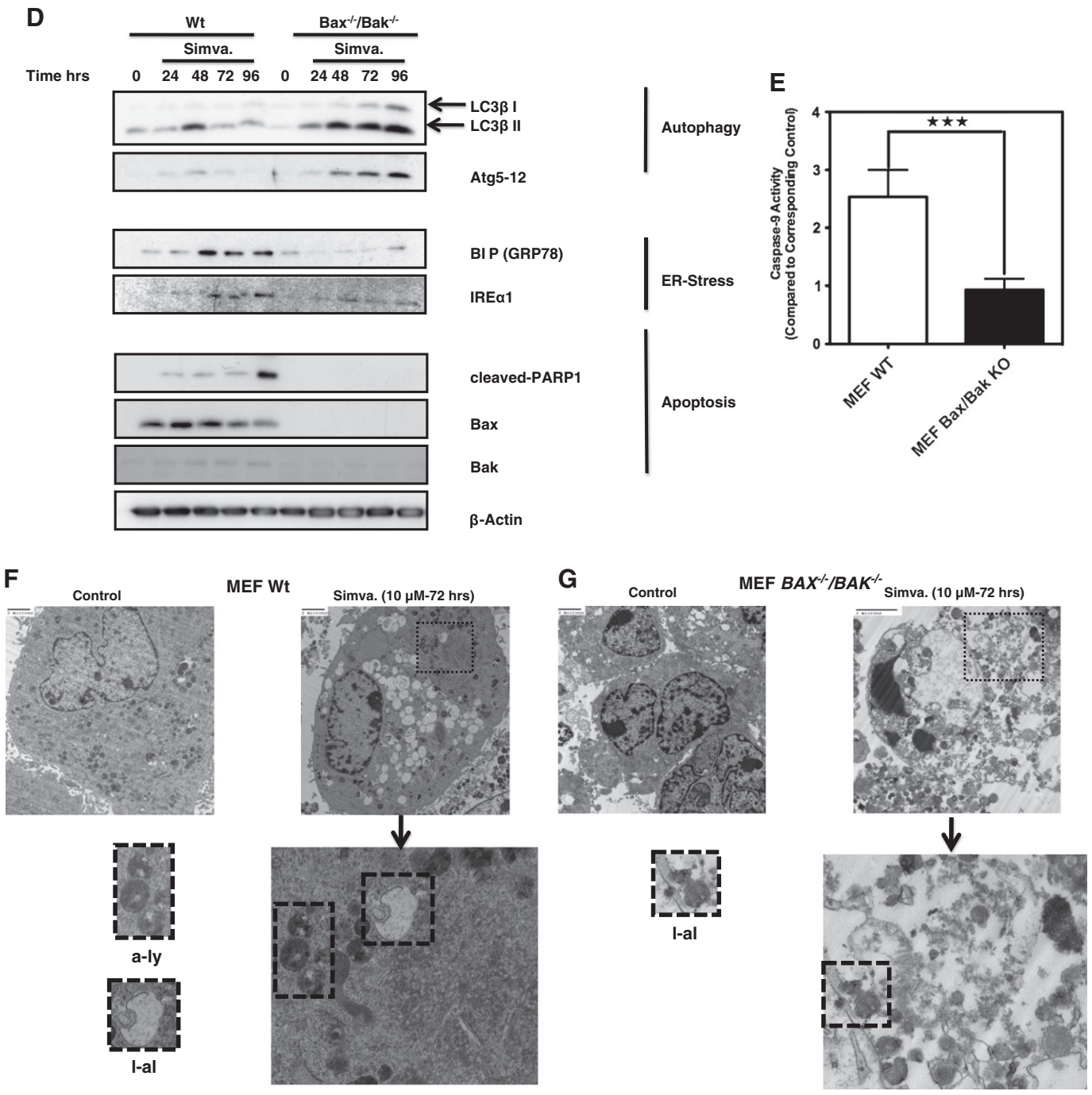
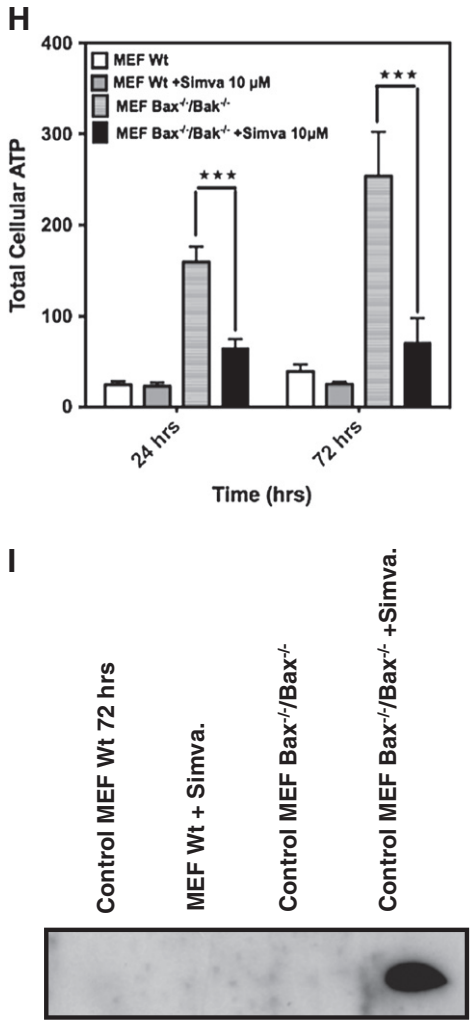


Fig. 5 (*continued*).

chaperone-like proteins, calnexin and Ero1, and late activation of the ER stress pathways involving IRE1, ATF6 and ATF4. This suggests that mevalonate cascade inhibition-induced UPR is a multi-step process.

The precise upstream mechanisms for the impact of simvastatin on apoptosis, autophagy and ER stress are not fully evident from our study; however, that we did observe rescue from these pathways with mevalonate replacement suggests that depletion of intermediates of the mevalonate cascade is a principal driver. Consistent with this conclusion, cholesterol supplementation was not sufficient to prevent statin induction of these pathways and the resulting loss of cell viability. Indeed in a prior study we confirmed that in conditions similar to those used in the present study, though simvastatin suppresses HMG-CoA reductase activity, it does not lead to loss of total cellular cholesterol in human airway mesenchymal cells. These observations offer clues to highlight the importance of lipid modification of protein effectors, such as small GTPase proteins that require prenylation with C-15 farnesyl or C-20 gernylgeranyl moieties, that are compromised during statin exposure [20,26]. This suggests that future research effort to assess the impact of selective inhibitors of prenyltransferases, which catalyze the prenylation of intracellular proteins, warrant investigation with respect to lung mesenchymal cell apoptosis, autophagy, UPR and cell fate.

Our data demonstrate a number of coordinated intracellular responses to statin exposure that determine cell death and survival. The triggering of ER stress and UPR activation has been associated previously with caspase-4 mediated activation of caspase-9 and the



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induction of apoptosis [40]. Our data reveal the involvement of caspase-4 in mevalonate cascade inhibition-induced cell death, as a specific inhibitor of caspase-4 suppressed simvastatin-triggered effector caspase activation and prevented loss of cell viability. We recently reported that concomitant induction of autophagy tempers statin-triggered apoptosis in human airway mesenchymal cells [7]. Autophagy eliminates damaged organelles [60] and cytosolic compounds to maintain cellular homeostasis and is protective during cellular stress (e.g. nutrient deficiency), thus, preventing cell death, including that stemming from ER stress [61–70]. Here we show that loss of cell viability induced by mevalonate cascade inhibition in MEFs and human airway smooth muscle cells is potentiated by silencing of pro-autophagy genes, *ATG3* or *ATG7*. Notably, this effect coincided with an increase in both UPR (IRE1α) and apoptosis markers (cleaved caspase-3 and -7). Thus, autophagy induced by mevalonate cascade inhibition modulates both the induction of apoptosis and ER stress that leads to UPR.

Bcl-2 family members, major regulators of apoptotic signaling, can localise to the ER and initiate ER damage that modulates apoptotic pathways linked to mitochondria and the ER [71–73]. Bax and Bak are pro-apoptotic Bcl-2 family members that are necessary and sufficient to mediate intrinsic apoptosis pathways triggered by diverse stimuli [74]. We have previously shown that Bax and Bak regulate mevalonate cascade inhibition-induced apoptosis in lung mesenchymal cells [20]. Here, using MEFs deficient in both Bax and Bak, we investigated the dependence of ER stress, apoptosis, and autophagy induced by simvastatin on these proteins. As expected, apoptosis was blunted, however we also found that ER stress was suppressed, but autophagy was potentiated and promoted programmed necrosis. Of note, Bax and Bak associate with, and are essential for IRE1α function at the ER membrane [75]. Indeed, ER stress generally activates Bax and Bak which in turn leads to decreased mitochondrial membrane potential and mitochondria-dependent apoptosis [72]. In the cells that lack Bax and Bak, formation of mitochondrial permeability pores is thwarted, thus, preventing apoptosome formation and activation of downstream executioner caspases. This also makes the mitochondria more susceptible to sudden rupture [76], and subsequent depletion of cellular ATP that can induce necrosis due to deficient energy stores [72]. This scenario appears to be bourn out in our studies and contribute to excessive statin-induced cell death, via necrosis. Collectively our data reveal the coordination of apoptosis, autophagy and ER stress pathways in the face of mevalonate cascade inhibition determines the death and/or survival response of mesenchymal cells.

In summary, our findings demonstrate that autophagy holds a protective function against apoptosis and ER-stress in human airway fibroblasts and smooth muscle cells exposed to simvastatin. The importance role of the ER in cell response to mevalonate cascade inhibition was evident by the fact that ER-activated caspase-4 is required for simvastatin-induced cell death and apoptosis. Further, the ER membrane anchored pro-apoptotic Bcl-2 family proteins, Bax and Bak, mediate mevalonate cascade inhibition-induced ER stress and apoptosis as the absence of these proteins is permissive for cell death by necrosis. We provide evidence for the interconnection between apoptosis, autophagy, and ER stress, and shed new light on some of the direct cellular effects of statins that may be of relevance to the clinical use of these compounds.

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