Protein Phosphatase 2A Promotes Endothelial Survival via Stabilization of Translational Inhibitor 4E-BP1 Following Exposure to Tumor Necrosis Factor-α

Carla Janzen, Suvajit Sen, Janis Cuevas, Srinivasa T. Reddy, Gautam Chaudhuri

Objective—Tumor necrosis factor-α (TNFα) may change from a stimulator of reversible activation of endothelial cells (ECs) to a killer when combined with cycloheximide (CHX). The means by which endothelial cells are destined to either the survival pathway or the apoptotic pathway are not fully understood. We investigated the role of p38 mitogen-activated protein kinase (MAPK) and protein phosphatase 2A (PP2A) activation and their regulation of 4E-BP1 stability in ECs to determine whether this pathway contributes to apoptosis induced by TNFα and CHX.

Methods and Results—Apoptosis was induced in human umbilical vein ECs (HUVECs) by treating them with a combination of TNFα and CHX (TNFα/CHX). Activation of p38 MAPK was increased in HUVECs undergoing apoptosis, which was associated with degradation of eukaryotic initiation factor 4A regulator 4E-BP1 in a p38 MAPK–dependent manner. CHX attenuated a TNFα-stimulated increase in the expression and activity of PP2A. Silencing PP2A expression with small interfering RNA transfection mimicked CHX sensitization, increasing HUVEC apoptosis with TNFα stimulation and suggesting a protective role for PP2A in the apoptotic process.

Conclusion—Our data suggest that (1) TNFα stimulates PP2A and HUVECs elude apoptosis by PP2A-dependent dephosphorylation of p38 MAPK, and (2) CHX-induced inhibition of PP2A leads to maintenance of p38 activity and degradation of 4E-BP1, resulting in enhanced TNFα-induced apoptosis. (Arterioscler Thromb Vasc Biol. 2011; 31:2586-2594.)

Key Words: apoptosis ■ atherosclerosis ■ cytokines ■ endothelial function ■ endothelium

Tumor necrosis factor-α (TNFα), a proinflammatory cytokine that mediates apoptosis in endothelial cells, is implicated in the pathogenesis of atherosclerosis.1 Although it has been well established that endothelial cell (EC) apoptosis is an important process underlying the pathogenesis of atherosclerotic plaque,2 the molecular mechanisms responsible for apoptosis of ECs in the setting of TNFα exposure remains elusive, because TNFα simultaneously stimulates pathways for apoptotic response (eg, caspase activation) and pathways for survival (eg, activation of the transcription factor NFκB).3,4 The ultimate fate of the cell is determined by the balance between pro- and antiapoptotic stimuli and is a consequence of cross-talk between the major TNFα-induced signaling pathways.5,6

A clue to solving the identity of protective factors that are activated with TNFα in ECs has come from the cell culture model; ECs in vitro will not undergo apoptosis in the presence of TNFα unless they are sensitized by exposure to an inhibitor of protein synthesis such as cycloheximide (CHX),7 a well-known ribotoxin.8 Surprisingly, although CHX sensitizes ECs to apoptosis with TNFα exposure, CHX on its own does not induce EC apoptosis and has been shown in several in vivo studies to protect the endothelium from the formation atherosclerosis.9–11 This is therefore an interesting paradox: although TNFα on its own does not cause apoptosis of ECs and CHX alone has beneficial effects on atherosclerosis, a combination of the 2 leads to EC apoptosis. Various hypotheses have been put forward to explain this phenomenon.

In view of the fact that inhibition of protein synthesis by CHX exposure alone does not induce apoptosis in ECs, we investigated a novel mechanism as to whether TNFα/CHX treatment can modulate 4E-BP1, the inhibitor of eukaryotic initiation factor 4E (eIF4E), and thereby lead to apoptosis of the EC. In addition to its well-known role in initiation of cap-dependent translation, eIF4E has been more recently identified as a master regulator of cell survival.12 Several investigators have identified 4E-BP1 as an important regulator in maintaining cellular viability during conditions of cellular stress, such as hypoxia.13 Energy homeostasis is maintained by 4E-BP1 by its sequestration of eIF4E and resulting reduction of translation initiation. Thus, 4E-BP1 is a well-conserved metabolic brake. In addition, cleavage of the
full-sized 4E-BP1 polypeptide by caspase generates a peptide fragment that sequesters and inhibits eIF4E even more potently than the full-length 4E-BP1. Peptide products sequester eIF4E by binding to its conserved binding site, involving trypotphan. Studies suggest that increasing intracellular levels of peptides containing a conserved eIF4E-binding motif found within 4E-BP1, with the ability to bind eIF4E, leads to rapid dose-dependent apoptosis that is not linked to inhibition of cap-dependent translation.

In agreement with other studies, we demonstrate that the p38 pathway plays a role in regulating the TNFα/CHX-induced EC apoptosis. In addition, our data suggest a novel mechanism by which CHX decreases EC resistance to apoptosis with TNFα stimulation. This work presents evidence that CHX, through inhibition of protein phosphatase 2A (PP2A) activity, leads to uninhibited upregulation of p38 in ECs, which in turn increases the degradation of 4E-BP1. Ultimately, we have provided new insights into the mechanisms modulating the vascular endothelial apoptotic response with TNFα/CHX treatment.

Methods

Reagents

Recombinant human TNFα was purchased from R&D Systems (Minneapolis, MN). CHX, rapamycin, and okadaic acid (OA) were purchased from Sigma-Aldrich (St Louis, MO). SB203580, was from Calbiochem (San Diego, CA). Cell culture media were purchased from Lonza (Walkersville, MD). Antibodies used for this study were anti-p38, anti-phospho-p38 (New England Biolabs, Beverly, MA), anti-4E-BP1, anti-phospho-4E-BP1 (Cell Signaling Technology, Beverly, MA) and anti-GAPDH (Chemicon, Temecula, CA).

Cell Culture

Primary cultures of human umbilical venous ECs (HUVECs) were obtained as described and used at passage 3 or 4. HUVECs were cultured in EC basal medium-2 and the medium was supplemented with Endothelial Growth Medium-2 Bullet kit (Lonza). HUVECs were cultured until 80% confluent and then serum starved overnight. Cells were incubated in 100-mm culture dishes and then trypsinized. Floating cells were collected, pelleted, and pooled with trypsinized cells, and viability was assessed by trypan blue exclusion.

Fluorescence-Activated Cell Sorter Analysis

Cells were cultured as described previously, and after treatment, aliquots of cells were resuspended in 500 μL of binding buffer and stained with 5 μL of fluorescein isothiocyanate–labeled annexin V according to the manufacturer’s instructions. Propidium iodide (5 μL) was added to the samples after staining with annexin V; samples were then put in the dark for 30 minutes. Flow cytometry (FACScalibur, BD Biosciences) was performed immediately after staining.

Western Analysis

Cells were lysed on ice in cell lysis buffer. Protein concentration was measured using Bio-Rad protein assay reagent. Lysates (30 μg) were boiled in Laemmli sample buffer containing β-mercaptoethanol and resolved electrophoretically on 10% SDS-polyacrylamide gel. The gels were electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad) using a tank blot procedure. Membranes were incubated with primary antibodies and the respective horseradish peroxidase–linked to secondary antibody. Immunoreactive bands were visualized by an enhanced chemiluminescence detection system (Amersham).

Immunoprecipitation

For immunoprecipitation analysis, the Dynabead Protein A kit was used (Invitrogen). Briefly, cell lysates were incubated with anti-eIF4E or anti-eIF4G antibody that was bound to the magnetic beads in 200 μL of PBS/Tween 20, and the kit’s instructions were followed. Target antigen was eluted and prepared for Western blotting.

Determination of Caspase Activity

Caspase-3 activity was analyzed by a fluorescence spectrophotometric assay, using a caspase-3 fluorogenic substrate kit (BD Pharmingen, San Diego, CA) as a substrate. After appropriate cell treatment, adherent cells were harvested.

RNA Interference to Silence Expression of PP2A

Small interfering RNA (siRNA) oligonucleotides, as well as control oligonucleotides, were purchased from Santa Cruz Biotechnology (catalog no. sc-43509). Briefly, each well of a 6-well plate containing a subconfluent HUVEC culture was transfected with 100 nmol/L siRNA using Lipofectin (Invitrogen) according to manufacturer’s protocol. The cells were then incubated at 37°C overnight. After 8 hours, the cells in each well were replaced with fresh growth medium. At 72 hours after transfection, the cells transfected with control and experimental siRNA were used for experiments, harvested separately for extraction of total protein, and used for Western blot analysis.

Statistics

All data are expressed as means±SEM, and the Student t test was used for statistical analysis of the differences. For statistical analysis of more than 2 groups, data were analyzed by ANOVA, and pairwise comparisons were performed by the Bonferroni/Dunn post test. Analysis was accomplished with PRISM (GraphPad, La Jolla, CA). The experiments were repeated 3 to 5 times, and data from representative experiments are shown.

Results

Cotreatment With TNFα/CHX Induces Apoptosis in HUVECs

HUVECs are resistant to the cytotoxic effects of either TNFα or CHX alone at low doses; however, TNFα and CHX work synergistically to induce apoptosis when cells are incubated with TNFα and CHX together (Figure 1). No significant difference was observed on cell viability when HUVECs were treated with TNFα or CHX alone. However, when the cells were treated with a combination of TNFα and CHX, cell viability decreased significantly by 8 hours (Figure 1A). TNFα alone increased the activity of caspase-3 after 4 hours of treatment, but a decrease of caspase-3 activity was seen subsequently at 8 hours, with no significant changes in cell viability compared with untreated cells throughout the incubation period (Figure 1B). CHX had no significant effect if given alone, but simultaneous treatment with TNFα increased caspase-3 activation with a peak at 8 hours. Figure 1C shows annexin V and propidium iodide staining assays with flow cytometry, which quantified the effect of TNFα and CHX on apoptosis induction after 8 hours of treatment. The percentage of annexin V–positive cells increased significantly in TNFα/CHX compared with other groups (10.5±3.0%).

Increased Binding of eIF4E to 4E-BP1 in TNFα/CHX-Induced Apoptosis

During cellular conditions favoring apoptosis, 4E-BP1 is hypophosphorylated (active form), allowing its binding to eIF4E, thus impeding binding to eIF4G and formation of an
active initiation complex. We first examined the levels of initiation complexes in HUVECs treated with TNFα/CHX through immunoprecipitation with anti-eIF4E antibody and anti-eIF4G antibodies. Figure 1D shows that at 4 hours of TNFα/CHX treatment, 4E-BP1-eIF4E complexes were increased (top) with a simultaneous reduction in eIF4E-eIF4G complexes (bottom), suggesting that formation of cap-dependent translation initiation machinery, the eIF4E-eIF4G protein complex, was reduced when cells were treated with a combination of TNFα and CHX.

4E-BP1 is phosphorylated at multiple sites, including Thr46, Ser64, and Thr69. p38 mitogen-activated protein kinase (MAPK) has been shown to phosphorylate several sites on 4E-BP1 including Ser-64. We next analyzed the phosphorylation state of 4E-BP1 by Western blot at these sites to determine whether 4E-BP1 hypophosphorylation...
correlates with increased binding to eIF4E. Figure 1E shows that with 4 hours of treatment, CHX had a stimulatory effect on the level of phosphorylation of 4E-BP1, particularly on Ser64 and Thr69 (Figure 1E, compare lanes labeled media and C). Figure 2A shows a time course revealing that although treatment of HUVECs with TNFα/CHX led to an initial increase of Thr37/46 phosphorylation, there was an overall reduction of 4E-BP1 phosphorylation at these sites by 8 hours (top). Remarkably, there was a concurrent and significant reduction of total (full-length) 4E-BP1 at 8 hours following TNFα/CHX treatment (Figure 2A, bottom). Interestingly, this reduction in total level of 4E-BP1 proteins seen with TNFα/CHX treatment was not observed with TNFα or with CHX alone (Figure 2B). Moreover, TNFα/CHX-mediated 4E-BP1 degradation was not due to inhibition of the classic mammalian target of rapamycin pathway because total 4E-BP1 protein levels were stable in the presence of rapamycin, a direct inhibitor of mammalian target of rapamycin (Figure 2B).

**p38 MAPK Inhibitor SB203580 Decreases TNFα/CHX-Induced Apoptosis**

The p38 pathway has been shown previously (1) to regulate the 4E-BP1 and eIF4E complexes and (2) to be required for TNFα-mediated apoptosis in ECs. We therefore examined whether p38 signaling is also involved in TNFα/CHX-induced apoptosis in HUVECs. Treatment of HUVECs with TNFα induced phosphorylation of p38 within 15 minutes (Figure 3A). Figure 3A also shows that p38 phosphorylation was not significantly increased with CHX at 15 minutes, but in cells cotreated with TNFα and CHX, there was a significant increase in p38 phosphorylation compared with cells treated with TNFα alone. To further investigate the involvement of p38, we tested the effects of the p38 inhibitor SB203580 on activation of caspase-3. As compared with TNFα/CHX cotreatment, SB203580 significantly attenuated p38 activation (Figure 3A). In addition, SB203580 treatment led to protection from cell death (Figure 3B, left) and a 45% decrease of caspase-3 release at 8 hours (Figure 3B, right), suggesting that p38 plays a proapoptotic role in TNFα/CHX-induced apoptosis in HUVECs. The increased level of TNFα-induced p38 phosphorylation seen with the addition of CHX (Figure 3A) indicated that CHX facilitates the maintenance of p38 activation. Furthermore, pretreatment with p38 inhibitor SB203580 abrogates 4E-BP1 degradation mediated by TNFα/CHX treatment (Figure 4), suggesting a role for p38/4E-BP1 pathway in TNFα/CHX-mediated apoptosis of HUVECs. Rapamycin pretreatment also partially prevented the TNFα/CHX-induced decrease in 4E-BP1 protein levels. These data, taken together, suggest that increased phosphorylation of 4E-BP1 may contribute to its disappearance.

**Decreased Expression of PP2A With CHX Treatment**

Our results indicate that CHX may be sensitizing ECs to TNFα-induced apoptosis by enhancing the activation of p38 (Figure 3A). To determine the mechanism behind the enhanced activation of p38, we examined the known negative regulators of p38 phosphorylation. Although MAPK phosphatases (MKP1 and MKP2), primarily localized in the nucleus, play a key role in p38 inactivation, we chose to study type 2A phosphatases (PP2A) because it catalyzes the dephosphorylation of both p38 and 4E-BP1. The expression of PP2A subunit was increased with TNFα stimulation, persisting to 8 hours (Figure 5A, left). With TNFα/CHX cotreatment, there was an initial increase in total PP2A protein level by 30 minutes, but the total protein level of PP2A was reduced significantly by 4 to 8 hours (Figure 5B). However, CHX treatment and cotreatment with TNFα/CHX caused a significant increase in PP2A activity (200% compared with untreated cells; Figure 5B). Nevertheless, CHX treatment and cotreatment with TNFα/CHX significantly decreased PP2A activity to 50% of the level seen in untreated cells. To determine whether CHX was enhancing TNFα-promoted 4E-BP1 degradation through inhibition of PP2A, we examined the total 4E-BP1 protein levels in the presence of OA, an inhibitor of PP2A. We found a further decrease in the level of 4E-BP1 protein with OA pretreatment (Figure 5C). OA pretreatment also mimicked CHX treatment in sensitizing HUVECs to TNFα-induced apoptosis. That is, OA pretreatment led to increased HUVEC cell death and a 30% increase of caspase-3 release at 8 hours (data not shown).

To demonstrate that inhibition of PP2A sensitizes HUVECs to TNFα-induced apoptosis, we transiently transfected HUVECs with PP2A siRNA and treated with TNFα alone.
Figure 5D (middle) shows increased phosphorylation of p38 (top row) and increased levels of cleaved caspase-3 (middle row) when transfected cells were treated with TNFα for 2 hours, suggesting that HUVECs avoid TNFα-induced apoptosis through maintenance of negative feedback to activated pathways (p38) through dephosphorylation. Figure 5D (right) demonstrates that silencing of PP2A in the setting of TNFα led to degradation of 4E-BP1 (8 hours of TNFα stimulation). This supports a model in which CHX sensitizes HUVECs to apoptosis through inhibition of PP2A, which activates uninhibited p38 activity and increased degradation of 4E-BP1.

Discussion
In agreement with other investigators,3,4,6,7 we demonstrated that TNFα does not induce EC apoptosis unless the cells are made susceptible to apoptosis by the addition of CHX. Our results present a novel mechanism by which CHX sensitizes HUVECs to TNFα-mediated apoptosis. It is well known that p38 MAPK activation plays a critical role in TNFα-induced EC apoptosis.5 TNFα alone only transiently activates p38 MAPK, because its activity is quickly terminated following dephosphorylation by PP2A.20 As shown by other investigators, CHX maintains p38 phosphorylation through inhibition of phosphatases PP2A20 and MKP-1/2.24
Our model illustrates that CHX sensitizes ECs to TNFα-induced apoptosis by, first, inhibiting PP2A activity, allowing p38 MAPK to remain in its active phosphorylated state. Second, treatment of HUVECs with TNFα/CHX decreased the apparent half-life of 4E-BP1, a key component in the general translational machinery. This effect was also due to PP2A inhibition. Whereas 4E-BP1 is normally a stable protein that is long-lasting in the presence of CHX alone,21 the combination of TNFα and CHX uniquely led to a rapid degradation of 4E-BP1 in a p38 MAPK–dependent manner (Figure 4). Indeed, PP2A inhibition with both OA and siRNA in our system mimicked the effects seen with CHX treatment and increased the susceptibility of HUVECs to apoptosis. Our data support a protective role for PP2A. In our model, PP2A behaved as a counter-regulator of p38 MAPK signaling, as demonstrated by Cornell et al in epithelial cells.27 In this model, TNFα stimulation also activated both the p38 MAPK pathway and PP2A, which terminated ongoing p38 MAPK activation. We hypothesized that HUVECs resist apoptosis with stimulation by TNFα (alone) because of the ability of intact PP2A to quickly dephosphorylate TNFα-activated p38 MAPK. The addition of CHX (TNFα/CHX) disables PP2A’s ability to turn off p38 MAPK. Indeed, PP2A silencing with siRNA in our system mimicked the effect of CHX and increased the susceptibility of HUVECs to apoptosis. Our results agree with a report by Lee et al showing a direct protein interaction between PP2A and p38 MAPK in ECs and increased p38 MAPK activity with PP2A inhibition.28 In addition, the effect of PP2A may differ depending not only on the cell type but also temporally. Recent in vivo studies in rat hearts reveal that PP2A inhibition immediately before the onset of sus-

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**Figure 4.** The p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 abrogates 4E-BP1 degradation. Human umbilical vein endothelial cells (HUVECs) were treated with tumor necrosis factor-α (TNFα) (10 ng/mL) alone, CHX alone, or both together (10 μg/mL) for 8 hours, following rapamycin (100 nmol/L) pretreatment or SB203580 (20 μmol/L) pretreatment for 30 minutes as indicated. Total cell lysates were prepared and processed for Western blotting with total 4E-BP1 and GAPDH antibodies. Results are representative of 3 different experiments. Bottom, arbitrary densitometric units; mean of 3 different experiments; bars indicate SE. Data were analyzed by ANOVA, and pairwise comparisons were performed by the Bonferroni/Dunn post test, ***P<0.001. T indicates TNFα; C, CHX; R, rapamycin; SB, SB203580.
Figure 5. A, Left, Human umbilical vein endothelial cells (HUVECs) were exposed to tumor necrosis factor-α (TNF-α) (10 ng/mL) for 0 to 8 hours as indicated and then prepared for Western blotting, and membranes were probed with protein phosphatase 2A (PP2A) and GAPDH antibodies. Right, HUVECs were exposed to TNF-α (10 ng/mL) and cycloheximide (CHX) (10 μg/mL) for 0 to 8 hours as indicated and then prepared for Western blotting, and membranes were probed with PP2A and GAPDH antibodies. Results are representative of 3 different experiments. Bottom, arbitrary densitometric units; mean of 3 different experiments; bars indicate SE. Data were analyzed by ANOVA, and pairwise comparisons were performed by the Bonferroni/Dunn post test, ***P<0.001, **P<0.01 compared with control. B, HUVECs were treated with TNF-α (10 ng/mL) with or without CHX (10 μg/mL) for times indicated. Cells were then harvested and PP2A activity was measured as described in Methods. Results are expressed as mean of 3 different experiments ±SE. Data were analyzed by ANOVA, and pairwise comparisons were performed by the Bonferroni/Dunn post test, ***P<0.001, **P<0.01 compared with control. C, HUVECs were preincubated with or without okadaic acid (100 nmol/L) for 30 minutes and further treated for 8 minutes with TNF-α (10 ng/mL) with or without CHX (10 μg/mL). Total cell lysates were prepared and processed for Western blotting with total 4E-BP1 and GAPDH. Results are representative of 3 different experiments. Data were analyzed by ANOVA, and pairwise comparisons were performed by the Bonferroni/Dunn post test, ***P<0.001, **P<0.01 compared with control. D, HUVECs, transfected with either control or PP2A antisense oligonucleotides, were exposed to TNF-α (10 ng/mL) for 2 hours (left and middle) or 8 hours (right). Western blotting with PP2A, phospho-p38 (p38-P), anti-cleaved caspase-3, GAPDH, and total 4E-BP1 are shown. T indicates TNF-α; C, CHX; OA, okadaic acid; siRNA, small interfering RNA.
tained ischemia abolishes protection during reperfusion (this was associated with activation of p38 MAPK), whereas PP2A inhibition during reperfusion may have a cardioprotective effect (when no upregulation of p38 MAPK was observed).29

Protein synthesis inhibition noted in cells fated to undergo apoptosis is associated with a decrease in the proportion of ribosomes in polysomes,30 suggesting that there is an inhibition of the initiation phase, characterized by recruitment of the 40S ribosomal subunit to the mRNA. The initiation phase occurs through ribosomal recognition of the mRNA 5′ cap protein complex, created by interaction of 3 subunits: eIF4A, eIF4E, and eIF4G. The eIF4E polypeptide, which is involved in binding the mRNA cap to the ribosome, is the rate-limiting component of the initiation of protein translation.31 In addition to its role in translation initiation, recent studies have implicated eIF4E as a key antiapoptotic protein, likely because of its function in the exporting nuclear growth-related mRNAs from the cell nucleus to the cytoplasm.12 Indeed, there is evidence that eIF4E could represent a checkpoint protein by which cells sense the integrity of the translation machinery, and perturbing eIF4E function through binding rapidly and directly triggers the apoptotic machinery.16

eIF4E activity is controlled through reversible interaction with 4E-binding protein-1 (4E-BP1). 4E-BP1 competes with eIF4G for binding to eIF4E.32 In quiescent cells, hypophosphorylated 4E-BP1 binds to and inhibits eIF4E, but on exposure to a variety of extracellular stimuli, 4E-BP1 is phosphorylated, resulting in bond disruption, release of eIF4E, and binding of eIF4E to eIF4G to initiate mRNA cap-dependent translation.33 In MCF-7 and HeLa cells, translation inhibition preceding apoptosis is characterized by dephosphorylation of 4E-BP1.34

Multiple stimuli affect the phosphorylation of 4E-BP1. The protein kinase mammalian target of rapamycin plays a key role in phosphorylation of 4E-BP1, but other kinases, such as phosphatidylinositol 3-kinase/Akt,35 and p38 MAPK,18 have been implicated. Dephosphorylation of 4E-BP1 is catalyzed by PP2A.36

It is well known that 4E-BP1 can bind and inhibit eIF4E when 4E-BP1 is in its hypophosphorylated state. However recent studies have revealed a different mechanism by which the cellular apoptotic machinery can use 4E-BP1 to inhibit eIF4E during cellular stress: through degradation of the full-length 4E-BP1 protein. For instance, early in apoptosis, caspase activation leads to cleavage of full-sized 4E-BP1 polypeptide. This cleavage generates a fragment that sequesters and inhibits eIF4E even more potently than the full-length 4E-BP1.14

Increased degradation of 4E-BP1 can also occur through 4E-BP1 hyperphosphorylation and subsequent ubiquitination leading to proteosomal degradation.21 p38 MAPK, which plays a key role in a variety of cellular responses to stresses such as viral infection, osmotic shock, and UV irradiation,37–39 may be increasing turnover of 4E-BP1 protein by hyperphosphorylation. This effect has also been described in HSV-1 infection of primary human epithelial cells in which p38 MAPK is activated. HSV-1 infection results in hyperphosphorylation of 4E-BP1 and a reduction of 4E-BP1 steady-state protein levels because of an increase in proteosomal degradation.38 In the quiescent state of the cell, 4E-BP1 binding to eIF4E not only might prevent eIF4E from associating with eIF4G but may also maintain 4E-BP1 protein levels. Unbound 4E-BP1 is free to become phosphorylated and thus susceptible to degradation. Rapid degradation of phosphorylated 4E-BP1 by viral invasion ensures that PP2A is unable to dephosphorylate 4E-BP1, restoring its ability to bind eIF4E.39 Similarly, the addition of CHX in our model may lead to 4E-BP1 degradation through hyperphosphorylation. Thus, our model may serve as a potential illustration of a common mechanism of viral inflammation leading to EC dysfunction and apoptosis.

In conclusion, we have shown a novel mechanism by which (1) ECs resist apoptosis with exposure to TNFα and (2) CHX induces vulnerability to apoptosis in ECs exposed to TNFα. We postulate that CHX in our model may elucidate the role of putative factors found in atherosclerotic plaque, such as lipid peroxidation products, which contribute to apoptosis by modulating TNFα activation in the endothelium.40 Oxidative stress and the resulting lipid peroxidation products, such as 4-hydroxynonenal, have been shown to play a powerful role in the modulation of cell signaling and inhibition of protein synthesis, respectively, thereby facilitating the apoptotic actions of TNFα on ECs in vivo.41 Cotreatment of HUVECs with TNFα and CHX led to rapid degradation of the eIF4E regulator 4E-BP1 in a p38 MAPK-dependent manner. As shown by others, 4E-BP1 is an important regulator of cell stress and is important for maintaining cell viability.13 Degradation of 4E-BP1 is likely to promote apoptosis through several mechanisms. First, loss of the full-length 4E-BP1 may impair energy homeostasis through dysregulation of translation initiation.13 In addition, degradation of 4E-BP1 gives rise to a truncated 4E-BP1 peptide that binds to the strongly antiapoptotic protein eIF4E, thereby leading to potent inhibition of eIF4E.14 Modulation of eIF4E activity may therefore be a mechanism by which CHX potentiates TNFα-mediated EC apoptosis.

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Disclosures

None.

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