Cigarette smoke extract induces prolonged endoplasmic reticulum stress and autophagic cell death in human umbilical vein endothelial cells

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Aims
Consumption of cigarette smoke (CS) is a well-known risk factor for early atherosclerosis; yet, the underlying mechanisms of smoking-associated atherosclerosis are poorly understood. Based on the previous results indicating that CS-induced endothelial cell death neither shows typical features of apoptosis nor of necrosis, we investigated the role of autophagy in CS extract (CSE)-induced cell death of human umbilical vein endothelial cells (HUVECs).

Methods and results
Here, we demonstrate that overexpression of the classical apoptosis inhibitor BCL-XL had no protective effect on CSE-induced cell death, whereas the autophagy inhibitor 3-methyladenin and an shRNAi-mediated knockdown of the autophagy mediator ATG5 significantly delayed cell death. Our results indicate that CSE induces an excess accumulation of misfolded proteins in the endoplasmic reticulum (ER) and consequently the onset of the unfolded protein response. We provide evidence that the ER-resident kinase PERK is a major transducer of ER stress leading to phosphorylation of eIF2α and attenuation of protein synthesis. Finally, we show that prolonged ER stress in cells subjected to CS is followed by activation of an autophagic programme. CSE-induced autophagy is characterized by an increase in LC3 II/I ratio and activation ATG12. The autophagic signalling pathway via energy depletion and consequent activation AMP-activated protein kinase could be excluded.

Conclusion
Our results confirm and extend previous findings reporting on the induction of autophagy by CSE in the lung. We show that protein damage caused by CSE activates autophagy, ultimately resulting in necrotic death of HUVECs. Via this mechanism, cigarette smoking may contribute to the deterioration of vascular endothelial function and the initiation of atherosclerosis.

Keywords
Cigarette smoke • HUVECs • ER stress • Autophagy • Atypical cell death • LC3 • eIF2α

1. Introduction
Despite compulsory inclusions of health warnings on cigarette packages, and increased governmental efforts to reduce the prevalence of smoking, consumption of cigarette smoke (CS) remains worldwide one of the most prevalent modifiable risk factors for atherosclerosis and its clinical consequences such as stroke and myocardial infarction. Our group performed experimental as well as clinical studies on the molecular mechanisms underlying the association between smoking and cardiovascular disease.1,2 We identified oxidative stress, generated by the combustion process itself and perpetuated by heavy metals present in CS, as the main stressor for endothelial cells subjected to CS constituents.3 A recent Affymetrix gene chip analysis of the immediate response of human umbilical vein endothelial cells (HUVECs) to CS challenge further corroborated the key role of oxidative stress and associated protein damage in CS-mediated endothelial cell damage.

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CS challenge of endothelial cells led to up-regulation of multiple heat shock proteins and endoplasmic reticulum (ER)-associated chaperones, and this effect was completely abrogated if cells were challenged with CS that was previously depleted of its reactive oxygen species (ROS) or metal content. Additionally, our in vivo study with young smokers revealed increased serum cadmium and strontium levels in this cohort of people as opposed to their non-smoking counterparts. Another study of our group identified increased serum cadmium levels as a novel and independent risk factor for increased carotid intima–media thickness in young people. Besides these immediate effects, we observed a peculiar type of cell death occurring in a protracted manner and exhibiting a delayed time course. Analysis of classical hallmarks of apoptosis (such as surface expression of phosphatidylserine or activation of effector caspases) at multiple time points gave no hint of an ongoing apoptotic programme, and the delayed nature of the ongoing cell death clearly spoke against necrosis in the classical sense.

In the present study, we examined whether ROS-mediated ER stress may be the underlying mechanism of CS-induced cell death in HUVECs. Although ER stress-mediated cell death has been shown to proceed primarily through apoptosis, in the case of failure of apoptosis, other forms of cell death such as autophagy can be engaged. Yet, the exact mechanism linking ER stress to induction of autophagy remains to be clarified. Formation of autophagic vacuoles during ER stress primarily serves the function of mitigating protein misfolding and suppressing cell death by sequestration and clearance of protein aggregates. However, in severe cases, autophagy might promote a form of cell death that is referred to as type-II cell death. Consequently, it depends on the nature and severity of the initial insult whether autophagy will serve a protective purpose, or it will be deleterious, culminating in cellular self-digestion. For instance, it has been demonstrated that autophagy augments the functional recovery of cardiomyocytes during ischemia, while it enhances cellular demise during the reperfusion phase. On a cellular level, in bax-bak mouse embryonic fibroblasts (MEFs), it has been shown that autophagic cell death mediates ER stress-induced cell death. Interestingly, autophagy also occurs in wild-type MEFs to a similar extent, though seemingly as a protective measure counteracting apoptosis. These findings highlight the pleiotropic roles of autophagy in cell death, which are strongly dependent on the cellular context.

Since in our previous work, we found that CS induces in HUVECs a type of cell death that lacks the molecular characteristics of apoptosis, the goals of this study were (i) to establish whether CS-induced cell death is preceded by prolonged ER stress; (ii) to determine the effects of inhibition of apoptosis as against autophagy on cell death kinetics; (ii) to examine whether autophagy has a role to play as an inducer of cell death or mediator of survival in cells subjected to CS.

2. Methods

2.1 Materials

All chemicals used were purchased from Sigma-Aldrich (Vienna, Austria) unless stated otherwise and were of analytical grade.

2.2 Preparation of the aqueous CS extract

CS extracts (CSEs) were prepared as described previously. Briefly, using a CS sampling device developed and validated in the laboratory, the smoke of two commercially available filter cigarettes (Marlboro, Philip Morris Products S. A., Neuchatel, Switzerland; nicotine, 0.8 mg; tar, 10 mg; carbon monoxide, 10 mg) was ‘puffed’ through 8 mL of pre-warmed (37°C) cell culture medium without additives (control: air drawn through the medium). The extracts were routinely chemically fingerprinted by HPLC-MS. Before use, the extracts were filtered through a 0.22 µm pore filter unit. The pH of the extracts was routinely checked and adjusted if the pH value differed from 7.2.

2.3 Cell culture

HUVECs were isolated from umbilical cords (kindly donated by the Gynaecology and Obstetrics Department, Innsbruck Medical University) by enzymatic detachment using collagenase as described elsewhere. Cells were routinely passaged in 0.2% gelatine-coated (Sigma, Steinheim, Germany) polylysine culture flasks (Becton Dickenson, Meylan Cedex, France) in Endothelial Cell Basal Medium (CC-3121, BioWhittaker, Inc., Walkersville, MD, USA) supplemented with EGM SingleQuots Supplements and growth factors (CC-4133, BioWhittaker, Inc.) in a humidified atmosphere containing 5% CO₂. Foetal bovine serum (2%) was routinely used for cell culturing and experiments. All experiments were conducted with HUVECs in passage 5. For cell death analyses, 3 x 10⁵ HUVECs per well were seeded onto gelatine-coated six-well plates (Becton Dickenson). Prior to each experiment, the medium was replaced by a fresh medium.

The isolation and analysis of HUVECs was approved by the Ethics committee of the Medical University of Innsbruck and confirms with the principles of the Declaration of Helsinki.

2.4 Generation of lentiviral vectors

For constitutive overexpression of human BCL-XL in HUVECs, BCL-XL-encoding cDNA was polymerase chain reaction (PCR)-amplified and recombined into pDONR-207 (Invitrogen, Carlsbad, CA, USA) using Invitrogen’s BP recombination kit. A sequence-verified clone was used for LR recombination with pHR-SFFV-Dest-ires-Puro thereby generating the lentiviral expression plasmid pHR-SFFV-BCL-XL-ires-Puro. For a stable knockdown of ATG5 in HUVECs, shRNA oligonucleotides (Metabion, Martinsried, Germany) were annealed and cloned into the lentiviral pLVTHM vector carrying a GFP cassette for monitoring virus production and infection. The sequence GCT GAA ACC TTT GGC CTA AGA was targeted to silence ATG5 by shRNA expression. For shRNA-control HUVECs were infected with lentiviral particle encoding shRNA targeting the Firefly luciferase gene (pHR-THT-luciferase-SFFV-eGFP, kindly provided by S. Geley).

2.5 Lentiviral infection

For the generation of BCL-XL-overexpressing HUVECs, human HEK 293T cells were transiently transfected with lentiviral plasmids containing cDNAs coding for human BCL-XL or eGFP, along with the packaging plasmids pCMV 8.91 and pVSV-G [kindly provided by Didier Trono, The Swiss Federal Institute of Technology Lausanne (EPFL), Switzerland]. Forty-eight and 72 h after transfection, lentiviral supernatant was sterile-filtered (0.2 µm), supplemented with polybrene to a final concentration of 4 µg/mL, and added to the target cells for a 12 h treatment. For the generation of HUVECs stably expressing ATG5 or luciferase targeting shRNA, 293T cells were transfected with the corresponding lentiviral constructs along with viral packaging vectors (pSPAX2 and pMD2G) by calcium phosphate transfection. ATG5 or luciferase shRNA-containing viruses were harvested from the supernatant 48 h.p. and applied to HUVECs for lentiviral infection in the presence of polybrene (5 µg/mL).

2.6 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using the LightCycler FastStart DNA Master SYBR Green kit (Roche, Vienna, Austria) on the LightCycler 1.0 system. Synthesis of the first strand of cDNA from total RNA (500 ng) was achieved using First Strand cDNA...
Synthesis Kit (Roche) according to the manufacturer’s recommendations. The cDNA was diluted 10-fold before equal amounts were added to duplicate (or triplicate) qRT-PCRs. To ensure the highest possible accuracy, a master mix containing all reagents except the primers was created, which, with the primers for the gene of interest, or a housekeeping gene, was added directly to the glass capillary. Each reaction proceeded for 40 amplification cycles and was followed by a melting curve analysis to ensure the specificity of each reaction, controlled by the supplied LightCycler software (version 3.5, Roche). Crossing point and melting curve analyses for each reaction were also performed using the LightCycler software.

2.7 Western blot analysis

Western blot analysis of cells harvested from each well of the six-well plates was carried out with the procedures we have described. Experimental details are provided in Supplementary material online.

2.8 Quantification of cell death

For the detection and/or quantification of cell death, Annexin-V-FITC/propidium iodide staining was used. Briefly, 2.5 × 10^5 cells were incubated with FITC-labelled Annexin-V (Apotec, San Diego, CA, USA, product number: APO-50-040F-T100) and propidium iodide, washed, and analysed on a FACScan (forward/sideward light scatter, red fluorescence for propidium iodide and green fluorescence for FITC-labelled Annexin-V) as described previously.11

2.9 ATP assay

ATP measurement was performed using a bioluminescence assay from Molecular Probes (Molecular Probes, Eugene, OR, USA. Kit A-22066) as described by the manufacturer.

2.10 Staining of cells and fluorescence microscopy

To analyse for autophagosome formation, the subcellular localization of LC3 protein was analysed. To do so, HUVECs were treated with 8% CSE for 12 h, washed with phosphate-buffered saline (PBS; pH = 7.2), and fixed by a 5 min incubation with 4% paraformaldehyde. Fixed monolayers were permeabilized (0.3% Triton X-100, 2 mM EDTA, 5 mM PIPES in a.d.; 15 min at 4°C). Following an additional washing step with PBS, non-specific-binding sites were blocked with 0.1% bovine serum albumin in PBS for 30 min at room temperature, followed by staining with rabbit anti-human LC3 antibody (Sigma-Aldrich) for 1 h. After washing three times with PBS and the application of Alexa 488-labelled goat anti-rabbit IgG (Molecular Probes, Lofer, Austria) for 1 h (incubation at room temperature; dark), cells again were washed three times with PBS, and nuclear staining was performed using propidium iodide (Invitrogen) for 5 min at room temperature in the dark. After three more washing steps, cells were mounted in ProLong Gold (Invitrogen) and analysed using an LSM 510 Meta attached to an Axioplan 2 imaging MOT using ZEN software (Zeiss, Oberkochen, Germany).

2.11 Metabolic protein labelling

Metabolic protein labelling was performed with 75 μCi/mL of 35S-methionine/cysteine mix (Hartmann Radiochemicals, Germany) for 8 h. Thereafter, cells were washed twice with cold PBS, suspended in Western loading buffer, and separated in SDS–polyacrylamide gels as described.7 Gels were dried for 1 h at 80°C and analysed by exposure to X-ray films.

2.12 Statistical analysis

Statistical analyses were performed using SPSS 17.0 software. Values of all groups were tested (per group) for a Gaussian distribution (Kolmogorov–Smirnov’s test) and equality of variances (Levene’s test). Further analyses were performed using ANOVA (post hoc Bonferroni). A comparison of two groups was performed by t-test.

3. Results

3.1 Inhibition of autophagy, but not of apoptosis, delays CSE-induced cell death

Our previous study showed that CSE induced in HUVECs an atypical kind of cell death lacking the characteristic features of apoptosis.7 Autophagy has been described as a non-apoptotic type of cell death in the context of oxidative stress. To find out if autophagy might be the cell death mechanism underlying the above-mentioned atypical mode of CSE-induced cell death, in the present study, we investigated the impact of interference with apoptotic vs. autophagy signalling pathways on the kinetics and extent of CSE-mediated cell death. Pharmacological inhibition of apoptosis by a general caspase inhibitor had no protective effect on cells exposed to CSE, whereas application of the inhibitor of autophagy 3-methyladenine (10 mM), a PI3K-type III inhibitor, significantly delayed CSE-induced cell death (Figure 1A). Since uncertainty remains as to the specificity of the pharmacological agents applied, we constructed two lentiviral systems in an effort to increase specificity of our experimental approach. For inhibition of apoptosis, BCL-XL was overexpressed, whereas shRNAi-mediated knockdown of the key autophagy gene ATGS was used for blocking autophagic pathways. As depicted in Figure 1B and C, our lentiviral systems yielded profound overexpression of BCL-XL as well as a marked knockdown of ATGS. Empty vector-infected cells served as an internal control. Figure 1D demonstrates that a massive overexpression of BCL-XL completely failed to reduce the extent of CSE-induced cell death. In contrast to this, knockdown of ATGS significantly delayed cell death compared with empty vector control and Firefly luciferase-targeting shRNA controls (Figure 1E), strongly indicating that apoptotic pathways are not engaged in the mechanism of CSE-mediated cell death in HUVECs, whereas autophagy clearly appears to have a major role to play in the type of cell death observed.

3.2 CSE leads to formation of LC3-II and prolonged ER stress

Formation of LC3-II, arising from the myosin light chain-associated protein 3 I (LC3-I), is a well-accepted hallmark of formation of autophagic vacuoles, which can be detected by immunofluorescence or by western blot analysis. The presence of cathepsin inhibitors has been reported to lead to accumulation of LC3-II (Figure 2B) which is otherwise rapidly degraded by lysosomal enzymes in the course of formation of autophagic vacuoles. As shown in Figure 2A, co-application of CSE and the cathepsin inhibitor E64d led to an increase in the formation of LC3-II, whereas this effect was not visible in cells treated with CSE alone. Immunofluorescence analyses revealed a conversion of the diffuse cellular staining pattern of LC3-I (Figure 2C) to a punctuate staining of LC3-II (Figure 2D).

We then raised the question as to the molecular pathways leading to activation of autophagy and associated modes of cell death in cells under CSE treatment. Given the high oxidant content of CSE and its hazardous effects on endothelial cells observed in our previous studies, we examined whether severe ER stress precedes induction of autophagy. Phosphorylation of the eukaryotic initiation factor 2α (eIF2α) serves as a hallmark of ongoing ER stress. Figure 2E and F
shows that cells subjected to CSE exhibited ER stress already after 1 h of incubation, increased until 24 h, and was reduced at 48 h. Phosphorylation and inactivation of eIF2α meliorated ER stress by abrogation of cap-dependent translation, leading to the reduction in the amount of protein circulating through the ER. To check whether protein synthesis is attenuated or abrogated in cells subjected to CSE, we performed radioactive labelling of newly synthesized proteins with 35S-methionine/cysteine. CSE treatment led to a complete abrogation of protein synthesis (Figure 2G) with a time course corresponding to the inactivation of eIF2α. Subsequently, we set out to explore the kinase that mediates phosphorylation of eIF2α. Figure 2E shows increased phosphorylation of the ER-resident kinase PKR-like ER kinase (PERK) in cells subjected to CSE followed by gradual up-regulation of the early autophagic protein ATG12 (Figure 2F). Overall, these data suggest that cells subjected to CSE undergo a prolonged phase of severe ER stress and that PERK-mediated phosphorylation of eIF2α appears to underlie the observed translational stop preceding the onset of autophagy.

3.3 Cells subjected to CSE preserve cellular ATP levels
To further characterize the mode of cell death, we examined the impact of CSE on cellular ATP levels. Activation of caspases during apoptosis is an energy-dependent process that critically presupposes the availability of sufficient amounts of cellular ATP. Thus, higher ATP levels would appear to be consistent with an apoptotic mechanism of cell death. Depletion of cellular energy levels by severe oxidative challenge prevents caspase activation and shifts the mode of cell death towards necrosis. The DNA damage response involves the enzyme poly-ADP-ribose-polymerase (PARP) that consumes large quantities of ATP for poly-ADP-ribosylation of proteins in the DNA repair machinery. To check whether ATP depletion has a role to play in CSE-mediated cell death, we employed a luciferin/luciferase-based system for determination of cellular ATP levels. As shown in Figure 3A, there was some decrease in ATP levels in cells exposed to CSE, but depletion of cellular ATP levels could clearly be ruled
out as a mechanism of necrosis induction in cells subjected to CSE. Thus, although we have shown that apoptosis is not the underlying mechanism of CSE-induced cell death, the lack of ATP depletion demonstrates that nor can a canonical necrotic mode of cell death be the mechanism under investigation. Up to this point, we demonstrated that in HUVECs, autophagy (Figure 1E) and prolonged ER stress (Figure 2D and E) are associated with CSE-induced cell death, whereas apoptosis (Figure 1D) and ATP depletion (Figure 3A) do not play a role in CSE-induced cell death.

### 3.4 CSE leads to inactivation of mammalian target of rapamycin

AMP-activated protein kinase (AMPK) is not only a sensitive sensor of cellular ATP levels, but also a critical regulator of autophagy during starvation and tissue ischaemia. In the case of sudden decrease of the ATP/AMP ratio, AMPK is activated and initiates autophagy by inhibition of mammalian target of rapamycin (mTOR). To examine whether the AMPK-mTOR axis has a functional role to play in CSE-induced autophagy, we examined the activity level of these enzymes by the application of phospho-specific antibodies (Figure 3B). Of note, we found reduced mTOR activation, but at the same time, also inactivation of AMPK, in cells subjected to CSE. Collectively, these data indicate that in HUVECs, CS-induced autophagy is independent of AMPK, which is consistent with our finding of reduced but not depleted energy levels in cells subjected to CS. Thus, the enzymes that mediate CS-induced inhibition of mTOR remain to be elucidated.

### 4. Discussion

CS exerts its atherogenic effects via multiple alterations of endothelial cell homeostasis ranging from altered endothelial cell morphology to increased endothelial cell death.3,7 The death pathways engaged by cells subjected to CSE have been investigated in different cell types, including endothelial and bronchial epithelial cells; yet, these studies came up with conflicting results, with the mode of cell death ranging from classical apoptosis to gross necrosis.13–15 Several reports suggested ROS to be essentially involved in CSE-mediated...
cell death induction. For instance, the study by Wickenden et al.\textsuperscript{13} found necrosis to be the primary type of CSE-induced cell death in HUVECs as well as alveolar epithelial cells, the ROS content of CSE actively inhibiting apoptosis and thereby shifting the mode of cell death to necrosis. However, as reported by Tagawa et al.\textsuperscript{14}, apoptosis was the main type of cell death in human bronchial epithelial cells challenged with CSE. In these cells, apoptosis was mediated by ER stress-induced up-regulation of the CCAAT/enhancer-binding protein homologous protein (CHOP) and subsequent activation of caspase-3 and -4.\textsuperscript{14} In a recent study, Kim et al.\textsuperscript{15} demonstrated hallmarks of autophagy and apoptosis in bronchial epithelial cells challenged with CSE, which were down-regulated by forced expression of hemeoxygenase-1. Along this line, Chen et al.\textsuperscript{16} found induction of autophagy and subsequent apoptosis in pulmonary epithelial cells subjected to CSE.\textsuperscript{16}

In the present study, we investigated the role of ER stress in CSE-induced cell death in HUVECs. ER stress constitutes an essential response in cells undergoing protein damage of any kind (under oxidative or reductive stress).\textsuperscript{17,18} Normally, proteins that fail to be properly folded are transported out of the ER to the cytosol for degradation by the proteasome in the so-called ER-associated degradation pathway (ERAD). However, in the case of overwhelming stress and failure of ERAD, the ER stress–response pathway is initiated. In essence, it involves abrogation of cap-dependent protein synthesis by phosphorylation of eIF2\textsuperscript{a} in its \(\alpha\)-subunit, while allowing for up-regulation of chaperones and heat shock proteins. Accumulation of misfolded protein in the ER is sensed by three distinct ER-membrane spanning signalling molecules, namely PERK, inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF6). Under physiological conditions, the luminal domains of these proteins are covered by BiP, whereas under ER stress, BiP is replaced by misfolded proteins, and thus signalling through these molecules is activated. Activation of PERK leads to phosphorylation of its target eIF2\textsuperscript{a} and consequently to translational block. Removal of BiP enables ATF6 to be processed in the Golgi and subsequently translocated to the nucleus, where it promotes up-regulation of ER-resident chaperones such as GRP94, BiP, CHOP, and \(\alpha\)-box-binding protein XPB1. XPB1 transcript is spliced by IRE-1, leading to its translation into protein and further up-regulation of chaperones in an effort to prevent overloading with misfolded proteins of the ER. Depending on the severity of ongoing stress, the translational block is either transient or persists in a prolonged manner.\textsuperscript{19,20} In the case of the latter
scenario, there is a functional coupling between ER stress and autophagy in cells subjected to severe protein stress, which is referred to as ER-activated autophagy (ERAA).

In our present study, we performed an in-depth analysis of a hitherto unpublished observation of a delayed type of cell death in endothelial cells exposed to CSE that neither exhibits classical hallmark of apoptosis nor corresponds to gross necrosis. Our finding of early and prolonged ER stress in HUVECs exposed to CSE led us to speculate that the ERAA pathway might serve as the essential executing cell death pathway. Further analysis revealed molecular evidence for the formation of autophagic vacuoles, whereas, at the same time, apoptosis could be ruled out as a mechanism contributing to cell death. Autophagy has been identified as a necrosis-promoting cell death pathway in apoptosis-incompetent cells, while it confers protection against cell death in cells capable of engaging the apoptotic cell death machinery. The reason for a lack of apoptosis in the context of CSE stress could either be a critical drop in cellular ATP levels, precluding apoptosis formation, or alternatively, induction of an apoptosis-non-permissive redox state, leading to oxidative damage of caspases and/or prevention of further caspase cleavage. In our study, we were able to rule out depletion of cellular energy levels as being causally related to a lack of caspase activation in cells subjected to CSE. Given the substantial oxidative activity of CS, it seems plausible that the overwhelming CSE-induced oxidative stress leads to a direct inhibition of apoptotic signalling (also see Figure 4). Consequently, cell fate is determined by alternative cell death pathways. Autophagy was initially identified as a means to adapt to ongoing cellular stress, thereby acting as a cell survival mechanism. However, in the case of overwhelming stress or when canonical pathways of cell death fail to be activated, autophagy has the ability to execute a distinct non-canonical type of cell death. Our finding of prolonged ER stress as reflected by persistent phosphorylation of the translation factor eIF2a indicates that this scenario applies to CS-induced cell death. The pathological significance of this finding appears to be two-fold: first of all, it underscores the degree of stress imposed on cells in the presence of CSE constituents. Although one has to be cautious in extrapolating the CSE-induced cell death mechanism in HUVECs to other endothelial cell types such as pulmonary vascular endothelial cells, and also the in vivo situation, it would appear that a shift from apoptosis towards a more necrotic-like type of cell death leads to increased local inflammation and structural damage of the endothelial cell layer, facilitating vascular plaque formation. Additionally, inhibition of apoptosis, necessitating the onset of alternative cell death pathways, might point to a pro-oncogenic mechanism of CS. Further experiments with bronchial epithelial cells are needed to corroborate this hypothesis.

The question remains as to the molecular pathway leading to CSE-induced autophagic cell death. At least four mammalian eIF2a kinases have been identified including AMPK, which is known to serve as a conserved sensor of cellular energy levels. In the case of starvation, AMPK is activated by phosphorylation, which leads to initiation of autophagy by phosphorylation of eIF2a and to inactivation of mTOR. In our study, we were unable to find phosphorylation of AMPK, which ties in with our finding of preserved energy levels in CSE-treated cells. However, we found increased phosphorylation of PERK during the course of ER stress in cells subjected to CSE, suggesting that PERK might serve as the essential link connecting ER stress to translational stop and autophagic cell death. Alternatively, promotion of autophagy via PARP-mediated activation of AMPK has been recently been described in cells subjected to oxidative stress. However, the absence of a dramatic drop of cellular ATP levels in cells subjected to CSE speaks against a significant contribution of PARP in the mode of cell in our experimental setting. Future experiments are likely to identify further ER-associated kinases capable of adapting the cell to increased stress and preparing cell death via autophagy. Although it is clear that CSE causes overwhelming oxidative stress in endothelial cells, it will be a challenging task to zero down to the protagonists of cell death induction among the thousands of compounds present in CS.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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