HMG-CoA reductase inhibitors activate the unfolded protein response and induce cytoprotective GRP78 expression

Jui-Ching Chen1, Mei-Lin Wu2, Kuo-Chin Huang3*,†, and Wan-Wan Lin1*,†

1Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan; 2Department of Physiology, College of Medicine, National Taiwan University, Taipei, Taiwan; and 3Department of Family Medicine, National Taiwan University Hospital, Taipei, Taiwan

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Aims Since apoptosis of macrophages induced by stress to the endoplasmic reticulum (ER) contributes to advanced atherosclerotic lesions, we sought to understand the effects of statins on the unfolded protein response (UPR).

Methods and results We used pharmacological, biochemical, and siRNA (small interfering ribonucleic acid) approaches to determine the signalling cascades of statin-induced 78 kDa glucose-regulated protein (GRP78) gene transcription and its role in cytoprotection. Exposure of RAW264.7 macrophages to statins increased the expression of GRP78, activating transcription factor 6, X box protein-1, and phosphorylated eukaryotic translation initiation factor 2α, while it had no effect on CCAAT/enhancer binding protein-homologous protein. GRP78 induction was abolished by co-treatment with mevalonate and 1,2-bis(o-aminophenoxy)ethane-N,N,N0,N0-tetraacetic acid, indicating the involvement of both 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase-dependent and -independent mechanisms. Studies on promoter activity measurements indicated that phosphoinositide turnover, cellular homologue of v-src (c-Src), protein kinase C (PKC), extracellular signal-regulated kinase (ERK), and p38 are involved in upregulating GRP78 gene transcription. We also observed that elevation of intracellular Ca2+ and interruption of small G proteins are two bifurcated but cooperative signalling pathways for c-Src activation, leading to downstream activation of phospholipase C, PKC, ERK, and p38. Functionally we demonstrated that fluvastatin could protect macrophages from hypoxia-induced cell death through GRP78 induction.

Conclusion We demonstrate a novel action of statins of inducing a cytoprotective UPR, providing new insights into the clinical potential of statins for ameliorating ER stress-related diseases.

KEYWORDS Statins; ER stress; GRP78; Macrophages; Signal transduction

1. Introduction

Endoplasmic reticulum (ER) is the primary site for protein synthesis, folding, and trafficking.1 Under a variety of stressful conditions, the accumulation of unfolded or misfolded proteins in the ER results in the onset of ER stress.1 Mammalian cells trigger an evolutionarily conserved mechanism, termed the unfolded protein response (UPR), to compensate for ER stress.2 The UPR is regulated by three ER intermembrane transducers: PKR-like ER kinase (PERK), inositol requiring 1 (IRE1), and activating transcription factor 6 (ATF6).3 Upon activation of the UPR, IRE1 mediates the splicing of X box protein-1 (XBPs1) messenger ribonucleic acid (mRNA) and results in the production of the XBP1s transcription factor.3 PERK phosphorylates the subunit of eukaryotic translation initiation factor 2α (eIF2α) and transiently attenuates general protein synthesis.3 Phosphorylated eIF2α induces the translation of ATF4 (activating transcription factor 4) and the expression of the ATF4 target gene, CHOP (CCAAT/enhancer binding protein-homologous protein), a proposed apoptotic mediator under ER stress.3 ATF4 undergoes proteolytic cleavage by proteases, allowing cytosolic domain p50ATF6 translocation to the nucleus and activation of UPR target genes.3

GRP78, a 78 kDa glucose-regulated protein, is a major ER chaperone and plays a critical role in regulating ER
homeostasis. The transcriptional activation of the GRP78 gene is mediated by the ER stress-response element (ERSE) present in its promoter. Three sequential cis-acting ERSEs are regulated by the transcription factors, p50ATF6 and XBP1s, and have been shown to be necessary for the induction of GRP78. Previous studies revealed that Ca²⁺, cellular homologue of v-src (c-Src), protein kinase C (PKC), extracellular signal-regulated kinase (ERK), and p38 mitogen-activated protein kinase (MAPK) are involved in the induction of GRP78. Induction of this stress protein protects cells from apoptosis induced by various insults.

Statins are inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase and are widely used as lipid-lowering agents. Besides their therapeutic use in hyperlipidaemia, possible pleiotropic effects of this class of drugs include anti-inflammatory and immunomodulatory benefits. The most studied anti-inflammatory benefits of statins rely on the reduction of cellular levels of mevalonate and mevalonate-derived isoprenoids, including farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which are necessary for post-translational modification of several small G proteins. In this context, we demonstrated the abilities of statins to inhibit inducible nitric oxide synthase, but to upregulate heme oxygenase-1 induction in macrophages. Both cellular responses are dependent on mevalonate, and provide two novel anti-inflammatory mechanisms of statins.

Although the UPR is considered a cytoprotective and survival pathway, severe ER stress leads to cell death via specific pathways. ER stress is a prominent feature in the pathogenesis of atherosclerosis. Trafficking of free cholesterol to the ER triggers ER stress and CHOP expression, leading to apoptosis of macrophages and advanced atherosclerotic lesions. In contrast, ER stress can also induce intracellular lipid accumulation and initiate cell death. Intracellular lipid accumulation and initiate cell death. Therefore, the fact that ER stress-induced cellular dysfunction is involved in the progression of atherosclerosis makes this pathway a plausible therapeutic target.

Given that HMG-CoA reductase is a transmembrane protein anchored to the ER, we herein examined whether statins, anti-atherosclerotic agents targeting HMG-CoA reductase, can regulate sensor proteins of the UPR. Intriguingly, our results clearly showed that statins are capable of inducing ER stress subpathways: increasing GRP78 protein expression and ATF6 and XBP1 activation, but not affecting CHOP or ATF4 protein expression. Moreover, the upstream signalling pathways contributing to the induction of GRP78 as well as the role of GRP78 in cytoprotection were demonstrated.

2. Methods
2.1 Cell culture
RAW264.7 macrophages and BV-2 microglia were cultured as previously described. Bone marrow-derived macrophages (BMDMs) were isolated from 6- to 10-week-old C57BL/6 mice, whose bone marrow was flushed out by Dulbecco’s modified Eagle’s medium, and cultured in DMEM containing 10% foetal bovine serum and 20% L929 cell-conditioning medium. Macrophages were obtained as a homogeneous population of adherent cells after 7 days of culture. For the hypoxic experiments, oxygen in the incubator was reduced from 21% O₂ (normoxia) to 1% O₂ (hypoxia).

2.2 Materials
Antibodies specific to GRP78, XBP1, eIF2α, CHOP, ATF4, β-actin, lamin B, c-Src, ERK, and p38 MAPK were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Antibodies specific to PKC isoforms were purchased from Upstate, Lake Placid, NY, USA. A monoclonal antibody specific to ATF6 was purchased from Imgenex, San Diego, CA, USA. All chemicals were purchased from Calbiochem, San Diego, CA, USA. The GRP78 promoters (−732/+24) were kindly provided by Dr Dai Ruey Chien (National Taiwan University, Taipei, Taiwan), who has amplified the 756 bp fragment of the GRP78 promoter from 9L genomic DNA, based on the sequence of the GRP78 gene (GenBank accession no.: M14866), and cloned it into the pGL3 basic vector. In order to analyse the involvement of the three ERSEs, which are required for p50ATF6 and XBP1 activation, wild-type (−132/+7) and mutant constructs cloned into the pGL3 basic vector were kindly provided by Dr Kazutoshi Mori (Kyoto University, Kyoto, Japan).

2.3 Western blot analysis
Cell lysates for SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) were prepared as previously described. For the PKC assay, lysis buffer (20 mM Tris (pH 7.6), 50 mM 2-mercaptoethanol, 0.1 mM EDTA, 1 mM PMSF, 1 μM leupeptin, and 100 μM NaF) was added to cells. Cells were separated into cytosolic and particulate fractions by centrifugation at 12 000 g for 20 min. The supernatant was recovered, and the membrane fraction was resuspended in lysis buffer containing 1% NP40 and 50 mM EGTA, followed by a brief sonication. The membrane fraction was recovered by centrifugation at 12 000 g for 20 min.

2.4 Real time reverse transcription–polymerase chain reaction with SYBR green detection
The isolated RNA subjected to real-time reverse transcription–polymerase chain reaction (RT–PCR) was treated with DNase to avoid amplification of DNA contaminants. The forward and reverse primers were as follows – GRP78: ACATGGACCTGTTCCGCTCTA and TGGCTCTTGGCATTGAAAGA; CHOP: CCACACACCTGAAAGCGAGA and GGTCGCCCAATTTCACT; ATF4: TTGCCCTCTTACCTCTTG and GAAGTGGTCTGGAGGAAG; and β-actin: CGGGAGACTGCTACCC and AGGAAGGCTGGAAGAGTGC. The C₄ method was used to analyse the results. The C₄ value, which is inversely proportional to the initial template copy number, is the calculated cycle number where the fluorescence signal emitted is significantly above background levels. The multiples of induction by real-time RT–PCR were measured relative to time-matched vehicle-treated controls and calculated after adjusting for β-actin using 2^ΔΔCt, where ΔCt = target gene – β-actin Ct, and ΔΔCt = ΔCt treatment – ΔCt control.

2.5 Promoter activity assay and small interfering ribonucleic acid
Using the LipofectAMINE PLUS reagent, cells were transfected with 1 μg of the necessary plasmids, together with 1 μg of the β-gal-LacZ expression vector. After a 24 h incubation, cells were incubated with the indicated concentrations of agents. After an 18 h incubation, the luciferase activity of cell lysates, normalized to β-gal-LacZ expression, was determined as we previously described. For gene silencing, small interfering ribonucleic acid (siRNA) for GRP78 (accession no.: NM_0223100) and siCONTROL non-targeting siRNA were synthesized by Dharmacon RNA
Technologies, Lafayette, CO, USA. Cells were transfected with siRNAs by Dharmafect.

2.6 Measurement of intracellular Ca$^{2+}$ in macrophages

Intracellular-free Ca$^{2+}$ was detected using the ratiometric Ca$^{2+}$ indicator dye, Fura-2, and a microspectrofluorometer, as we previously described.\textsuperscript{22}

2.7 Measurement of phosphoinositide turnover

Accumulation of [H]$\text{-}$inositol phosphate (IP) in the presence of LiCl as an index of phosphoinositide (PI) turnover was determined, as we previously described.\textsuperscript{23}

2.8 Data analysis and statistical analysis

Data are expressed as the mean ± SEM from several independent experiments as indicated. Analysis of variance was used to assess the statistical significance of the differences, and a value of $P < 0.05$ was considered statistically significant. The protein levels derived from western blotting were quantified by a densitometer, normalized to an internal control, and then expressed as percentages of the control group treated with vehicle only.

3. Results

3.1 Statins induce GRP78 gene expression

In RAW264.7 macrophages, lovastatin and fluvastatin time- and concentration-dependently induced GRP78 protein expression (Figure 1A). Thapsigargin (TG) and tunicamycin (TM), two well-known ER stressors whose actions are respectively ascribed to Ca$^{2+}$ release from the lumen of ER and accumulation of unglycosylated proteins,\textsuperscript{24,25} were also able to elicit GRP78 expression. The cell viability assessed by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenylenetetrazolium bromide assay did not change after incubation with these agents for 24 h (see Supplementary material online, Figure S1). In addition to the macrophage cell line, such GRP78 induction actions of statins were observed in primary BMDMs (Figure 1B) and BV-2 microglia (see Supplementary material online, Figure S2). To determine whether changes in protein levels of GRP78 were due to gene transcription, GRP78 mRNA was determined. As a result, statin-induced increases in GRP78 mRNA were detected at 3 h, and reached a maximum at 9 h (Figure 1C). Moreover, statins, TG, and TM also increased GRP78 promoter activity (Figure 1D).

3.2 Signalling pathways of statin-induced GRP78 expression

To correlate the effect of statins with HMG-CoA reductase, we incubated cells with mevalonate in the presence of fluvastatin. Figure 2A shows that mevalonate (200 μM) partially reversed the effect of fluvastatin on GRP78 induction, but did not reverse the TG-induced response. We further found that the squalene synthase inhibitor, YM53601, did not induce significant GRP78 expression (see Supplementary material online, Figure S3), suggesting that the lowering of cholesterol was not involved in statin-induced GRP78 expression. To further verify the involvement of the isoprenylation pathway in this phenomenon, we determined the effects of a farnesyl transferase inhibitor (FTI-277), a geranylgeranyl transferase inhibitor (GGTI-298), and a Rac inhibitor (NSC23766). FTI-277 (10 μM) was found to cause GRP78 protein induction in a time-dependent manner. Similarly, GGTI-298 (10 μM) and NSC23766 (100 μM) caused GRP78 protein expression after 12 and 24 h of incubation (Figure 2B). Furthermore, the extent of GRP78 induction by these inhibitors was less pronounced than that by statins, and co-treatment with FTI-277 + GGTI-298, FTI-277 + NSC23766, or GGTI-298 + NSC23766 did not lead to the same GRP78 expression level as with statins. These results suggest the involvement of both mevalonate-dependent and -independent mechanisms in the statin-induced GRP78 response.

To elucidate the role of intracellular Ca$^{2+}$, [Ca$^{2+}$], and its relationship with small G proteins in statin-induced GRP78 expression, cells were incubated with BAPTA [1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid] and/or mevalonate. Figure 2A shows that individually BAPTA/AM and mevalonate partially reversed the effect of fluvastatin on GRP78 induction, and the combined treatment almost completely abrogated the response to fluvastatin. Nevertheless, only BAPTA inhibited TG-induced GRP78 protein expression, and further addition of mevalonate did not increase the extent of inhibition (Figure 2A). These observations suggest that two independent pathways elicited by [Ca$^{2+}$], increase and inhibition of small G protein isoprenylation are coordinately involved in the action of statins, while only Ca$^{2+}$ plays a role in TG’s action.

To investigate the signal pathway(s) involved in GRP78’s induction, we examined several inhibitors. Results of Figure 2C show that treatment of cells with a PI-specific phospholipase C (PLC) inhibitor (U73122), but not an inactive analogue (U73343) or a phosphocholine-specific PLC inhibitor (D609), antagonized the inductive effect of fluvastatin. A PKC inhibitor (Ro31-8220), a c-Src inhibitor (PP2), a MEK inhibitor (U0126), and a p38 inhibitor (SB203580) reduced fluvastatin-induced GRP78 induction. These findings suggest the participation of c-Src, PI-PLC, PKC, ERK, and p38 in statin-induced GRP78 expression. Any of these inhibitors alone had no effect on GRP78 expression (data not shown). In parallel with GRP78 protein expression, BAPTA, mevalonate, and kinase inhibitors all diminished fluvastatin-induced GRP78 promoter activity (Figure 2D). Accordingly, the inhibitory effects of BAPTA and mevalonate on GRP78 gene induction were additive. Moreover, all inhibitors except mevalonate and D609 inhibited the TG-induced protein level and promoter activity of GRP78 (see Supplementary material online, Figure S4). All these results confirmed the existence of two signalling pathways for statin’s action, i.e. interruption of small G protein function through preventing isoprenylation, and an [Ca$^{2+}$], increase independent of the inhibition of HMG-CoA reductase.

3.3 Statin increases intracellular Ca$^{2+}$ and stimulates phosphoinositide turnover

Since BAPTA inhibited statin’s response, we next addressed the effect of fluvastatin on [Ca$^{2+}$]. The resting [Ca$^{2+}$] in RAW264.7 macrophages was 106 ± 5 nM, and the maximal [Ca$^{2+}$], with 2 min of fluvastatin
Figure 1 Statins induce GRP78 gene transcription. RAW264.7 cells (A, C) and bone marrow-derived macrophage (BMDM) (B) were treated with statins at the indicated concentrations, thapsigargin (TG, 1 μM), or tunicamycin (TM, 1 μg/mL) for different periods. The lower panel of (A) was determined at 24 h of incubation. GRP78 and β-actin proteins were determined by immunoblotting. (C) Real-time RT–PCR (reverse transcription–polymerase chain reaction) was performed to determine GRP78 messenger ribonucleic acid (mRNA). (D) GRP78 promoter activity was determined in RAW264.7 cells after incubation with statins (30 μM), TG, or TM for 18 h. The data are the mean ± SEM of three separate experiments performed in duplicate. Asterisk – P < 0.05 indicates a significant increase in GRP78 protein, mRNA and luciferase activities.
Figure 2 Effects of pharmacological agents on fluvastatin-induced GRP78 expression in RAW264.7 macrophages. (A) 1,2-Bis(o-aminophenoxy)ethane-N, N', N'-tetraacetic acid (BAPTA)/AM (30 μM) and/or mevalonate (200 μM) were used to pretreat macrophages for 30 min prior to incubation with fluvastatin (30 μM) or thapsigargin (TG, 1 μM) for 24 h. (B) Cells were treated with farnesyl transferase inhibitor (FTI-277) (10 μM), geranylgeranyl transferase inhibitor (GGTI-298) (10 μM), NSC23766 (100 μM), and/or fluvastatin for the indicated time periods. (C) Cells were pretreated with U73122 (5 μM), U73343 (5 μM), D609 (30 μM), Ro31-8220 (1 μM), PP2 (10 μM), U0126 (10 μM), or SB203580 (3 μM) for 30 min, then stimulated with fluvastatin for 24 h. Protein levels of GRP78 were measured in the cell lysates by immunoblotting. (D) The luciferase activity derived from GRP78 activation was determined in cells pretreated with the indicated agent for 30 min followed by fluvastatin stimulation for 18 h. The data are the mean ± SEM of three separate experiments performed in duplicate. Asterisk – *P < 0.05, indicates a significant stimulating response of statin, TG, and small G protein inhibitors. Hash mark – #P < 0.05 indicates significant inhibition of statin- and TG-induced responses.
(30 μM) incubation was $1451 \pm 130$ nM (Figure 3A, left panel). The peak response of $[\text{Ca}^{2+}]_i$ was lower when extracellular calcium was chelated by 3 mM EGTA in the incubation solution ($490 \pm 70$ nM) (Figure 3A, right panel). In Ca-free medium, a concentration-dependent increase in $[\text{Ca}^{2+}]_i$ by fluvastatin (1–30 μM) was observed (Figure 3B). This indicates that the transient increase in $[\text{Ca}^{2+}]_i$ was due to $\text{Ca}^{2+}$ release from intracellular stores, which was followed by extracellular $\text{Ca}^{2+}$ influx. We further investigated the role of the PI-PLC system in
fluvastatin-evoked Ca\(^{2+}\) release and if this event was mevalonate-dependent. In Figure 3B, with pretreatment of cells with U73122 or mevalonate for 30 min, the transient increase in [Ca\(^{2+}\)]\(_i\), by fluvastatin was inhibited. To analyse which [Ca\(^{2+}\)]\(_i\)] pool was responsible for statin’s action, we first depleted ER Ca\(^{2+}\) stores using TG, and then stimulated cells with fluvastatin. Figure 3C shows that after TG treatment, the fluvastatin-induced Ca\(^{2+}\) increase was markedly reduced from 490 ± 70 to 168 ± 2 nM. Nevertheless, after fluvastatin treatment, TG no longer induced an increase in Ca\(^{2+}\). Thus, TG- and inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive Ca\(^{2+}\) stores in the ER are the major intracellular sources of fluvastatin.

To verify our suggestion that PI–PLC activation is involved in statin’s actions, we measured intracellular IP accumulation. Figure 3D shows that in RAW264.7 cells, fluvastatin induced significant activation of PI turnover. Our previous study showed that activation of the pyrimidine receptor by uridine triphosphate (UTP) triggers PI turnover in RAW264.7 macrophages. Here we found the additive PI turnover through activation of receptor (i.e. UTP) and non-receptor- (i.e. statin) mediated pathways. To further dissect the upstream regulators of statin-induced PI–PLC activation, we pretreated cells with PP2, mevalonate, or U73122. Figure 3E shows that these pretreatments almost completely abolished fluvastatin-induced IP formation, suggesting that inhibition of small G protein isoprenylation and activation of Src are in the same upstream input for statin-induced PI–PLC activation.

3.4 Roles of intracellular Ca\(^{2+}\) increases and isoprenylation interruption in statin-induced cellular homologue of v-src, protein kinase C, extracellular signal-regulated kinase, and p38 activations

The above results indicated that statin-induced PI–PLC activation and GRP78 expression were markedly attenuated by pretreatment with PP2 and mevalonate. Mutoh et al.\(^{26}\) reported that simvastatin can induce tyrosine phosphorylation of PLC\(_y\)1 and generate IP formation in L6 myoblasts. Therefore, we hypothesized that the interruption of small G protein isoprenylation leading to c-Src/PI–PLC\(_y\)1/IP\(_3\)-PKC signalling activation is involved in statin-induced GRP78 expression. To verify this notion, we measured c-Src activation. After fluvastatin treatment for 2 and 8 min, phosphorylated c-Src was detected (Figure 4A); this action was abolished by PP2 and inhibited by mevalonate and BAPTA. Moreover, co-treatment with mevalonate and BAPTA caused its abolition (Figure 4B), suggesting that c-Src activation by statin is not only downstream of a mevalonate-restorable pathway, but also downstream of the increase in [Ca\(^{2+}\)]\(_i\). Next we assessed the effect of statins on PKC activation. Pretreatment of cells with BAPTA, mevalonate, or U73122 markedly attenuated the fluvastatin-induced PKC\(_\alpha\) and \(\beta\)1 translocation to the membrane fraction (Figure 4C). These results further support our notion that mevalonate-reversible action of statin as well as Ca\(^{2+}\)-regulated Src event can lead to PI–PLC activation, generation of the secondary messengers, diacylglycerol and IP\(_3\), and then activation of classical PKC isoforms.

As the above results suggested the involvement of ERK and p38 in statin-induced GRP78 expression, we dissected the upstream signalling cascade for the activation of both kinases. Figure 4D shows that fluvastatin-elicted ERK and p38 activations were attenuated by BAPTA, U73122, Ro31-8220, PP2, and mevalonate. Moreover, an additive inhibition was detected upon co-treatment with BAPTA and mevalonate.

3.5 Fluvastatin activates activating transcription factor 6, X box protein-1, and eukaryotic translation initiation factor 2a

Since ATF6 and XBP1 are involved in GRP78 gene transcription, we examined their expression levels after treatment with statin. p50ATF6, the processed and active form of ATF6, and the spliced (active) form of XBP1 were both increased by fluvastatin (Figure 5A). Fluvastatin also induced sustained eIF2\(\alpha\) phosphorylation after 6 h of incubation. Similar to fluvastatin, TG and TM also induced these activating markers of the UPR (Figure 5A). We next examined the effect of fluvastatin on the expression of the ER stress-inducible proapoptotic protein, CHOP, and its major transcription factor, ATF4.\(^4\) In contrast to the robust CHOP expression induced by TG and TM, fluvastatin did not induce CHOP (Figure 5A). Moreover, unlike TG and TM, fluvastatin had no effects on the protein level of ATF4 (see Supplementary material online, Figure S5).

It is well known that the ERSE is involved in transactivation of the GRP78 gene through the enhanced binding of transcription factors, such as p50ATF6 and XBP1. To elucidate the relative contributions of ERSE1, ERSE2, and ERSE3 contained in the GRP78 promoter in mediating statin’s action, the reporter activity driven by mutation of ERSEs in the GRP78 promoter was evaluated after statin treatment. A transfection experiment with a −132/+7 GRP78 promoter–luciferase reporter construct showed that fluvastatin, TG, and TM were able to increase luciferase activity. Mutation of ERSE1 diminished the effects of fluvastatin, TM, and TG. Similar extents of inhibition were achieved by the simultaneous mutation of ERSE1 and ERSE3. In contrast, when both the ERSE1 and ERSE2 sites were mutated, complete abolition was seen (Figure 5B). These results suggest that ERSE1 and ERSE2 are equivalently involved in fluvastatin- and TM-induced GRP78 gene expression, while ERSE1 is the major element in TG’s action.

Next we attempted to clarify the roles of the ERK and p38 pathways in fluvastatin-induced ATF6 and XBP1s expression, and activation of ERSE components for GRP78 gene expression. As shown in Figure 5C, fluvastatin- and TG-induced p50ATF6 and XBP1 expressions were markedly decreased by U0126. SB203580 also significantly inhibited the p50ATF6 response, but marginally affected the XBP1s response of both ER stress inducers. In agreement with these findings, fluvastatin-induced GRP78 (−132/+7) promoter activity, assayed on either the full-length or ERSE1-mutant construct, displayed partial inhibition of U0126 and SB203580 (Figure 5D).
Figure 4 Fluvastatin activates cellular homologue of v-src, protein kinase C (PKC), p38, and extracellular signal-regulated kinase (ERK). (A) RAW264.7 cells were treated with fluvastatin for different periods. (B–D) Cells were incubated with various inhibitors for 30 min at concentrations as described in the legend of Figure 2, prior to stimulation with fluvastatin for 2 min (B) or 30 min (C, D). Total cell lysates (A, B, D) or cellular fractions (C) were immunoblotted with specific proteins. Asterisk – $P < 0.05$ indicates a significant stimulating response of fluvastatin. Hash mark – $P < 0.05$ indicates significant inhibition of a fluvastatin-induced response.
Figure 5 Statin activates activating transcription factor 6 (ATF6), X box protein-1 (XBP1), and eukaryotic translation initiation factor 2a (eIF2a). (A) RAW264.7 cells were treated with fluvastatin or thapsigargin (TG) for different periods. (B, D) Schematic representation of intact (CCAATN9CCACG) or various disrupted endoplasmic reticulum stress-response element (ERSE) constructs. ERSEs disrupted by mutating nucleotides (shown in lowercase letters) are marked by crosses. Cells transfected with the indicated constructs and a β-gal-lacZ plasmid were pretreated with vehicle, U0126 (10 μM), or SB203580 (3 μM) for 30 min followed by fluvastatin, TG, or tunicamycin (TM) stimulation for 18 h. The luciferase activity derived from GRP78 activation was normalized to the transfection efficiency with β-gal-lacZ. (C) RAW264.7 cells were treated with SB203580 (3 μM) or U0126 (10 μM) for 30 min prior to stimulation by fluvastatin or TG. Nuclear extracts after 6 h of stimulation were used to analyse p50ATF6 and lamin B. Total cell lysates after 12 h of stimulation were prepared to determine XBP1 and β-actin. Asterisk – P < 0.05 indicates a significant induction of protein expression by fluvastatin, TM, and TG. Hash mark – P < 0.05 indicates a significant reduction in stimuli responses. (B) The increased luciferase activity in deleted constructs, expressed as percentages in full (−132/+7), is shown in parenthesis.
3.6 Statin-induced 78 kDa glucose-regulated protein confers cytoprotection

Since in many circumstances CHOP contributes to apoptosis following severe ER stress, for example in the case of hypoxia, we were interested in determining if statin-upregulated GRP78 can decrease CHOP induction and confer cell protection. To this end, we first found that RAW264.7 macrophages, pretreated with fluvastatin for 16 h and then washed out, could not achieve high CHOP protein induction following challenge with TM, TG, and hypoxia (Figure 6A). Compared with a normoxic condition, the survival rate of hypoxic RAW264.7 macrophages decreased to 40% within 24 h. When cells were pretreated with fluvastatin for 16 h and then washed out, such hypoxia-induced cell death was inhibited by fluvastatin (3–30 μM) in a concentration-dependent manner (Figure 6B, right panel). Next we used RAW264.7 cells in which GRP78 expression was knocked-down by RNA interference (Figure 6B, left panel), and found that hypoxia-induced cell death, which could be protected by pretreatment with fluvastatin, was significantly attenuated (Figure 6B, right panel).

To verify that ERK- and p38-dependent GRP78 induction indeed plays a role in cytoprotection, we pretreated cells with U0126 or SB203580 before fluvastatin. Results revealed that the significant cytoprotective effect of fluvastatin was inhibited by both inhibitors (Figure 6C). Even though fluvastatin pretreatment exerts cytoprotection against hypoxia, cell death caused by TG and TM was not affected by fluvastatin (data not shown).

4. Discussion

GRP78 upregulation is believed to increase the capacity to buffer stressful insults initiating from ER. In hyperlipidaemia patients, the accumulation of free cholesterol in the ER membrane of macrophages induces ER stress, resulting in the expression of CHOP, cell death, lesional rupture of atheroma plaques, and thrombogenicity. Beyond directly lowering the intracellular cholesterol level as a therapeutic benefit for atherosclerosis, in this study we found that statins at therapeutic concentrations can induce protective GRP78 expression, but not proapoptotic CHOP expression in macrophages, implying a beneficial role in reducing cell death occurring in atheroma lesions. Mevalonate, a metabolite of HMG-CoA reductase, is the substrate for cholesterol synthesis and isoprenylation products. We found that mevalonate reversed statin-elicited GRP78 induction, while a squalene synthase inhibitor could not, suggesting that impeding small G protein function, GRP78 induction, while a squalene synthase inhibitor could not, suggesting that impeding small G protein function, and Src’s activation after treatment with statins, we suggested that Src activation is subsequent to the inhibition of the small G protein, and mediates PI–PLCγ activation. Supporting this notion, previous studies showed that c-Src plays a crucial role in PI–PLCγ activation. However, based on the partial inhibition by U73122 and mevalonate, and a moderate but significant [Ca2+]i increase following depletion of ER Ca2+ stores, we suggest that statin can also mobilize [Ca2+]i stores other than those of the ER, and this action is independent of mevalonate. Accordingly, a previous study reported that simvastatin could trigger mitochondrial-dependent Ca2+ signalling in skeletal muscles.

In this study, we detected increased p50ATF6 and XBP1 after statin treatment, indicating the stimulating effects of statin on the UPR. Moreover, we demonstrated that induction of both transcription factors relies on ERK and p38 to different extents. ERK is the predominant kinase instead of p38 for mediating p50ATF6 expression and XBP1s mRNA splicing. Previous studies revealed that MEK1/2 activation is an early upstream event in the induction of XBP1 mRNA splicing, and ATF6 is a target of p38 phosphorylation, the phosphorylation of which augments the nuclear translocation and transactivation of ATF6. Despite ERK appearing to play a more-important role than p38 in the expression of p50ATF6 and XBP1s, results of GRP78 promoter activity and protein expression do not support similar effects. Both kinase inhibitors attenuated the latter responses to similar extents. We suggest that this controversy might be related to the redundancy of p50ATF6 and/or XBP1s, or functional integration with other transcription factors. Participation of ERSEs in expression of the GRP78 gene was reported. In agreement with most cases reported before, statin-induced GRP78 appeared to require p50ATF6 and XBP1s binding with both ERSE1 and ERSE2.

GRP78 is a multifunctional protein whose physiological role in attenuation of cell injury is just emerging. To verify GRP78’s expression as one mechanism for the therapeutic benefits of statins, we demonstrated the ability of statin to reduce ER stressor (TG, TM, and hypoxia)-induced CHOP production. In this aspect, we found that pretreatment with fluvastatin at a circulating concentration (3 μM) is sufficient to display cytoprotection against hypoxia-induced cell death, and this event was associated with GRP78 induction in macrophages. Thus statin-induced GRP78 leading to prevention of macrophage injury from hypoxia insult provides an additional mechanism to ameliorate signalling pathways, i.e. a Ca2+ increase and G protein-interruption, which induce GRP78 expression.

In accordance with previous studies which found that simvastatin elevates the [Ca2+]i level in L6 myoblasts, skeletal muscles, and endothelial cells, the present study demonstrated that statins mobilized [Ca2+]i in RAW264.7 cells, and this effect was partially dependent on the inhibition of HMG-CoA reductase. Acute application of statins resulted in a prominent [Ca2+]i increase in Ca2+-containing medium; however, the same treatment resulted in a minor increase in Ca2+-free medium. Thus, the Ca2+ response induced by fluvastatin was due to Ca2+ release from intracellular stores and Ca2+ influx. The present results also confirm that PI–PLC was activated by statin, and IP3 partially mediated Ca2+ mobilization from TG-sensitive ER stores. Since PP2 and mevalonate almost abolished PI turnover and Src’s activation after treatment with statins, we suggest that Src activation is subsequent to the inhibition of the small G protein, and mediates PI–PLCγ activation. Supporting this notion, previous studies showed that c-Src plays a crucial role in PI–PLCγ activation. However, based on the partial inhibition by U73122 and mevalonate, and a moderate but significant [Ca2+]i increase following depletion of ER Ca2+ stores, we suggest that statin can also mobilize [Ca2+]i stores other than those of the ER, and this action is independent of mevalonate. Accordingly, a previous study reported that simvastatin could trigger mitochondrial-dependent Ca2+ signalling in skeletal muscles.
Figure 6 Involvement of GRP78 in statin-mediated cytoprotection. (A) RAW264.7 cells were pretreated with fluvastatin for 16 h, washed out, and incubated with tunicamycin (TM, 1 μg/mL), thapsigargin (TG, 1 μM), or hypoxia for 24 h. CCAAT/enhancer binding protein-homologous protein (CHOP) expression was then determined. (B) Cells were transfected with small interfering ribonucleic acid (siRNA) of the control or GRP78 and cultured for 32 h. Then, cells were incubated with TM (1 μg/mL) or fluvastatin (30 μM) for 24 h, and cell lysates were prepared for determining GRP78 and β-actin. In some experiments, cells following siRNA and statin treatments were subjected to hypoxia for 24 h, and cell viability was analysed by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide assay. (C) Cells were pretreated with fluvastatin and/or U0126 (10 μM) or SB203580 (3 μM) for 16 h, washed out, and subjected to normoxia or hypoxia for 24 h. After incubation, the cell viability was analysed by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide assay. Data represent the mean ± SEM from four separate experiments. Asterisk – *P < 0.05 indicates a significant increase in GRP78 expression by TM and fluvastatin as well as the increased survival rate under hypoxic conditions induced by fluvastatin. Hash mark – #P < 0.05 indicates a significant reduction in stimulus-induced CHOP and GRP78 expression as well as survival induced by fluvastatin. (D) A schematic representation of signalling pathways for unfolded protein response (UPR) activation in RAW264.7 cells treated with statins.
the pathological progress of atherosclerosis. Interestingly, we found that such GRP78 induction by fluvastatin was still insufficient to protect macrophages from death induced by TG and TM. The complexity of the death pathways, some of which cannot be balanced by the function of GRP78, might explain these inconsistent results. In human prostate cancer cells, no effect of GRP78 in preventing apoptosis induced by TM has been reported.9

In conclusion, results obtained from the present study demonstrate for the first time that HMG-CoA reductase inhibitors are able to upregulate the canonical UPR targets ATF6, XBP1, and GRP78. A schematic summary of this UPR activation induced by statins is depicted in Figure 6D. These results taken together suggest that GRP78 might be involved in statin-mediated cytoprotection, providing new insights into the protective response associated with UPR activation and hence a new basis for the therapeutic development of statins.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

**Conflict of interest:** the authors declare no conflicts of interest.

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