

T-cadherin attenuates the PERK branch of the unfolded protein response and protects vascular endothelial cells from endoplasmic reticulum stress-induced apoptosis

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ABSTRACT

Endoplasmic reticulum (ER) stress activated by perturbations in ER homeostasis induces the unfolded protein response (UPR) with chaperon Grp78 as the key activator of UPR signalling. The aim of UPR is to restore normal ER function; however prolonged or severe ER stress triggers apoptosis of damaged cells to ensure protection of the whole organism. Recent findings support an association of ER stress-induced apoptosis of vascular cells with cardiovascular pathologies. T-cadherin (T-cad), an atypical glycosylphosphatidylinositol-anchored member of the cadherin superfamily is upregulated in atherosclerotic lesions. Here we investigate the ability of T-cad to influence UPR signalling and endothelial cell (EC) survival during ER stress. EC were treated with a variety of ER stress-inducing compounds (thapsigargin, dithiothreitol, brefeldin A, tunicamycin, A23187 or homocysteine) and induction of ER stress validated by increases in levels of UPR signalling molecules Grp78 (glucose-regulated protein of 78 kDa), phospho-eIF2 α (phosphorylated eukaryotic initiation factor 2 α) and CHOP (C/EBP homologous protein). All compounds also increased T-cad mRNA and protein levels. Overexpression or silencing of T-cad in EC respectively attenuated or amplified the ER stress-induced increase in phospho-eIF2 α , Grp78, CHOP and active caspases. Effects of T-cad-overexpression or T-cad-silencing on ER stress responses in EC were not affected by inclusion of either N-acetylcysteine (reactive oxygen species scavenger), LY294002 (phosphatidylinositol-3-kinase inhibitor) or SP6000125 (Jun N-terminal kinase inhibitor). The data suggest that upregulation of T-cad on EC during ER stress attenuates the activation of the proapoptotic PERK (PKR (double-stranded RNA-activated protein kinase)-like ER kinase) branch of the UPR cascade and thereby protects EC from ER stress-induced apoptosis.

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Abbreviations: apoE, apolipoprotein-E; ASK1, apoptosis signal-regulated kinase 1; ATF, activating transcription factor; BCL-2, B-cell leukemia/lymphoma-2; Bref.A., brefeldin A; CHOP, C/EBP homologous protein; DTT, dithiothreitol; EC, endothelial cells; ED, endothelial dysfunction; eIF2 α , eukaryotic (translation) initiation factor 2 alpha; ER, endoplasmic reticulum; ERAD, ER associated degradation system; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Grp78, glucose-regulated protein of 78 kDa; GSK3 β , glycogen synthase kinase 3 beta; Hcys, homocysteine; HUVEC, human umbilical vein endothelial cells; HMEC-1, human microvascular endothelial cell line; IRE1, inositol requiring kinase 1; JNK, Jun N-terminal kinase; LDLR, low density lipoprotein receptor; NAC, N-acetylcysteine; PERK, protein kinase RNA (PKR)-like ER kinase, phosphatidylinositol-3-kinase; ROS, reactive oxygen species; RT-PCR, real time PCR; T-cad, T-cadherin; Thapsi, thapsigargin; TRAF2, TNF receptor-associated factor 2; Tunicamycin, tunicamycin; UPR, unfolded protein response; XBP-1, X-box binding protein-1.

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1. Introduction

Endothelial dysfunction (ED) plays a key role in the pathogenesis of atherosclerosis [1]. ED is characterized by impairment of endothelium-dependent vasorelaxation and by a specific state of endothelial “activation” which manifests itself in increased endothelial cell (EC) production of proinflammatory and proliferative mediators, adhesion molecules and procoagulants. Thus injured EC significantly impair vascular function and trigger thrombotic and atherogenic reaction cascades.

Important pathophysiological roles have been attributed to different types of stress in the development of ED and the progression of atherosclerosis. Luminal endothelium which is in direct contact with plasma and blood components is the first target of pathogens and damaging toxic compounds including risk factors for atherosclerosis. Acute and chronic oxidative stress associated with lipoprotein oxidation, activation of the inflammatory response and generation of

reactive oxygen species (ROS) is well recognized as an early event which plays an initiating role in cardiovascular disorders, while the ability of EC to resist stressors determines the progression and outcome of the disease [2,3]. Endoplasmic reticulum (ER) stress caused by perturbations in ER function including accumulation of misfolded proteins, changes in redox status or intraluminal calcium concentration, nutrient deprivation, altered glycosylation and pathogen infections contributes to pathogenesis of many human pathologies including neurodegenerative disorders, diabetes, obesity and cancer [4–10]. ER stress triggers the unfolded protein response (UPR), an adaptive signalling cascade aimed at restoring normal ER folding capacity and promoting cell survival, or, if ER stress is severe or prolonged, at promoting death of damaged cells to ensure protection of the whole organism [4–10].

UPR has recently been demonstrated to be involved in ED and related cardiovascular pathologies. Accumulation of free cholesterol in macrophages was shown to trigger ER stress and C/EBP-homologous protein (CHOP)-induced apoptosis [11]. Upregulation of ER chaperone glucose-regulated protein of 78 kDa (Grp78), apoptosis signal-regulated kinase 1 (ASK1) and other ER stress-related genes were detected in hypoxic cultured myocytes and in a murine model of myocardial infarction [12,13]. Both cytoprotective and apoptotic components of ER stress signalling are upregulated in myocardial ischemia and heart failure [14,15]. High levels of apoptosis and expression of Grp78 and pro-apoptotic transcription factor CHOP were found in smooth muscle cells and macrophages within the fibrous caps of thin-cap atheroma and ruptured plaques of both autoptotic human coronary specimens and atherectomy specimens from patients with unstable angina pectoris [16]. Studies in apolipoprotein E knock-out (*apoE*^{-/-}) mice showed that UPR activation occurred at all stages of atherosclerosis, and evidence was provided to support the relevance of ER stress to macrophage apoptosis and enlargement of the necrotic core in advanced atherosclerotic plaques [17,18]. ER stress markers are also upregulated in atherosclerotic lesions of *apoE*^{-/-} mice fed a hyperhomocysteinemic diet [19]. Homocysteine, a well known inducer of ER stress, induces apoptosis *in vitro* in a number of cell types including EC [20–23] and is believed to adversely affect stability and thrombogenicity of atherosclerotic lesions. In EC ER stress is activated by shear stress *in vitro* [24] and *in vivo* at atherosclerosis-susceptible arterial sites [25]. Pharmacological inducers of ER stress including tunicamycin, thapsigargin, Sin-1 and A23187 have variously been demonstrated to activate apoptosis in EC *via* induction of UPR-related proapoptotic factors CHOP and T-cell death-associated gene 51 [22,26]. Taken together, these findings support an association of ER stress and induction of UPR induced-apoptosis of EC, smooth muscle cells and macrophages with progression of atherosclerosis and development of plaque vulnerability.

T-cadherin (T-cad), an unusual glycosylphosphatidylinositol-anchored member of the cadherin superfamily of adhesion molecules, is upregulated in vascular cells under pathological conditions associated with abnormal vascular tissue remodeling and stress including atherosclerosis [27] and restenosis [28]. We have previously demonstrated that T-cad is involved in regulation of EC survival under conditions of oxidative stress. T-cad is markedly upregulated in EC by ROS, and its overexpression decreases oxidative stress-induced apoptosis [29]. Both overexpression and ligation of T-cad on the EC surface activate signalling pathways (phosphatidylinositol-3-kinase (PI3-kinase), Akt, glycogen synthase kinase 3 beta (GSK3β)) important for cell survival and proliferation [29,30]. There is considerable interplay between ER stress and oxidative stress. Oxidative stress, which occurs when the production of ROS overwhelms the antioxidant defenses, may cause alterations in ER homeostasis and activate UPR-related signalling, while conversely ER stress may result in accumulation of ROS and initiate expression and activation of oxidation-related signalling mediators [7]. Together these data

suggest that T-cad upregulation represents a protective mechanism aiming at resisting stress conditions and might be involved in endothelial ER stress response. Moreover, our recent data on physical colocalization of T-cad and ER chaperon Grp78 in EC [31] further point to a possible role for T-cad in UPR signalling. In this study we investigate the ability of T-cad to influence UPR activation and EC survival during ER stress.

2. Experimental

2.1. Cell culture

Human umbilical vein EC (HUVEC), purchased from PromoCell GmbH (Heidelberg, Germany), were cultured on plates precoated with 0.1% gelatine in EC growth medium containing EC growth supplement (PromoCell). Human microvascular EC line (HMEC-1) was cultured in the same medium supplemented with 10% fetal calf serum (FCS). Cells were seeded either at 2×10^5 cells/well into 6-well plates (for immunoblotting) or at 2×10^4 cells/well into 96-well plates (for Homogenous Caspases ELISA) and allowed to adhere overnight. Culture medium was refreshed and cells were treated for selected intervals with a variety of ER stress-inducing agents including thapsigargin (500 nM), dithiothreitol (DTT, 1 mM), brefeldin A (5 µg/ml), tunicamycin (3 µg/ml), calcium ionophore A23187 (2 µM) and homocysteine (5 mM). Some experiments included ROS scavenger N-acetylcysteine (NAC, 15 mM), PI3-kinase inhibitor LY-294002 (10 µM) or Jun N-terminal kinase (JNK) inhibitor SP600125 (20 µM), which were added to cultures 1 h before stimulation protocols. All compounds were purchased from Sigma-Aldrich (Buchs, Switzerland) with the exception of SP600125 which was purchased from VWR/Merck (Dietikon, Switzerland). Agent vehicles at the appropriate final concentrations were tested in pilot studies and found not to affect any of the parameters measured in this study.

2.2. Overexpression and silencing of T-cad

Overexpression of T-cad in HMEC-1 was achieved by using Adeno-X Expression System (Clontech, Palo Alto, USA) as detailed previously [32]. Briefly, HMEC-1 in normal growth media were infected overnight with empty, LacZ or T-cad containing adenoviral particles at a final approximate concentration of 4 pfu/cell; expression of T-cad was monitored by immunoblotting (representative blots shown in Fig. 3). T-cad silencing in HMEC-1 was performed using MISSION[®] Lentiviral transduction system (Sigma-Aldrich Chemie, Buchs, Switzerland). Lentiviral particles expressing T-cad short hairpin RNA (product number TRCN0000055546) and non-target control transduction particles (product number SHC002V) were used at a multiplicity of infection of 4 lentiviral particles per cell according to manufacturer's recommendations. Infected cells were selected against puromycin for about 10 days. Efficiency of T-cad silencing was in the order of 80–90% as controlled by immunoblotting (representative blots shown in Fig. 4).

2.3. Homogenous caspases assay

Following exposure to ER stress-inducing agents caspase activity in treated cells was evaluated using Homogeneous Caspases ELISA that detects activated caspases 2, 3, 6, 7, 8, 9, and 10 (Roche Diagnostics GmbH, Mannheim, Germany) as described previously [29]. Substrate solution was added to wells without prior removal of any detached cells or medium change.

2.4. Immunoblotting

Immunoblotting was performed on whole cell lysates prepared by lysis with PBS containing 1% SDS and protease inhibitor cocktail

(Sigma), with inclusion of 1 mM orthovanadate and 5 mM NaF in samples analysed for protein phosphorylation status. In some experiments immunoblotting for CHOP protein was additionally performed on nuclear extracts prepared using the CHEMICON® Nuclear Extraction Kit according to the manufacturers' protocol (Millipore, Billerica MA, USA). Protein concentrations were determined using the Lowry method. Samples were loaded at 5–10 µg per lane and electrophoresed in 8% SDS-polyacrylamide gels under reducing conditions. The following antibodies were used for immunoblotting: goat anti-T-cad (R&D Systems Europe Ltd., Abingdon, UK), goat anti-GAPDH (Abcam, Cambridge, UK), mouse anti-Grp78 (BD Biosciences, Basel, Switzerland), rabbit anti-cleaved caspase 3 and rabbit anti-phospho-eIF2 α (Cell Signalling, New England Biolabs, Frankfurt, Germany), rabbit anti-CHOP (sc7351, Santa Cruz from Lab Force AG, Nunningen, Switzerland), mouse anti-lamin A/C (LaZ-1) (kind gift of Prof. Harald Herrmann, Department of Molecular Genetics, German Cancer Research Center, Heidelberg, Germany). Secondary HRP-conjugated goat anti-mouse IgG or anti-rabbit IgG (Southern Biotechnology (BioReba AG, Reinach, Switzerland) or donkey anti-goat IgG (Santa Cruz) together with Amersham ECL (Amersham Biosciences, Little Chalfont, UK) were used for detection of immunoreactive proteins. Scanned images of autoradiograms were analyzed using AIDA Image or Scion (NIH) Image software.

2.5. RT-PCR

Isolation of RNA, reverse transcription, and real-time PCR analysis were performed as described previously [33]. The expression of target molecules was normalized to the expression of β_2 -microglobulin or to GAPDH. Primer sequences are available on request.

2.6. Statistical analysis

All experiments were performed on at least three separate occasions. Results are given as mean \pm SD unless otherwise stated. Differences were variously determined with 1-way or 2-way repeated measures ANOVA with Tukey's or Bonferroni's multiple comparison test, respectively, using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). A *P* value of <0.05 was considered significant.

3. Results

3.1. Activation of the UPR and apoptosis in EC by ER stress inducers

In order to establish the experimental model we examined the kinetics of responses of HMEC-1 to a variety of pharmacological

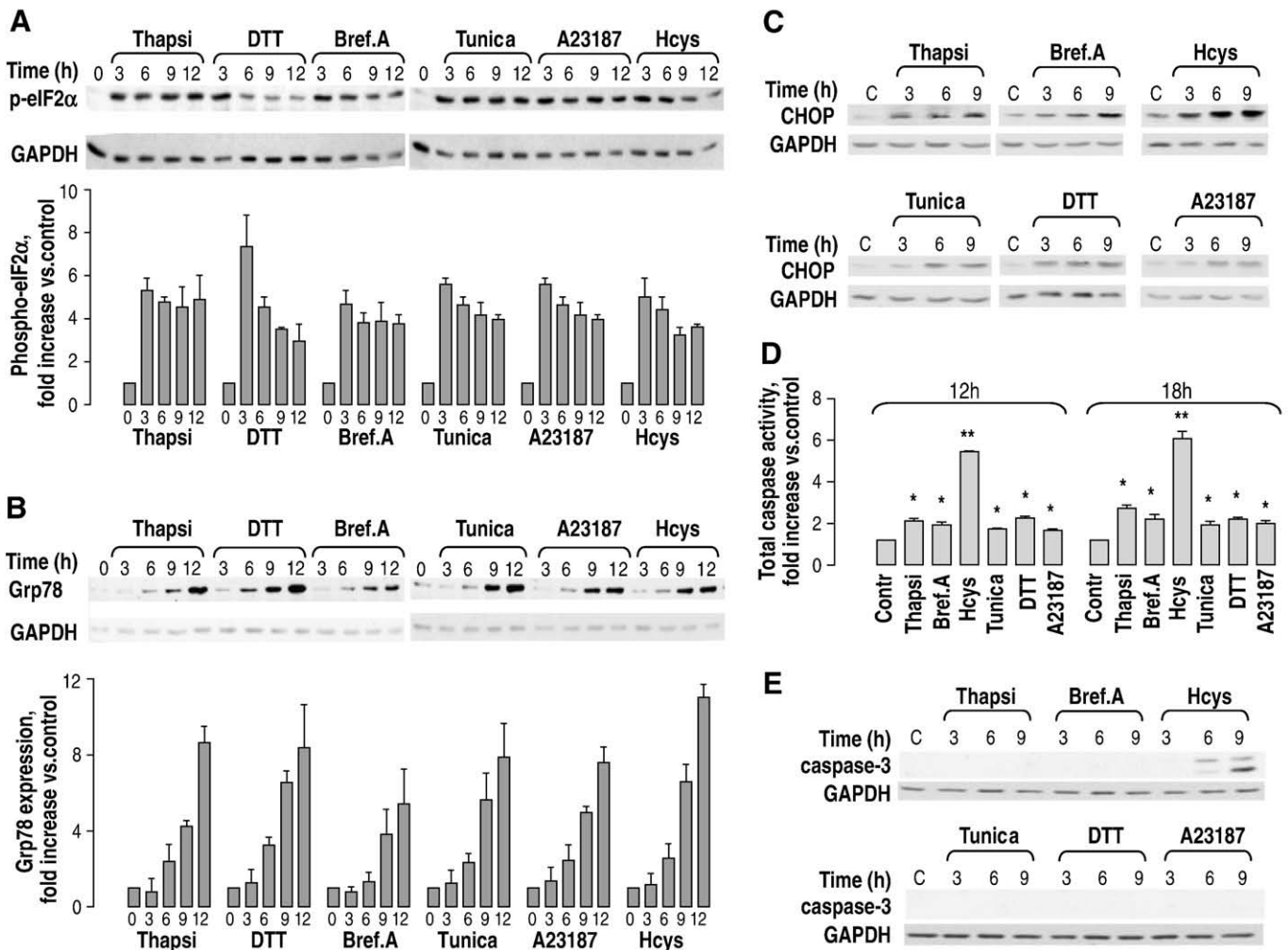


Fig. 1. Induction of ER stress in HMEC-1. HMEC-1 cultures were exposed for the indicated periods of time to the different ER stress inducers: thapsigargin (Thapsi), DTT, brefeldin A (Bref.A), tunicamycin (Tunica), A23187 and homocysteine (Hcys). Cells were processed for immunoblot analysis of phospho-eIF2 α (A), Grp78 (B) or CHOP (C). Blots were probed with GAPDH to control for equivalence of protein loading. Changes in levels of phospho-eIF2 α , Grp78 and CHOP are expressed relative to their respective levels in untreated cells (0h). Total caspases activity in HMEC-1 cultures was measured using homogenous caspases assay kit (D). Data (mean \pm SD, *n* = 3) are expressed relative to the baseline fluorimetric level in untreated control cells. Activity of caspase-3 in HMEC-1 lysates were analysed by immunoblotting (E). The histograms show data (mean \pm SD) obtained from 3 to 4 independent experiments. Blots are representative of 3–4 separate experiments.

compounds that are known to induce ER stress by different mechanisms, namely disturbance of calcium homeostasis (ER calcium pump inhibitor thapsigargin, calcium ionophore A23187), inhibition of N-glycosylation in the ER (tunicamycin), inhibition of ER/Golgi transport (brefeldin A) and reduction of disulfide bonds (dithiothreitol, homocysteine). Cells were treated with the compounds for up to 12 h. As read-outs for UPR activation we determined the phosphorylation status of the α -subunit of eukaryotic translational initiation factor 2 (eIF2 α) as an index of translational attenuation and the level of Grp78 protein as an index of transcriptional induction of ER chaperone genes. All the chemicals tested induced an increase in phosphorylation of eIF2 α within 3 h that remained generally steady during treatment for up to 12 h (Fig. 1A). Levels of Grp78 increased between 3 and 6 h of exposure to the chemicals and thereafter continued to rise steadily during treatment for up to 12 h (Fig. 1B). Similar responses were elicited in HUVEC (Supplemental Fig. S1).

Since excessive or prolonged ER stress is linked to the triggering of programmed cell death [4–10] the effects of the ER stressors on indices of apoptosis in HMEC-1 were investigated. CEBP homology protein (CHOP), a major ER stress-inducible pro-apoptotic transcription factor which operates as a downstream component of ER-stress pathways, at the convergence of the inositol-requiring kinase 1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) [34] was induced by all the ER stressors (Fig. 1C). The specificity of the anti-CHOP antibodies used in this study (Sc-7351 from Santa Cruz) and our use of whole cell lysates for immunoblotting [35] were validated by comparison of immunoreactivity in whole cell lysates and nuclear extracts prepared in parallel

following treatment of parental HMEC-1 with thapsigargin, brefeldin A or homocysteine (Supplemental Fig. S2). Analysis for caspases activity with the Homogeneous Caspases ELISA revealed a ~5-fold increase in cells treated with homocysteine and a lesser ~2-fold increase in cells treated with thapsigargin, brefeldin A, A23187, tunicamycin and DTT (Fig. 1D). Immunoblot analysis for active caspase-3 revealed its presence only in cells treated with homocysteine (Fig. 1E). This, taken together with the ~2–3-fold greater induction of caspases activity by homocysteine as compared with the other stressors (Fig. 1D), might reflect that, under our experimental conditions, homocysteine elicited the most potent proapoptotic response in EC.

3.2. Induction of the UPR in EC is associated with upregulation of T-cad

Next we investigated the effects of the ER stressors on induction of T-cad mRNA and protein. All ER stressors induced a 1.5 to 3-fold upregulation of T-cad transcripts within 2 h that remained elevated for up to at least 6 h (Fig. 2A). Likewise all compounds induced a 1.5 to 3-fold increase in T-cad protein expression within 3 h, which generally remained stable for up to at least 9 h (Fig. 2B). HUVEC responded similarly to conditions of ER stress (Supplemental Fig. S1).

3.3. T-cad expression modulates ER stress responses of EC

To determine whether T-cad plays a role in ER stress response in EC we examined the consequences of overexpression and silencing of T-cad on UPR activation. These gain-of-function and loss-of-function

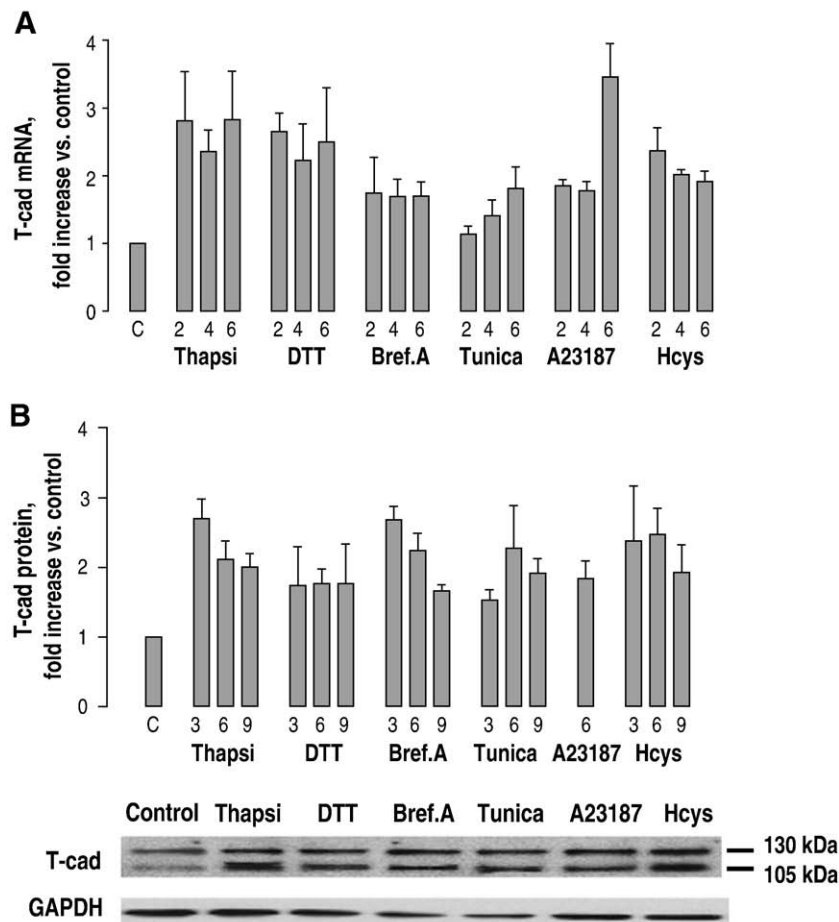


Fig. 2. Induction of T-cad expression in HMEC-1 in response to ER stress. HMEC-1 cultures were exposed for the indicated periods of time to the different ER stress inducers: thapsigargin (Thapsi), DTT, brefeldin A (Bref.A), tunicamycin (Tunica), A23187 and homocysteine (Hcys). Cells were processed for RT-PCR analysis of T-cad transcript levels (A) or for immunoblot analysis of T-cad protein (B). The immunoblot shows samples from cells treated for 6 h. Changes in T-cad mRNA and protein expression are expressed relative to their respective levels in untreated cells. The histograms present data (mean \pm SD) obtained from 3 to 4 independent experiments.

experiments were performed using HMEC-1. Homocysteine, thapsigargin and brefeldin A were selected as the test ER-stress and UPR activation agents. Compared with the responses in control empty vector- or LacZ-transduced cells, T-cad overexpression significantly attenuated the elevation in levels of phospho-eIF2 α , Grp78 and proapoptotic C/EBP homologous protein CHOP induced by the ER stressors (Fig. 3). In contrast, and compared with control shRNA-transduced cells, T-cad silencing significantly augmented the stimulatory effects of the ER stressors on phospho-eIF2 α , Grp78 and CHOP levels (Fig. 4).

3.4. T-cad promotes EC survival during ER stress

In order to determine if modulation of UPR signalling by T-cad translates into a functional response and influences apoptosis rates during ER stress we studied effects of T-cad overexpression and silencing on caspase activation. T-cad overexpression blunted the induction of total caspases activity by thapsigargin, brefeldin A and homocysteine (Fig. 5A) and upregulation of cleaved caspase-3 level by homocysteine (Fig. 5B). In T-cad silenced cells these proapoptotic responses to the ER stressors were amplified (Fig. 5C, D).

3.5. T-cad effects on UPR signalling in EC do not depend on PI3-kinase pathway, JNK activity or protection against oxidative stress

We have previously demonstrated that T-cad-dependent activation of prosurvival signalling protects EC from oxidative stress. Since generation of ROS can be a cause of disturbances in ER function we investigated whether T-cad may prevent ER stress-induced apoptosis by acting not on UPR pathways directly but rather by limiting the impact of oxidative stress. As described above, upregulation of proapoptotic factor CHOP by thapsigargin and homocysteine was much more prominent in EC transduced with T-cad shRNA as compared to control shRNA-expressing cells. ROS scavenger N-acetylcysteine did not prevent the stress-induced increase in CHOP or Grp78 levels in T-cad-silenced cells (Fig. 6A), suggesting that the ability of T-cad to attenuate ER stress is distinct from its ability to protect EC from oxidative stress [29]. ER stress attenuation in T-cad overexpressing cells could not be prevented by PI3-kinase inhibitor LY-294002 suggesting that activation of PI3-kinase pathway by T-cad [29] does not contribute to protection of cells from excessive UPR (Fig. 6B). Inclusion of anthracycline SP600125 to inhibit JNK, which is a target of ASK1 and plays an important role in ER stress-

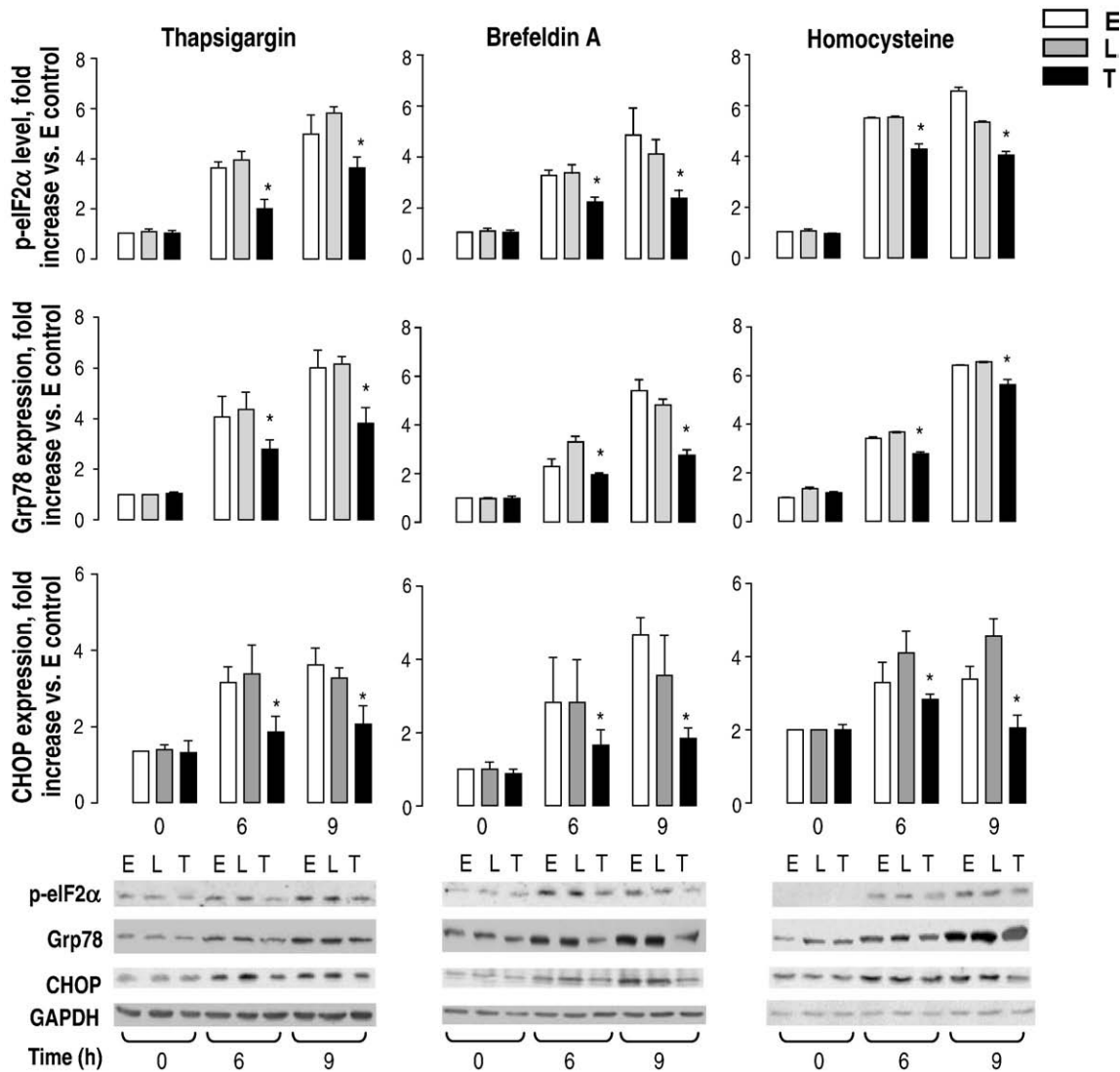


Fig. 3. T-cad overexpression attenuates ER stress response. Empty- (E, open bars), LacZ- (L, grey bars) and T-cad- (T, black bars) lentivector transduced HMEC-1 were treated with thapsigargin, brefeldin A or homocysteine for the indicated times. Whole cell lysates were prepared for immunoblot analysis of eIF2 α phosphorylation and expression of Grp78 and CHOP proteins. Changes are expressed relative to levels in untreated E-transduced HMEC-1. The histograms present data (mean \pm SD) obtained from 3 independent experiments. Representative blots, including the controls for T-cad overexpression, are shown. Asterisks indicate a significantly decreased response in T vs. E or L (P at least <0.05).

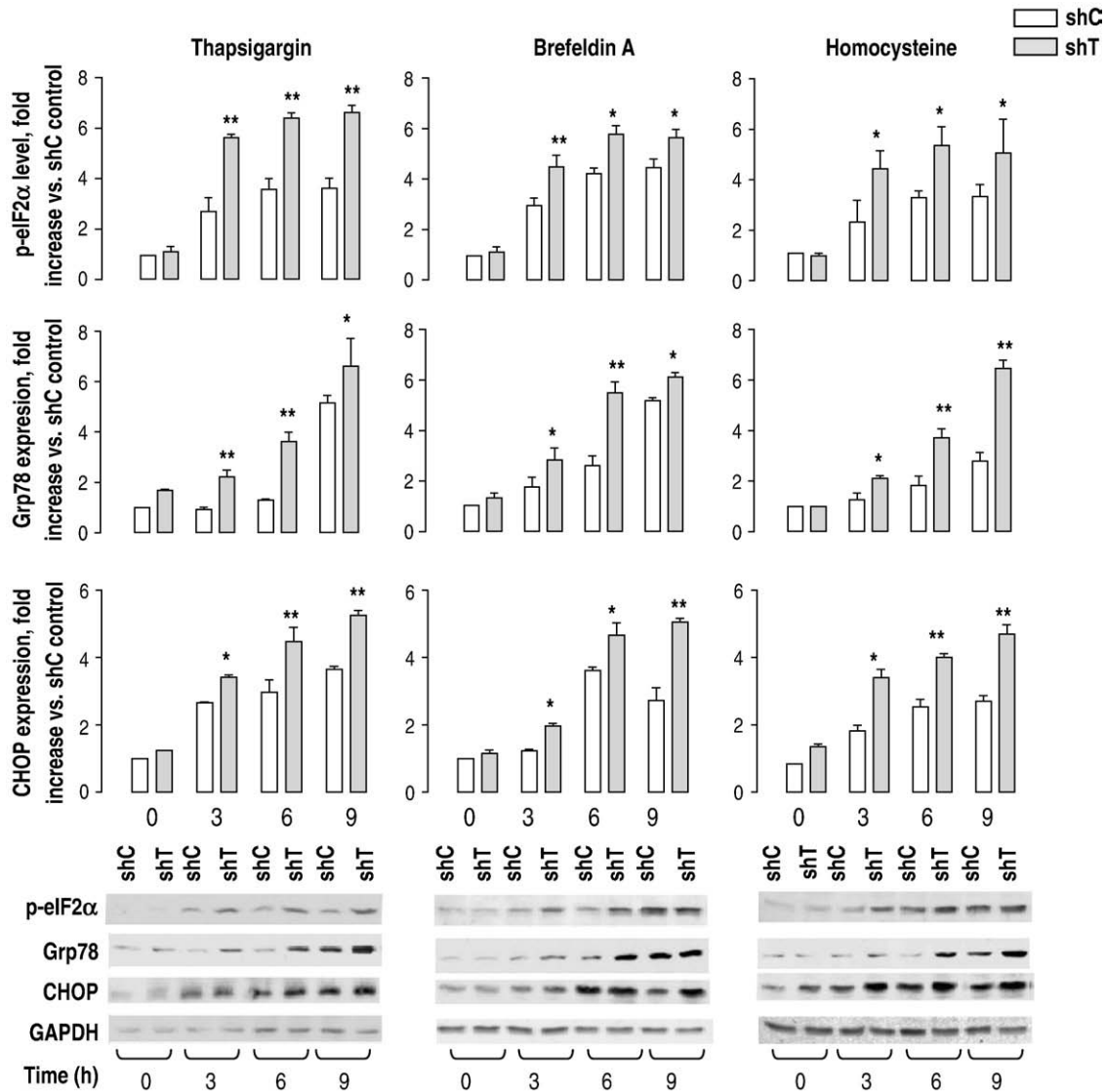


Fig. 4. Silencing of T-cad expression augments ER stress response. HMEC-1 transduced with lentiviral vectors expressing T-cad-specific (shT, grey bars) or control (shC, open bars) shRNAs were treated with thapsigargin, brefeldin A or homocysteine for the indicated times. Whole cell lysates were prepared for immunoblot analysis of T-cad, eIF2 α phosphorylation and expression of Grp78 and CHOP proteins. Changes are expressed relative to levels in the control shRNA-transduced HMEC-1. The histograms present data (mean \pm SD) obtained from 3 independent experiments. Representative blots, including the controls for T-cad silencing, are shown. Asterisks indicate a significantly increased response in shT vs. shC (* P <0.05, ** P at least <0.01).

induced apoptosis, also did not prevent the stress-induced increase in CHOP or Grp78 levels in T-cad-silenced cells (Fig. 6C).

4. Discussion

We have previously reported that upregulation of T-cad in EC occurs *in vivo* [27,28] and *in vitro* under conditions associated with oxidative stress [29] and functions as a protection mechanism promoting EC survival through activation of the PI3K/Akt/mTOR survival signal pathway and concomitant suppression of the p38 MAPK proapoptotic pathway [29]. The present study has demonstrated that upregulation of T-cad in EC also takes place during ER stress. T-cad overexpression and silencing studies collectively suggest that upregulation of T-cad attenuates ER stress by restricting activation of the proapoptotic PERK branch of the UPR cascade and thereby limits ER stress-induced apoptosis.

The cellular ER stress/UPR signalling cascade falls into several phases with different effector functions (reviewed in [4–9]). It is triggered by an accumulation of misfolded proteins in the ER which bind to ER chaperone Grp78, causing dissociation of Grp78 from the

three major ER stress sensors PERK, ATF6 and IRE1 and a resultant launching of the UPR [4–9]. The first response aims at re-establishing homeostasis and normal ER function. PERK-dependent phosphorylation of eIF2 α results in translational attenuation reducing the load of new protein synthesis on the ER. IRE1 induces expression of X-box binding protein XBP-1, while ATF6 is translocated to the Golgi and activated by proteolysis. XBP-1 and ATF6 act together with eIF2 α -downstream target ATF4 as transcription factors activating expression of ER chaperones and components of ER associated degradation system (ERAD) eliminating misfolded proteins. In a later phase, immune and anti-apoptotic responses are activated via the NF κ B pathway. When the adaptive mechanisms fail to compensate in the face of protracted or excessive ER stress apoptotic cell death is induced to protect the organism by eliminating the damaged cells. Several apoptosis pathways are known to be involved, the central role being played by the proapoptotic transcription factor CHOP which blocks expression of antiapoptotic protein Bcl-2. Transcriptional induction of CHOP mostly depends upon activation of PERK/eIF2 α ; however, IRE1 and ATF6 pathways also stimulate CHOP transcription, meaning that CHOP operates at the convergence of all UPR branches. ER stress-induced

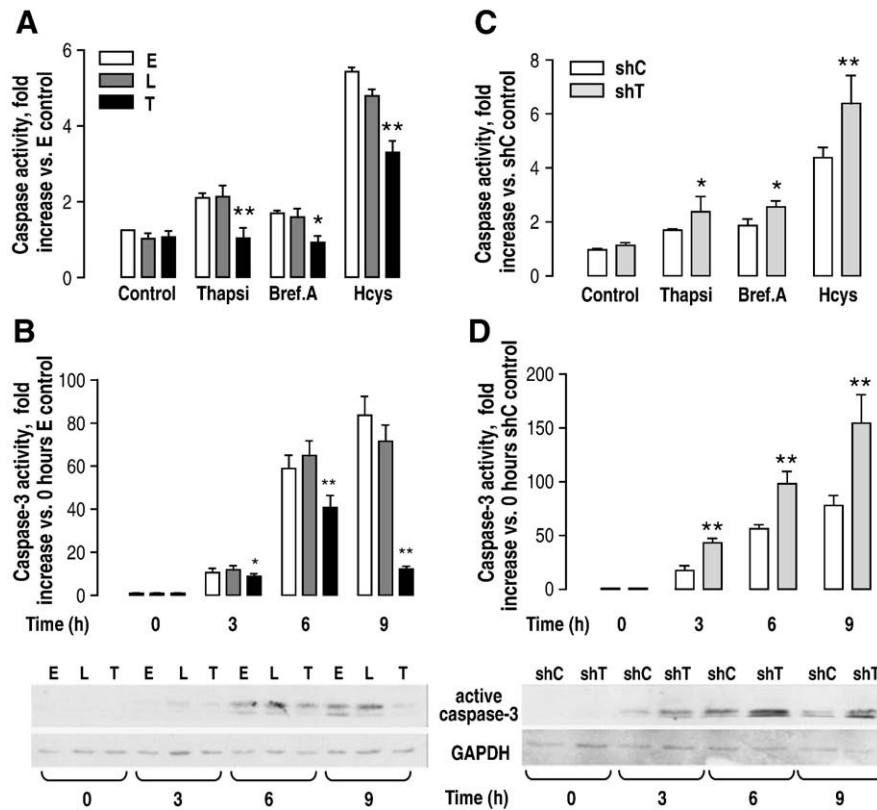


Fig. 5. Effects of T-cad overexpression and silencing on ER stress-induced apoptosis. Apoptosis was measured by homogenous total caspase activity assay (A, C) or by immunoblotting with anti-cleaved caspase-3 antibody (B, D) in empty- (E, open bars), LacZ- (L, grey bars) and T-cad- (T, black bars) lentivector transduced HMEC-1 (A, B) or in HMEC-1 transduced with lentiviral vectors expressing T-cad-specific (shT, grey bars) or control (shC, open bars) shRNAs (C, D) after treatment with thapsigargin (Thapsi), brefeldin A (Bref.A) or homocysteine (Hcys) for 15h. Changes in caspase activity or level of caspase 3 are expressed relative to baseline levels (untreated) in respective control-transduced HMEC-1. The histograms present data (mean \pm SD) obtained from 3 independent experiments. Asterisks in A and B indicate a significantly decreased response in T vs. E or L (*, $P < 0.05$; ** P at least < 0.01). Asterisks in C and D indicate a significantly increased response in shT vs. shC (* $P < 0.05$, ** P at least < 0.01).

apoptosis can also occur *via* IRE1-dependent activation of TRAF2/ASK1/JNK cascade and *via* Ca^{2+} -dependent activation of caspase-12.

Experimental evidence suggests that all stress sensors activate both protective and proapoptotic signalling. The final outcome of UPR will depend on a complex interplay of various factors such as balance between activity of different pathways and persistence of stress conditions, so any influence that can shift the phase of UPR between adaptation and apoptosis is likely to affect the cell fate decision between survival and death (reviewed in [4–9]). In terms of cardiovascular disease this phenomenon of “the double-edged sword” is illustrated by several studies demonstrating that UPR can be either beneficial or damaging for vascular and cardiac cells. Upregulation of Grp78 by endothelin-1 or salvianolic acid protects hypoxic cardiomyocytes and HUVEC from ER-stress [36]. Overexpression of Grp78 inhibits homocysteine-induced ER stress [37] associated with endothelial injury and increased risk of thrombosis. On the other hand, downregulation of UPR markers including Grp78 by darbepoetin and kaempferol exerts cardioprotective effect in autoimmune cardiomyopathy [5,38]. Increasing eIF2 α phosphorylation status by pharmacological inhibition of its dephosphorylation may either protect cells [4] or potentiate ER stress-induced apoptosis caused by fatty acids [39]. Many studies suggest that prevention of UPR-induced apoptosis is an attractive target for minimizing tissue damage under stress conditions. In an experimental model of myocardial infarction ASK1 $^{-/-}$ mice show reduced cardiomyocyte apoptosis rates and myocardial injury. Inactivation of CHOP gene exerted a survival benefit during diabetes [40] and prevented plaque growth, apoptosis and necrosis in apoE $^{-/-}$ and LDLR $^{-/-}$ mouse models of atherosclerosis [41].

In this study we have demonstrated that T-cad overexpression-dependent attenuation of UPR signalling is beneficial and results in

the shift of the balance between UPR signalling branches in favour of prosurvival signalling, while T-cad silencing promotes apoptotic cell death. We have previously demonstrated that T-cad upregulation improves survival of EC during oxidative stress *via* concomitant activation of PI3-kinase/Akt signalling and inhibition of p38 MAPK signalling [29]. There is bi-directional cross-talk between oxidative and ER stress: protein folding and generation of ROS as a by-product of protein oxidation in the ER are closely linked events, and activation of the UPR upon exposure to oxidative stress is an adaptive mechanism to preserve cell function and survival [7]. Therefore, it was logical to consider that protective effects of T-cad against ER stress might be related to its ability to minimize ROS-induced damage [29]. However, we were unable to prevent the amplified ER stress-induced apoptosis of T-cad-silenced EC by treatment with ROS scavenger N-acetylcysteine. Inhibition of PI3-kinase did not eliminate the attenuating effects of T-cad overexpression on Grp78 and CHOP expression, supporting that T-cad limits ER stress in EC by mechanisms distinct from those (i.e. PI3-kinase/Akt signalling axis) mediating T-cad-dependent protection of EC from oxidative stress. Furthermore, JNK inhibitor failed to prevent the amplified ER stress-induced apoptosis of T-cad-silenced EC, indicating that anti-apoptotic effects of T-cad upregulation under conditions of ER stress are also unlikely to involve inactivation of ASK1 signalling (and by inference p38 MAPK [5,10]). On the other hand, we have clearly demonstrated T-cad-dependent modulation of phospho-eIF2 α and CHOP which represent the proapoptotic PERK branch of the UPR cascade [4–10]. Thus, the protection afforded by T-cad against ER stress-induced apoptosis likely involves main ER stress pathways directly rather than modulation of related anti-stress signalling which converge with UPR.

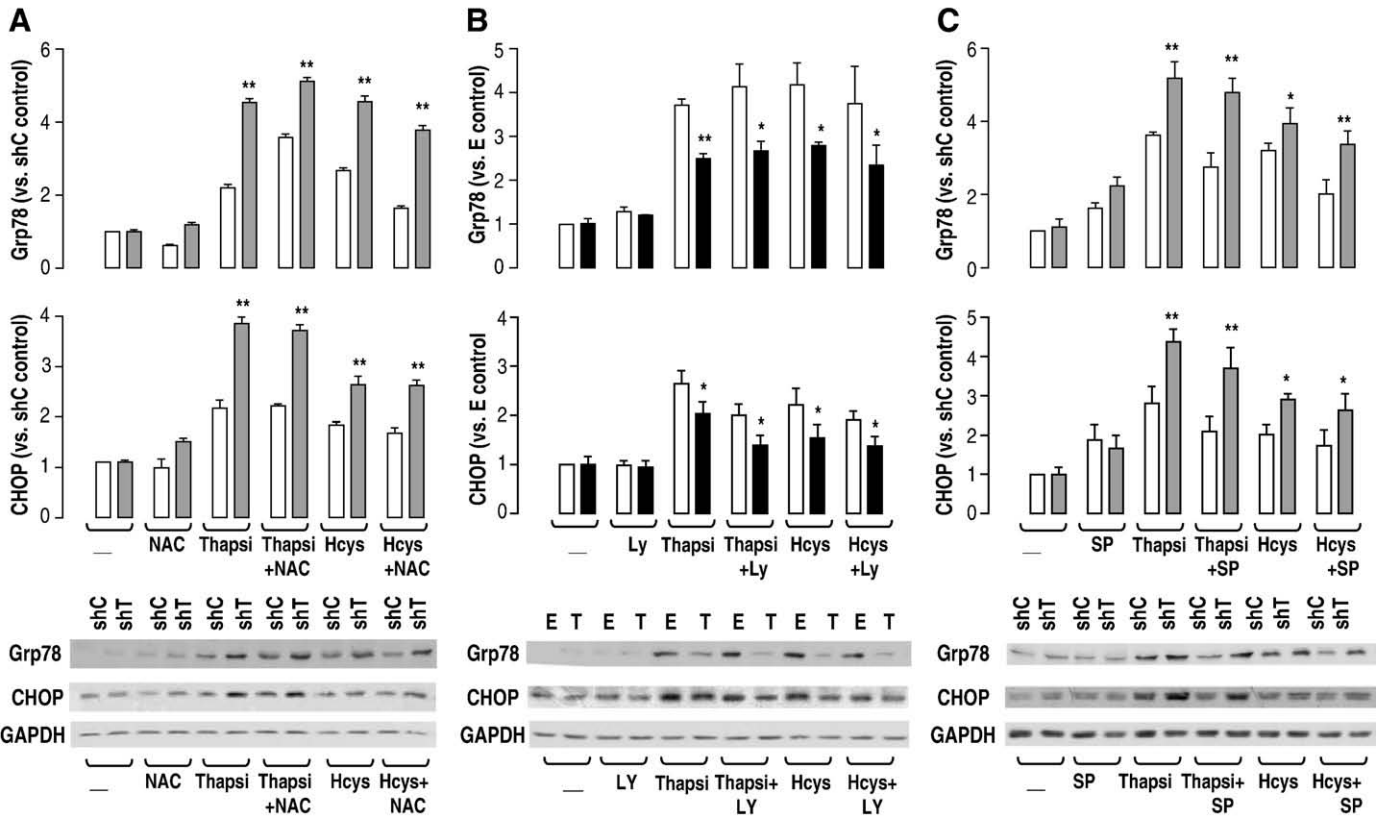


Fig. 6. T-cad dependent effects on ER stress response do not involve oxidative stress, PI3-kinase and JNK. HMEC-1 transduced with lentiviral vectors expressing T-cad-specific (shT, grey bars) or control (shC, open bars) shRNAs (A, C) or Empty- (E, open bars) and T-cad- (T, black bars) lentivector transduced HMEC-1 (B) were treated with thapsigargin (Thapsi) or homocysteine (Hcys) for 6 h in the absence or presence of ROS scavenger N-acetylcysteine (NAC; 15 mM), PI3-kinase inhibitor LY-294002 (LY; 10 μM) or JNK inhibitor SP6000125 (SP; 20 μM). Levels of Grp78 and CHOP protein were determined by immunoblot analysis of whole cell lysates. Changes are expressed relative to baseline levels (untreated) in the respective control-transduced HMEC-1. The histograms present data (mean ± SD) obtained from 3 independent experiments. Asterisks indicate significant differences between shC and shT or between E and T (**P*<0.05, ***P* at least <0.01).

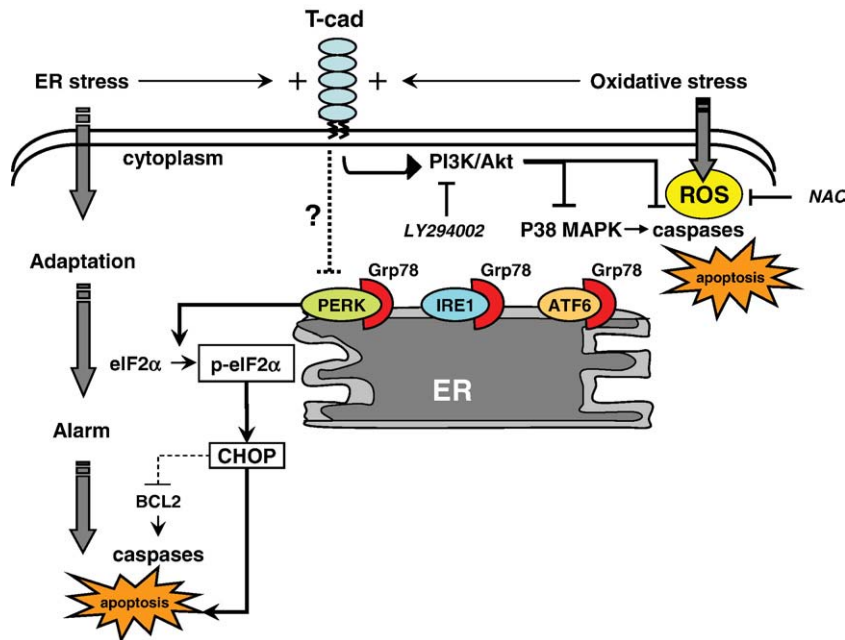


Fig. 7. Schematic diagram illustrating different mechanisms whereby upregulation of T-cad induced in response to cellular stress protects EC from apoptosis. In EC GPI-anchored T-cad is upregulated in response to ER stress and oxidative stress. The diagram shows the three major ER stress sensors (PERK, IRE1 and ATF6) which, for the sake of simplicity, are all depicted in association with Grp78. Our results suggest that T-cad protects against ER-stress-induced apoptosis by restricting the PERK arm of the UPR response, and in a manner that is insensitive to PI3-kinase inhibitor LY-294002 or ROS scavenger N-acetylcysteine. This is distinct from the PI3-kinase/Akt-dependent mechanism whereby T-cad protects EC from oxidative stress-induced apoptosis. How T-cad communicates signals to the ER stress machinery is not yet known.

The exact mechanisms linking T-cad to UPR mediators have yet to be elucidated. Of interest in view of the present data is our recent finding that T-cad associates with Grp78 in EC [31]. However, we detected colocalization not with ER-localized Grp78, which is a well-recognized trigger of UPR, but with its cell surface pool [31]. Surface Grp78 has been demonstrated to be present on the plasma membrane of several cell types including vascular endothelium [42,43]. Increased levels of surface Grp78 have been detected in different pathological conditions, for example on highly metastatic cancer cells [44], on endothelial and monocyte/macrophage-like cells in atherosclerotic lesions [42], and on procoagulant microparticles shed from the plasma membrane of activated EC [45]. When expressed on the cell surface, Grp78 is able to initiate various functional responses including inhibition of tissue factor procoagulant activity [42], mediation of signal transduction from activated α 2-macroglobulin in peritoneal macrophages [44] and 1-LN prostate cancer cells [44,46,47], binding of dengue virus [48] and coxsackievirus [49], regulation of EC apoptosis induced by K5 kringle domain of plasminogen [43] and association with MHC class I molecules [50] and with Ro-52 antigen on mouse splenocytes presumably contributing to autoimmunity during rheumatoid arthritis [51]. There is also some evidence for participation of surface Grp78 in ER stress responses; in 1-LN cells ligation of activated α 2-macroglobulin by cell-surface expressed Grp78 was associated with induction of UPR signalling [47]. Our study did not address the contribution of surface Grp78 to the protective effects of T-cad against adverse effects of ER stress. One might speculate that association of T-cad with surface Grp78 may either regulate downstream signalling or modulate localization or trafficking of Grp78 in the cell thus affecting intracellular levels of Grp78, which is the main sensor and trigger of the UPR.

5. Conclusions

The study reveals a novel regulatory mechanism for modulation of ER stress responses in the context of cardiovascular disease. We have demonstrated that upregulation of T-cad in EC occurs early following induction of ER stress, and that it functions to protect EC from adverse outcomes (e.g. apoptosis) caused by excess and chronic UPR. The mechanism of protection primarily involves an attenuation of signalling through the PERK arm of the UPR and is distinct from the mechanism whereby T-cad protects EC from oxidative stress-induced apoptosis [29] (Fig. 7). Upregulation of T-cad on the surface of vascular cells in atherosclerotic lesions may represent a protective mechanism for limiting tissue damage during cardiovascular disease progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2010.04.008.

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