Advanced glycation end-products induce endoplasmic reticulum stress in human aortic endothelial cells

Abstract

Background: Advanced glycation end products (AGEs), the final products of the Maillard reaction, have been shown to impair endothelial proliferation and function, thus contributing to endothelial cell injury present in diabetes, inflammatory and cardiovascular diseases. Endoplasmic reticulum (ER) stress triggered under hyperglycemic, hypoxic and oxidative conditions has been implicated in endothelial dysfunction through activation of the unfolded protein response (UPR). The present study investigates the role of AGEs in ER stress induction in human aortic endothelial cells exposed to variable AGE treatments.

Methods: Human aortic endothelial cells (HAEC) were treated with increasing concentrations (100, 200 μg/mL) of AGE-bovine serum albumin (AGE-BSA) at different time-points (24, 48, 72 h). The induction of ER stress and the involved UPR components were investigated on mRNA and protein levels. Apoptosis was quantitatively determined by flow cytometry detecting propidium iodide expression and annexin V binding simultaneously.

Results: AGEs administration significantly reduced HAEC proliferation in a time- and dose-dependent manner. An immediate induction of the ER chaperones GRP78, GRP94 and the transcriptional activator, XBP-1 was observed at 24 h and 48 h. A later induction of the phospho-elF2α and proapoptotic transcription factor CHOP was observed at 48 h and 72 h, being correlated with elevated early apoptotic cell numbers at the same time-points.

Conclusions: The present study demonstrates that AGEs directly induce ER stress in human aortic endothelial cells, playing an important role in endothelial cell apoptosis. Targeting AGEs signaling pathways in order to alleviate ER stress may prove of therapeutic potential to endothelial dysfunction-related disorders.

Keywords: advanced glycation end-products (AGEs); apoptosis; endoplasmic reticulum (ER) stress; human aortic endothelial cells; unfolded protein response (UPR).

Introduction

Advanced glycation end products (AGEs) are highly reactive molecules resulting from the irreversible post-translational modification reactions of glucose with the amino groups on proteins, lipids or nucleic acids [1, 2]. Increased circulating AGE levels are a common finding and a pathogenic mediator of hyperglycemic, oxidative and inflammatory conditions including diabetes, polycystic ovarian syndrome, neurodegenerative and cardiovascular diseases [3–6]. However, exogenous intake of AGEs from high fat and/or protein diets, beverages or cigarette smoke may also contribute to increased AGEs tissue deposition under normal physiology predisposing individuals to metabolic, cardiovascular disorders and progressive aging [7, 8]. AGEs have been shown to accumulate in almost all major organs including vascular tissues mediating both extracellular and intracellular functions [9].

Endothelial cells (ECs) have been proposed as primary targets of AGEs, affecting their proliferation, migration and adhesion [10]. Extracellular effects of AGEs in endothelium involve irreversible crosslinking formation with stable, long-lived extracellular matrix proteins such as collagen, elastin or laminin [11]. At the same time they can induce cell activation, proinflammatory cytokine release and oxidative stress through interaction with their receptor, RAGE, leading to endothelial injury and dysfunction [12, 13]. The role of AGEs in the induction of aortic endothelial apoptosis has not been adequately addressed. It has been suggested that AGEs trigger oxidative stress and thus
accelerate apoptosis of bovine aortic ECs contributing to vascular complications associated with diabetes mellitus [14]. This effect has been attributed to AGE-RAGE interaction leading to increased intracellular accumulation of reactive oxygen species and elevation of intracellular Ca\(^{2+}\) concentration. Additionally, AGE-RAGE dependent activation promotes apoptosis of rat endothelial progenitor cells and inhibits their migration [15]. Recently methyglyoxal, the main AGE precursor has also been shown to induce apoptosis of human aortic endothelial cells (HAEC), through oxidative stress and by triggering the mitochondrial route of apoptotic machinery [16].

Relevant studies challenge the central role of mitochondria in apoptosis and suggest that some apoptotic signals may bypass mitochondria to directly activate caspases [17]. Endoplasmic reticulum (ER) stress presents such an alternative mechanism triggering apoptosis without the involvement of mitochondria [18]. ER is the major organelle involved in the synthesis and folding of secreted and membrane bound proteins, calcium homeostasis and lipid biosynthesis [19]. The lumen of ER is characterized by high Ca\(^{2+}\) concentration and a very oxidizing environment [19]. Stressors that interfere with the ER functions, such as oxidant or reducing agents, result in the disruption of Ca\(^{2+}\) homeostasis or glucose deprivation leading to the accumulation of unfolded and/or misfolded proteins in the ER lumen, known as ER stress [20].

The mammalian ER stress response known as unfolded protein response (UPR) can be separated into two phases, adaptation and apoptosis. The cells initially adapt to the accumulation of unfolded proteins by increasing the concentration of chaperones in the ER lumen, namely the glucose-regulated protein 78 (GRP78) and glucose-regulated protein 94 (GRP94). The UPR response is triggered by the activation of three main sensors of ER stress: protein kinase-like ER kinase (PERK), inositol requiring kinase 1 (IRE1) and the activating transcription factor 6 (ATF6) [20]. All these proteins are maintained in the inactive form by binding the N-terminus of GRP78 that serves both as chaperone and sensor of ER stress. When misfolded proteins accumulate, GRP78 is required for its chaperone function and releases these sensors to initiate the UPR [21]. PERK, upon activation, phosphorylates the eukaryotic translation initiation factor 2α (eIF2α), decreases mRNA translation and induces UPR-related genes [20]. Similarly, activation of IRE1α and ATF6 also leads to regulation of various UPR-related genes, such as protein chaperones and calnexin [22]. However, if this adaptation does not prove sufficient, the apoptotic response is initiated.

Previous studies have shown that ER stress is implicated in endothelial dysfunction associated with diabetes mellitus and cardiovascular diseases indicating that ER stress-induced apoptosis can be an important pathophysiological factor [23, 24]. Furthermore, AGEs have been shown to induce ER stress-mediated apoptosis in mouse podocytes [25], human neuroblastoma cells and cortical neurons [26]. However, the role of AGEs in ER stress-induced apoptosis in human aortic ECs remains to be elucidated. The present study aims to delineate ER stress induction in human ECs exposed to different AGE concentrations at variable time-points, monitoring the series of events leading to apoptosis.

### Materials and methods

#### Reagents

All reagents were purchased from Life Technologies (Carlsbad, CA, USA) unless otherwise stated. AGE-bovine serum albumin (AGE-BSA) was obtained from Abcam plc (ab51995; Cambridge, UK). Tunicamycin was purchased from AppliChem (Darmstadt, Germany). BSA, TNF-α and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Aldrich (St Louis, MO, USA).

#### AGE-BSA characteristics

According to manufacturer’s instructions, glycated BSA has been produced by reacting BSA with glycoaldehyde under sterile conditions followed by extensive dialysis and purification steps. Fluorescence of AGEs was confirmed by fluorescence spectrophotometry with Ex./Em.=370/440 nm. Glycated BSA showed 7000% increase in fluorescence when compared to control BSA. The purity of the final stock is >95% as analyzed by SDS-PAGE and filter sterilized using 0.22 µm filter.

#### Culture of endothelial cells

HAEC were obtained as cryopreserved cells from European Collection of Cell Cultures (ECACC) and were cultured in endothelial cell growth medium M200 supplemented with Low Serum Growth Supplement containing Fetal Bovine Serum (2% v/v), hydrocortisone, (1 g/mL), human epidermal growth factor (10 ng/mL), basic fibroblast growth factor, (3 ng/mL), heparin (10 g/mL), gentamicin (50 µg/mL) and amphotericin B (50 ng/mL). Cells were cultured at 37°C in a humidified 95% air –5% CO₂ atmosphere and were split according to standard procedures. HAEC were used in all assays between passage four and eight.

#### Cell proliferation assay

For the proliferation assay, HAEC were plated on 96-well microplates at a density of approximately 1×10⁴ cells per well, and then treated
with either unmodified BSA or AGE-BSA at concentrations of 100 and 200 μg/mL for 24, 48 and 72 h or with tunicamycin at concentration of 5 μg/mL for 24 h. Co-treatment with both tunicamycin at the same concentration and AGE-BSA at concentrations of 100, 200 and 400 μg/mL was also performed. Cell viability was subsequently determined by the MTT assay as described previously [27]. Each experiment was conducted in triplicate.

RT-PCR analysis

Total RNA was isolated from cultured cells using RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. cDNA was synthesized in a two-step reaction using iScript cDNA synthesis kit (Biorad, Hercules, CA, USA).

For semi-quantitative PCR, cDNA was amplified using gene specific primer pairs in 25 cycles. Detailed PCR conditions have been described previously [28]. PCR fragments were resolved on EtBr agarose gel. Densitometric analysis of PCR fragments was performed using image analysis software Image J (La Jolla, CA, USA) after normalization to actin levels. All experiments have been performed at least three times. Data and densitometric analysis from one representative experiment are presented.

Real-time quantitative PCR

Real-time quantitative PCR was performed using an iCycler real time instrument (Biorad). RT-PCR product was amplified using the iQ SYBR Green Supermix (Biorad) in a total reaction volume of 20 μL. Primer efficiencies were calculated from a standard curve of serially diluted cDNA. Product identity was confirmed by a single pick in the melt curve. Relative expression values were calculated using the 2^(-ΔΔCt) formula. The data are presented as fold change in gene expression normalized to GAPDH and relative to the untreated control. The primers used for the amplification of CHOP were forward 5′-agtgcacggagaaagctaa-3′, and reverse primer, 5′-ccatacagcagcctgagtga-3′, and for GAPDH were forward primer 5′-gggtgtgaaccatgagaagt-3′, and reverse primer 5′-catgcctgagtctccgcttc-3′.

Western blot analysis

For immunoblot analysis cells were solubilized with ice-cold RIPA buffer (Thermo Scientific, Rockford, IL, USA) supplemented with protease inhibitor cocktail (Thermo Scientific). The protein concentration in the lysates was determined by using Bradford assay (Biorad). Equal amounts of total protein were resolved by SDS-PAGE and immunoblotted with anti-GRP78 (#3177S; Cell Signaling), anti-CHOP (sc-71817; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p-eIF2α (#3398S; Cell Signaling), anti-XBP-1 (sc-7160; Santa Cruz) and anti-Actin (MAB-150; Millipore, Bedford, MA, USA). Relative protein amounts were evaluated by a densitometric analysis using Image J software and normalized to the corresponding actin levels. All experiments have been performed at least three times and representative results and the corresponding quantification data of one experiment are shown.

Assessment of apoptosis by flow cytometry

For the apoptosis assay, HAEC were seeded in six-well plates at a density of approximately 2×10^4 cells per well and then treated with BSA or AGE-BSA at a concentration of 200 μg/mL for 48 h and 72 h, with tunicamycin at a concentration of 5 μg/mL for 24 h and with both AGE-BSA and tunicamycin at the same concentrations for 24 h. Cells were then harvested, rinsed twice with PBS, collected by centrifugation at 2000 rpm for 5 min and annexin V-FITC apoptosis detection kit (Trengren, Gaithersburg, MD, Germany) was used according to the manufacturer’s protocol. Samples were scanned with a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, USA), and the data were analyzed with CellQuest software (Becton Dickinson). A total of 10,000 events were measured per sample.

Plots in the annexin V-positive/propidium iodide-negative quadrant were counted as apoptotic cells. Each experiment was performed in triplicate.

Statistics

The data are presented as mean ± SE for the number of experiments indicated. Statistical analysis of the results was performed using Student’s t-test. A p-value of <0.05 was considered to indicate statistical significance.

Results

AGE-BSA administration inhibited HAEC proliferation in a time- and dose-dependent manner

In order to elucidate the impact of AGEs in endothelial cell proliferation, HAEC were treated with increasing concentrations (100, 200 μg/mL) of unmodified bovine serum albumin (BSA) or AGE-BSA for different periods of time (24, 48, 72 h). MITT proliferation assay indicated that AGES administration significantly reduced HAEC viability in a time- and dose-dependent manner (48 h–100 μg/mL AGE-BSA: p<0.01; 48 h–200 μg/mL AGE-BSA: p<0.05; 72 h–100 μg/mL AGE-BSA: p<0.05; 72h–200 μg/mL AGE-BSA: p<0.001) (Figure 1A). In addition the administration of the ER stress-inducer tunicamycin had the same effect with the higher concentration dose of AGE-BSA (200 μg/mL) and longer incubation time (72 h) (p<0.001). The combined administration of tunicamycin and increasing concentrations of AGE-BSA indicated minor reduction of HAEC proliferation suggesting a non-synergistic effect between these two agents (Figure 1B).
AGE-BSA treatment-induced ER stress in HAEC

Based on recent studies that describe the involvement of AGEs in ER stress signaling, we investigated the possible induction of ER stress and the expression of UPR components upon AGE-BSA administration. Semi quantitative PCR analysis demonstrated the up-regulation of chaperones GRP78 and GRP94, the major indicators of ER stress induction in all conditions under study (Figure 2). We also observed an increase in XBP-1 mRNA levels at early time points (24 h, 48 h) and lower concentrations (~100 μg/mL) and of the spliced form of XBP-1 at 72 h, suggesting the early activation of IRE-1 pathway upon AGEs presence (Figure 2B–D).

Furthermore, we assessed the protein levels of the early GRP78, XBP-1 and late UPR effectors, p-eIF2α and CHOP following AGE-BSA incubation. GRP78 expression was found to be up-regulated in all dose- and time-points (Figure 3) being in accordance with the elevated mRNA levels (Figure 2). Similar trend was also observed in XBP-1 protein expression levels that remained elevated at early time points (24 h, 48 h) and decreased at 72 h at both AGE-BSA concentrations (Figure 3). In order to investigate the involvement of late UPR effectors in AGES-induced ER stress, key molecules of the PERK pathway were studied. As shown in Figure 3, AGE-BSA administration in HAEC increased the phosphorylated form of eIF2α and of the pro-apoptotic transcription factor CHOP expression in a time- and dose- dependent manner. Higher levels were observed in 48 h (200 μg/mL AGE-BSA) and in 72 h (at both concentrations).

AGE-BSA administration-induced ER stress-mediated apoptosis in HAEC

In order to confirm the proapoptotic transcription factor CHOP induction upon AGES treatment, we monitored CHOP mRNA expression levels using quantitative real time PCR analysis. Our results demonstrated an even higher statistically significant CHOP mRNA fold induction compared with protein expression levels in 72 h treatment at both concentrations (Figure 4).

This finding indicated the development of CHOP-mediated apoptosis in HAEC upon AGES treatment that was further investigated by annexin V/propidium iodide flow cytometric analysis (Figure 5). High concentration of AGES (200 μg/mL) for 48 h and 72 h incubation resulted in a significant increase of the early apoptotic fraction of HAEC (four- and eight-fold, respectively). In addition tunicamycin and AGE-BSA co-treatment did not further increase the early apoptotic cells compared to tunicamycin alone (39.64% vs. 37.51% of gated cells). This result is consistent with the proliferation data (Figure 1B), providing further evidence for a common mechanism between AGES and tunicamycin in inducing HAEC apoptosis via activation of UPR signaling.

Discussion

Endothelial cells present primary dynamic structures that actively regulate basal vascular tone and reactivity in physiological and pathological conditions. They respond to neurohumoral mediators and mechanical forces as to maintain the balance between vasodilatation and
vasoconstriction, restore blood fluidity and vessel wall integrity [29]. Loss of endothelial function may be a critical and initiating factor for several conditions including inflammation, diabetic micro- and macrovascular complications as well as cardiovascular diseases [29].

AGEs have been shown to contribute to endothelial dysfunction and this has been clearly demonstrated in diabetes, where levels of serum AGEs in patients with type 2 diabetes were negatively associated with the degree of endothelium-dependent and endothelium independent vasodilation [30]. Several mechanisms have been suggested to explain the association. Among them, bioavailability and activity of the endothelium-derived NO has been demonstrated to be reduced by AGEs [31]. AGE associated induction of oxidative stress may quench and inactivate endothelium-derived NO and uncouple endothelial NO synthase (eNOS) activity through receptor-mediated phosphorylation and increased eNOS degradation [32, 33]. In addition, AGEs may impair endothelial balance by reducing the endothelial production of prostacyclin [34] and enhancing the expression of endothelin-1 [35]. They are also capable of increasing endothelial permeability to macromolecules [36, 37] and modify the functions of endothelial progenitor cells by promoting their apoptosis and migration [38]. Endothelial cell apoptosis due to AGEs has been demonstrated in several studies using bovine and human aortic ECs and has been mainly attributed to the induction of oxidative stress and mitochondrial dysfunction [14, 16].

Figure 2 AGE-BSA administration induces mRNA levels of UPR components in HAEC.
(A) ER stress induction in HAEC following AGE-BSA treatment. RT-PCR analysis of GRP78, GRP94 and XBP-1 mRNA expression after AGE-BSA administration at increasing concentrations (100, 200 μg/mL) for different periods of time (24 h, 48 h, 72 h). The lower band represents the spliced form of activated XBP-1 (arrow).
(B) The panel represents results of densitometric analysis of GRP78, GRP94 and XBP-1 PCR fragments after normalization to actin levels. TNF-α (50 ng/mL for 4 h) and tunicamycin (5 μg/mL for 24 h) treatments were used as positive ER stress induction controls. All experiments have been performed at least three times and representative results as well as corresponding quantification data of one experiment are shown (B–D).
Here, we show AGEs-induced apoptosis in ECs through ER stress pathway, providing a novel molecular mechanism for endothelial dysfunction in metabolic and cardiovascular disorders. ER is exquisitely sensitive to glucose availability and depends on blood glucose levels for the energy supply required for the protein folding process [39]. ECs always exposed to elevations and reductions of blood nutrients, are very dynamic, metabolically active cells, with a high volume of protein synthesis which predispose them to ER stress [40]. In particular ECs cannot tolerate the continued high glucose exposure, and thus ER stress is commonly initiated in a diabetic milieu [41]. It was thus hypothesized that ER stress may also be induced in HAECs under conditions of increased exogenous glycated proteins (AGEs) uptake. Indeed, incubation of HAEC with high AGE levels for prolonged durations (48 h and 72 h) was capable to significantly reduce cell proliferation. This reduction was accompanied by increased mRNA and protein levels of ER stress sensors (GRP78, GRP94) indicative of ER stress induction. Recent studies have also shown the direct induction of ER stress by AGEs in murine podocytes, human neuroblastoma cell lines and human chondrocytes where they are implicated in inflammatory and oxidative processes as well as cell death pathways [25, 26, 42].

**Figure 3** AGE-BSA administration increases GRP78 and other UPR component protein levels. GRP78, p-eIF2α, XBP-1 and CHOP protein levels were assessed by Western blot analysis after AGE-BSA treatment of HAEC at increasing concentrations (100, 200 µg/mL) for various time points (24 h, 48 h, 72 h) (A). The densitometric quantification of these results (normalized to the actin levels) is shown in the graph (B). Tunicamycin (5 µg/mL for 24 h) treatment was used as positive ER stress induction control. All experiments have been performed at least three times and representative results as well as corresponding quantification data of one experiment are shown.

**Figure 4** Induction of proapoptotic transcription factor CHOP upon AGE-BSA treatment. CHOP mRNA levels were monitored by quantitative real time PCR analysis in HAEC following treatment with AGE-BSA at increasing concentrations (100, 200 µg/mL) for different time points. TNF-α (50 ng/mL for 4 h) and tunicamycin (5 µg/mL for 24 h) treatments were used as positive ER stress induction controls. *p<0.05, **p<0.01, ***p<0.001.
Figure 5 AGE-BSA induced apoptosis in HAEC in a time-dependent manner. Apoptosis of HAEC was determined by annexin V/PI binding. HAEC were untreated (A), treated with AGE-BSA (200 μg/mL) for 48 h (B) and 72 h (C), with tunicamycin (5 μg/mL) for 24 h (D) and with both tunicamycin (5 μg/mL) and AGE-BSA (200 μg/mL) for 24 h (E). Percentage of gated cells is shown in each quadrant (UL: % of necrotic cells, UR: % of apoptotic cells, LL: % of viable cells, LR: % of early apoptotic cells).

Notably, at early time points (24 h, 48 h) of AGE-BSA administration, activation of the transcriptional activator, XBP-1 was observed that is a mediator of the adaptive UPR response. The three UPR branches (IRE1, PERK, and ATF6) have been reported to initially promote cell survival by reducing misfolded protein levels but when ER stress is not alleviated UPR signaling promotes apoptotic cell death. This is also possible to occur under AGEs accumulation, being capable of triggering both protective and cell death responses depending on their concentration and the duration of their presence. In accordance with this hypothesis, we found that IRE1 activity (as demonstrated by XBP-1 expression) was increased at early AGE-induced ER stress (24 h) and attenuated by persistent ER stress (48 h, 72 h). IRE1 is a transmembrane kinase (endoribonuclease, RNase) that upon activation, initiates the non-conventional splicing of Xbp-1 mRNA [43, 44]. Spliced Xbp-1 mRNA encodes a transcription activator that drives transcription of genes such as ER chaperones, whose products directly participate in ER protein folding [45]. By contrast, our data show that PERK signaling including translational inhibition (by phosphorylation of eIF2α) and proapoptotic transcription regulator CHOP induction was maintained at 48 h and 72 h incubation with AGEs. PERK is a transmembrane kinase that phosphorylates the eukaryotic translation initiation factor 2α (eIF2α), thus reducing protein synthesis and counteracting ER protein overload [46]. Additionally, CHOP is a B-ZIP transcription factor induced by the PERK branch of the UPR that promotes cell death [47]. Chop mRNA levels were significantly increased at 48 h incubation with 200 μg/mL AGEs compared to untreated control and remained elevated at 72 h at both concentrations, indicative of proapoptotic signaling. These data were further confirmed by double staining flow cytometric analysis of AGE-stimulated HAEC using the early apoptotic marker, annexin V and nucleic acid stain, propidium iodide. Significantly increased cell numbers of early apoptotic cells were detected following AGEs treatment for 48 h and 72 h. The modest increase in early and late apoptotic cell population after treatment with AGEs combined with tunicamycin compared to tunicamycin alone indicated that both agents share common
UPR signaling pathways in the induction of apoptosis in HAEC.

Several studies in other cell systems suggest that apoptosis mediated by AGEs could be induced by an ER pathway independent from mitochondria [25, 48]. More specifically, AGE-induced GRP78 expression as an ER stress sensor was associated with elevated intracellular Ca²⁺ and apoptosis in murine podocytes [25]. Additionally, 3-deoxyglucosone, a precursor for AGEs, that is highly upregulated in skin explants of diabetic cutaneous wounds, was found to mediate ER stress-induced apoptosis through ROS formation in human dermal fibroblasts via a RAGE-independent mechanism [48]. This is particularly interesting since previous studies limited AGE-induced apoptosis in being mediated through increased oxidative stress following interaction of AGEs with their receptor RAGE [14–16].

It is therefore evident that due to the heterogeneity of AGE compounds and the wide range of their precursor molecules; different signaling may be selected depending not only upon the cell type but the state of AGE molecules being either in circulating or protein-bound form. Further research is required in elucidating the necessity of RAGE in endothelial ER stress induction under high AGE environments. Additionally, since ER stress regulates mitochondrial bioenergetics, a potential crosstalk between oxidative stress in ER stress-induced apoptosis cannot be excluded and should be explored in detail.

However, our data show that ER function can be modulated by AGEs affecting critically both ECs functionality and cell fate. Thus, chemical enhancement of ER function to cope with cell apoptosis provides an interesting approach to managing endothelial dysfunction. Pharmaceutical or chemical chaperones such as trimethylamine N-oxide dehydrate, 4-phenyl butyric acid and taurine-conjugated ursodeoxycholic acid are representatives of a group of low-molecular weight compounds known to stabilize protein conformation that improve ER folding capacity and facilitate trafficking of mutant proteins [49, 50]. The effectiveness of these molecules in alleviating endothelial injury either alone or in conjunction with standard medications remains to be investigated.

Conflict of interest statement

Authors’ conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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