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ER stress is associated with reduced ABCA-1 protein levels in macrophages treated with advanced glycated albumin – Reversal by a chemical chaperone

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**A B S T R A C T**

ATP-binding cassette transporter A1 mediates the export of excess cholesterol from macrophages, contributing to the prevention of atherosclerosis. Advanced glycated albumin (AGE-alb) is prevalent in diabetes mellitus and is associated with the development of atherosclerosis. Independently of changes in ABCA-1 mRNA levels, AGE-alb induces oxidative stress and reduces ABCA-1 protein levels, which leads to macrophage lipid accumulation. These metabolic conditions are known to elicit endoplasmic reticulum (ER) stress. We sought to determine if AGE-alb induces ER stress and unfolded protein response (UPR) in macrophages and how disturbances to the ER could affect ABCA-1 content and cholesterol efflux in macrophages. AGE-alb induced a time-dependent increase in ER stress and UPR markers. ABCA-1 content and cellular cholesterol efflux were reduced by 33% and 47%, respectively, in macrophages treated with AGE-alb, and both were restored by treatment with 4-phenyl butyric acid (a chemical chaperone that alleviates ER stress), but not MG132 (a proteasome inhibitor). Tunicamycin, a classical ER stress inducer, also impaired ABCA-1 expression and cholesterol efflux (showing a decrease of 61% and 82%, respectively), confirming the deleterious effect of ER stress in macrophage cholesterol accumulation. Glycoxidation induces macrophage ER stress, which relates to the reduction in ABCA-1 and in reverse cholesterol transport, endorsing the adverse effect of macrophage ER stress in atherosclerosis. Thus, chemical chaperones that alleviate ER stress may represent a useful tool for the prevention and treatment of atherosclerosis in diabetes.

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1. Introduction

In diabetes mellitus (DM), cardiovascular disease is the major cause of morbidity and mortality (Earl, 2011). In particular, alterations along the reverse cholesterol transport (RCT) system that are associated with the development of atherosclerosis have been described in DM (Tan, 2009). The RCT system allows excess of cholesterol to be removed from arterial macrophages by lipid-free Apo A-1 and HDL and then transported to the liver for bile excretion. The removal of cellular cholesterol is mainly driven by the ATP cassette transporter type 1 (ABCA-1) which mediates cholesterol export to plasma Apo A-1 by utilizing free energy from ATP hydrolysis (Tang and Oram, 2009). ABCA-1 is a short-lived, 220 kDa plasma membrane protein. Its expression and activity are primarily regulated by the oxysterols-activated liver X receptor (LXR), although posttranslational modifications also influence its final protein content (Tall et al., 2002; Wellington et al., 2002).

In DM, cellular stressors, including reactive oxygen species (ROS), inflammatory cytokines, hexosamine pathway derivatives and lipid accumulation, lead to endoplasmic reticulum (ER) stress. ER stress is known to activate the unfolded protein response (UPR) as an adaptive response to the accumulation of misfolded proteins in the lumen of the organelle (Ron and Walter, 2007). During the UPR, sequential activation of ER-resident proteins mediates a cascade of events that promote cell adaptation and survival. As a short-term response, cell translational machinery is shut off via PERK-mediated phosphorylation of eIF2α, which helps to prevent the entrance of additional misfolded proteins into the ER. Next,
IRE1α and ATF6 activation lead to an increase in the expression of Grp78 and Grp94 chaperones, and subsequently triggers an antioxidant defense that aids in the proper folding of proteins. In addition, unfolded proteins are continuously cleared by ubiquitination and proteasomal cleavage in a process known as endoplasmic reticulum-associated degradation (ERAD). Finally, if translational homeostasis is not satisfactorily achieved, the pro-apoptotic ATF- 6/CHOP-mediated pathway can be elicited, resulting in cell death (Zhang and Kaufman, 2008). In both humans and animal models, UPR markers are enhanced in atherosclerotic lesions and have also been associated with plaque instability and rupture in atherosclerosis (Myoishi et al., 2007; Zhou et al., 2005; Tsukano et al., 2010).

Advanced glycation end products (AGE) are prevalent in DM and mainly associate with serum albumin (AGE-alb), which corresponds to 80% of the total glycated proteins in circulation (Cohen, 2003). AGE-alb enhances ROS generation and induces intracellular lipid accumulation by impairing the expression of ABCA-1 (de Souza Pinto et al., 2012). However, this effect is not related to alterations in ABCA-1 gene transcription or mRNA levels (Passarelli et al., 2005; Isoda et al., 2007). Considering that the activation of ER stress following AGE-alb treatment has not been reported in macrophages, the aim of this study was to evaluate whether ER stress mediates the reduction in ABCA-1 protein expression and in cholesterol efflux induced by AGE-alb. In addition, we sought to assess the effect of a chemical chaperone that alleviates ER stress (4-phenyl butyric acid; 4-PBA) on ABCA-1 cell content and cholesterol efflux. Understanding how ER stress modulates ABCA-1 levels in cells under glycoxidative stress will help to elucidate the role of hyperglycemia in foam cell formation in DM.

2. Material and methods

2.1. Preparation of AGE-albumin

AGE-alb was prepared by incubating fatty acid free albumin (FAPA, 40 mg/mL) with 10 mM glycolaldehyde (GAD; Sigma Chem. Com. St. Louis, MO) for 4 days, at 37 °C under sterile conditions and a nitrogen atmosphere in a water bath shaker in the dark. Control albumin (C-alb) was prepared under the same conditions, however incubated with PBS only. After extensive dialysis, the samples were sterilized in a 0.22 μm filter. All albumin samples contained <50 pg endotoxin/mL as determined using the chromogenic Limulus amebocyte assay (Cape Cod, Falmouth, MA).

2.2. AGE determination in albumin samples

The determination of AGE in albumin samples was performed according to Pageon et al. (2007). Briefly, AGE and C-alb samples were diluted to 1 mg/mL and fluorescence intensity was measured at 440 nm (λ emission) and 370 nm (λ excitation).

2.3. Cell culture

Mouse peritoneal macrophages were harvested after the injection of 6 mL of PBS into the peritoneal cavity. After a soft abdominal massage, cells were collected into sterile tubes, centrifuged and resuspended in RPMI 1640 containing 10% fetal calf serum (FCS), 1% penicillin–streptomycin and 4 mM l-glutamine. The cells were then plated in Petri dishes or 48 well-plates. J774 cells (a tumoral murine macrophage cell line) were cultured in RPMI 1640 containing 10% FCS, 1% penicillin–streptomycin and 4 mM l-glutamine and maintained in a 5% CO2 incubator at 37 °C. During the different experimental treatments described below, cells were maintained in DMEM, and cell viability was assured by exclusion of trypan blue (higher than 99%) and lactate dehydrogenase release to cell culture medium (In vitro toxicology assay kit, Sigma Aldrich, St Louis, MO, USA) in all experimental conditions. No change in apoptosis was observed after exposure to AGE-alb, as assessed by the number of Annexin V–FITC positive cells (data not shown).

2.4. Assessment of carbonyl content in macrophages

To determine the carbonyl content in macrophages, lysates from C or AGE-alb-treated cells were incubated with 10 mM of dinitrophenylhydrazine in 2.5 M HCl for 1 hour at room temperature. The reaction was blocked by the addition of 20% trichloroacetic acid (TCA). The pellets were washed twice with absolute ethanol/ethanolacetate (1:1) and once with 10% TCA. The protein pellets were dissolved in 6 M guanidine hydrochloride and the absorption at 370 nm was determined. Carbonyl content was calculated using the molar absorption coefficient of aliphatic hydrazones of 22,000 M−1 cm−1 and expressed as nanomoles carbonyl per milligram of protein.

2.5. Immunoblotting

Mouse peritoneal macrophages were treated with C- or AGE-alb for different intervals and were also treated with C- or AGE-alb in the absence or presence of 2.5 or 5.0 mM 4-PBA (Alpha Aesar, Ward Hill, MA). Following treatment, cells were scraped into Tris buffered saline containing protease inhibitors, and equal amounts of cellular protein were applied to a polyacrylamide gel (SDS-PAGE). The expressions of Grp78, Grp94, elf2α-P, ATF-6, ubiquitin, PDI, CHOP and β-actin were determined using the following antibodies, respectively: anti-KDEL (Stressgen, Ann Arbor, MI, USA), anti-elf2α-P (Upstate Biotechnology), anti-ATF6 (Abcam, Cambridge, MA, USA), anti-ubiquitin (ZYMED® Laboratories Carlsbad, CA, USA), anti-PDI (Stressgen), anti-CHOP (ABR-Affinity Bioreagents, Fisher Scientific, USA) and anti-β-actin (Fitzgerald Industries International Inc., Concord, MA). Tunicamycin (2 μg/mL) was used as a positive control for the induction of ER stress. Proteins were detected with horseradish peroxidase-conjugated antibodies and an enhanced chemiluminescence reagent (Pierce, Rockford, IL). Immunoblot band density was quantified using an ImageQuant 300 Imager (GE Healthcare), and differences between the bands were analyzed in pixels, using the ImageQuant TL software (GE Healthcare). The results were expressed as arbitrary units, analyzing AGE-alb versus C-alb normalized to β-actin expression.

2.6. ABCA-1 protein content

Due to the need for a higher number of cells, J774 macrophages were used in flow cytometry assays instead of mouse peritoneal macrophages. J774 macrophages were pre-incubated with 0.5 mM 8-bromoadenosine-3’, 5’-cyclic monophosphate (cAMP) (Sigma Aldrich) and were treated for 8 h with C- or AGE-alb in the presence or absence of the ER stress inhibitor 4-PBA (2.5 or 5.0 mM) or the proteasome inhibitor MG132 (1 μM). MG132 was added to the culture in the last 2 h of incubation with albumins, while 4-PBA was added simultaneously with the albumins. In some incubations, cells were treated with tunicamycin (2 μg/mL for 18 h). To assess the ABCA-1 content on the macrophage surface, a total of 1 × 10⁶ cells were trypsinized, washed extensively with PBS and fixed in 4% paraformaldehyde. After fixation, cells were incubated with anti-ABCA-1 antibody (Novus Biologicals, Inc., Littleton, CO – 1:250 dilution) for 1 h at room temperature, rinsed in PBS and incubated with 4 μg/mL Alexa Fluor 488 antibody (Invitrogen, USA). Cellular fluorescence intensity was evaluated by flow cytometry using a FACS Calibur and CellQuest Software (B.D., San Jose, CA). All of the conditions were corrected to basal cellular fluorescence (cells without antibody labeling). The total cellular ABCA-1 content was determined by immunocytochemistry. Briefly,
mouse peritoneal macrophages were seeded on coated cover slips and allowed to adhere for 2 days. After pre-treatment with 0.5 mM cAMP macrophages were incubated with C- or AGE-alb for 8 h in the presence or absence of 4-PBA and MG132. Cells were then washed with PBS and fixed with ice-cold 70% ethanol for 10 min and rehydrated with PBS. Cells were then permeabilized with 0.05% Tween 20 and incubated in a blocking solution containing 1% FAFA for 30 min at 37 °C. After blocking, cells were incubated for 2 h at 37 °C with the anti-ABCA-1 antibody (Novus Biologicals 1:250), washed and labeled with horseradish peroxidase conjugated-secondary antibody (Novus Biological 1:800) for 90 min at 4 °C. Finally, cells were stained with 3,3′-diaminobenzidine (DAB) for 3 min and after chromogen development, the slides were washed in distilled water and counterstained with hematoxylin (30 s). The samples were blindly analyzed, and brownish yellow granular deposits were considered positive areas. QWin V3 Standard was used to perform semiquantitative evaluation under a light microscope, and the integrated optical density of the positively stained area was measured at a magnification of 400×. Measurements were taken from 4 random fields in each slide, and 4 slides were assessed for each condition. Data were expressed as integrated optical density per group.

2.7. ABCA-1 mRNA by real time RT-PCR

Total RNA from macrophages treated with C- or AGE-alb was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quality was assessed using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Real-time RT-PCR was conducted using TaqMan two step RT-PCR (Applied Biosystems, Foster City, CA, USA). The following TaqMan Gene Expression Assay was used: Mouse ABCA-1 (Mm00442663_m1) in the Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The results were corrected to the internal β-actin gene (Mm00607939_s1), and relative quantification analysis was performed with StepOne Software 2.0 (Applied Biosystems, Foster City, CA, USA) using the comparative cycle threshold (Ct) \(2^{-\Delta\Delta Ct}\) method.

2.8. LDL isolation and acetylation

Low-density lipoprotein (LDL, \(d = 1.019–1.063 \text{ g/mL}\)) was isolated after sequential preparative ultracentrifugation of fresh plasma drawn from healthy donors and purified by discontinuous gradient ultracentrifugation. After dialysis, the protein concentration was measured by the Lowry protocol (Lowry et al., 1951) and acetylation performed according to Basu et al. (1976), followed by extensive dialysis and sterilization in a 0.22 μm filter.

2.9. Cholesterol efflux assay

Mouse peritoneal macrophages were cultured in RPMI 1640 containing 10%, penicillin and streptomycin and were maintained in a 5% CO\(_2\) incubator at 37 °C. Cells were enriched with acetylated LDL (50 μg/mL) and 0.3 μCi/mL of \(^{14}\text{C}\)-cholesterol (Perkin Elmer, Boston, MA, USA) for 48 h. After two washes with PBS containing FFAA, cells were maintained for 24 h in DMEM containing FFAA and cAMP, followed by 8 hours of incubation with 2 mg/mL C- or AGE-alb both in the absence or presence of 5 mM 4-PBA. In some experiments, macrophages were treated with tunicamycin (2 μg/mL) for 18 h. Macrophages were then incubated with...
Fig. 2. ABCA-1 protein level, but not mRNA, is reduced in macrophages treated with AGE-alb. Macrophages were treated for 8 h with C- or AGE-alb (2 mg/mL). (A) ABCA-1 content was determined by flow cytometry in J774 macrophages (n=6) and by (B) immunocytochemistry in mouse peritoneal macrophages (representative images are shown below; n=4). Inner panel: abca-1 mRNA levels were determined by RT-PCR.

30 μg/mL Apo A-I (Sigma Aldrich) for 8 h to determine the 14C-cholesterol efflux, as previously described (Machado et al., 2006).

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism 4.0 software (GraphPad Prism, Inc., San Diego, CA), and the Student’s t test was used to evaluate each group. Summary data are reported as the mean values ± standard deviation. A p-value <0.05 was considered statistically significant.

3. Results

Glycolaldehyde-modified albumin presented an increased fluorescence profile regarding total AGE when compared to C-alb samples (53,330 ± 15,824 vs. 1388 ± 368; p = 0.0047). In addition, previous studies from our group showed derivatization by carboxymethyllysine following albumin treatment in the same conditions as the present investigation (de Souza Pinto et al., 2012). The carbonyl content was higher in macrophages incubated with AGE-alb in comparison to cells treated with C-alb (respectively, 24.11 ± 2.01 and 17.10 ± 1.07 nanomoles of carbonyls per milligram of protein, n=4; p=0.0209).

Mouse peritoneal macrophages treated for 8 h with AGE-alb displayed increased expression of Grp78, which is considered a hallmark of ER stress activation (Fig. 1A). A time-dependent increase in the expression of Grp78 and Grp94 (Fig. 1B and C) was observed, with the most substantial increase at 8 hour after treatment, followed by a slight decrease overtime. At 18 h, AGE-alb-induced chaperone expression was still much higher compared to C-alb-treated macrophages. As expected, tunicamycin induced a 15% and 25% increase in Grp78 and Grp94, respectively. Similarly, the expression of ATF6 and eIF2α-P was also induced in a time-dependent manner in macrophages treated with AGE-alb.
Fig. 3. 4-PBA alleviates ER stress and restores ABCA-1 content and Apo A-I-mediated 14C-cholesterol efflux. Mouse peritoneal macrophages were treated for 8 hours with C- or AGE-alb (2 mg/mL) in the absence or presence of 2.5 or 5.0 mM 4-PBA. Equal amounts of cellular protein were loaded onto a polyacrylamide gel, and electrophoresis and immunoblotting were performed to measure the expression of: A) Grp78 (n=3), B) ATF6 (n=3) and C) eIF2α-P (n=3). Data are expressed as arbitrary units corrected to β-actin levels. (D) ABCA-1 content was determined by flow cytometry (n=6) in J774 macrophages and by (E) immunocytochemistry in mouse peritoneal macrophages (representative images are shown below; n=4). (F) Acetylated-LDL and 14C-cholesterol enriched-mouse peritoneal macrophages were treated for 8h with C- or AGE-alb (2 mg/mL), and cholesterol efflux to Apo A-I was assessed (30 μg/mL of Apo A-I for 8h).

(Fig. 1D and E). In agreement to our previous results, following an 18 hour-treatment, expression of these UPR markers was much higher compared to cells exposed to C-alb. No changes were observed in protein desulfide isomerase (PDI) or CHOP expression when macrophages were exposed to AGE-alb (data not shown).

Macrophages treated with AGE-alb did not alter the ABCA-1 mRNA levels when compared with C-alb-treated cells (Fig. 2
stress and the impairment of cholesterol efflux was also confirmed by incubation with tunicamycin, which reduced ABCA-1 protein content in 61% (Fig. 4A) and reduced Apo A-I mediated cholesterol efflux by 82% (Fig. 4B).

An exponential increase was observed in the total ubiquitin content of macrophages treated with AGE-alb over time (Fig. 5A). Incubation of macrophages with the proteasomal inhibitor, MG132, was unable to restore the diminished expression of ABCA-1 (Fig. 5B). Similar results were observed by immunocytochemistry (Fig. 5C). Therefore, the possibility of a proteasomal-mediated pathway targeting ABCA-1 degradation in macrophages treated with AGE-alb was excluded.

4. Discussion

In the setting of hyperglycemia, intracellular cholesterol accumulation, oxidative stress, inflammation and the accumulation of several different metabolites predict the ER stress response, which has been described as an important contributor to DM complications (Sage et al., 2010; Alhusaini et al., 2010).

In the present study, we demonstrated that in macrophages, AGE-alb induces ER stress, which is related to the impairment in macrophage RCT. The expression of endogenous chaperones that aid in proper protein folding, such as Grp78 and Grp94, as well as UPR mediators, such as ATF-6 and eIF2α, was elevated in a time-dependent manner in macrophages treated with AGE-alb compared to C-alb-treated cells, confirming ER-stress induction under glycoxidative conditions. Macrophage ER stress can be induced by several intracellular mechanisms elicited by AGE-alb, including cholesterol accumulation, inflammation and oxidative stress.

Oxaldehydes such as GAD are generated in inflammatory reactions, specifically mediated by the myeloperoxidase system. This can contribute to GAD generation in atherosclerotic lesions and to the local modification of albumin and other macromolecules. In addition, CML adducts have been found in atherosclerotic lesions from diabetic and dyslipidemic humans and animal models (Anderson et al., 1999).

ER membranes are highly sensitive to free cholesterol accumulation, which leads to cytotoxic effects in cells (Feng et al., 2003). In this regard, it has previously been shown by our group and others that AGE-alb treatment reduces the expression of both ABCA-1 and ABCG-1, impairing Apo A-I and HDL-mediated cholesterol efflux and leading to intracellular cholesterol accumulation (de Souza Pinto et al., 2012; Machado et al., 2006).

We previously identified a reduction in ABCA-1 macrophage content in response to both AGE-alb and serum albumin isolated from poorly controlled diabetes mellitus patients (Machado-Lima et al., 2010). This was related to the oxidative stress elicited by AGE-albumin via NAPDH oxidase and by the mitochondrial respiratory chain (de Souza Pinto et al., 2012) and also to the accumulation of 7-ketocholesterol that has been found in glycoxidized macrophages (Iborra et al., 2011).

Moreover, AGE-alb induces the expression of the receptor for advanced glycation end-products (RAGE) and scavenger receptors involved in the uptake of modified LDL by macrophages (Iwashima et al., 2000). RAGE activation elicits NAPDH oxidase activation and the production of ROS, which ultimately lead to NF-κB activation and the transcription of inflammatory genes (Basta et al., 2005). Inflammation contributes considerably to atherogenesis and also triggers ER stress via JNK activation (Hotamisligil, 2010; Hetz et al., 2006). Unpublished data from our group indicate that in macrophages, endotoxin-free AGE-alb primes these cells for an inflammatory response elicited by S100B calgranulins or lipopolysaccharides, which then damages the cholesterol efflux.

inner panel). However, the ABCA-1 protein level, assessed by flow cytometry and immunocytochemistry, was reduced by 33% and 25%, respectively, in AGE-alb-treated macrophages compared with cells treated with C-alb (Fig. 2A and B). Thus, both surface ABCA-1, as determined by flow cytometry, and total ABCA-1 content, as assessed by immunocytochemistry, were reduced in macrophages treated with AGE-alb.

AGE-alb-induced ER stress was alleviated by incubation with 4-PBA, as was demonstrated by the reduction in Grp78 expression (Fig. 3A). In agreement with this result, the expression of UPR markers (ATF6 and eIF2α-P) was also reduced when macrophages were incubated with 4-PBA (Fig. 3B and C). Furthermore, 4-PBA (2.5 mM) prevented the reduction of ABCA-1 protein levels induced by AGE-alb (Fig. 3D). These results were confirmed using immuno-}

A 47% reduction in Apo A-I-mediated cholesterol efflux was observed in cells treated with AGE-alb compared to C-alb; however, in the presence of 5 mM 4-PBA this difference was no longer observed between cells treated with AGE-alb and C-alb (Fig. 3F). These data agree with the recovery of the ABCA-1 protein level after inhibition of ER stress with 4-PBA. The association between ER...
Aside from the observed increase in ATF6 expression in macrophages treated with AGE-alb, we did not find any alterations in CHOP expression. In agreement, cellular apoptosis, assessed by Annexin V, was not different between cells treated with C- and AGE-alb (data not shown).

The level of ABCA-1 protein was reduced by 33% in macrophages treated with AGE-alb, and treatment with 4-PBA was able to completely restore ABCA-1 levels and Apo A-I-mediated cholesterol efflux in macrophages. The chemical chaperone 4-PBA is known to improve ER folding capacity and to facilitate the trafficking of misfolded proteins. These effects help to prevent protein aggregation stabilizing their conformation (Engin and Hotamisligil, 2010). In diabetic and obese animal models, Ozcan et al. (2006) have shown that oral administration of 4-PBA was able to normalize plasma glucose levels and restore systemic insulin sensitivity, separate from other anti-diabetic effects that were observed even in the absence of body weight loss.

The association between ER stress and ABCA-1 reduction, which has not been described so far, was further strengthened by the finding that tunicamycin – a classical ER stress inductor – also diminishes ABCA-1 and cholesterol efflux.

We have also obtained preliminary data demonstrating that aminoguanidine (an antioxidant and anti-AGE compound) diminishes the AGE-alb-induced expression of Grp78. Aminoguanidine is
able to reduce the generation of ROS in macrophages treated with AGE-alb and also recovers ABCA-1 protein content (de Souza Pinto et al., 2012). Thus, in our experimental conditions, ER stress is the most likely phenomenon linking the redox imbalance to the reduction in ABCA-1 levels in macrophages under glyoxidative stress, especially considering the important role this organelle plays in protein synthesis/stability.

Since ERAD is part of the UPR, specifically mediated by the IRE1 and ATF6 branches, and targets misfolded proteins for degradation (Kincaid and Cooper, 2007), we investigated whether inhibition of the proteasome had any effect on ABCA-1 protein levels in AGE-alb-treated macrophages. We found that proteasome inhibition by MG132 was not able to prevent the ABCA-1 reduction in AGE-alb-treated macrophages. In addition, the elevated level of total ubiquitin found in macrophages suggests proteasomal inhibition according to recent findings. Moheimi et al. (2010) demonstrated an impairment in the activity of proteasomal enzymes in macrophages incubated with GAD-modified albumin, suggesting a deleterious effect of oxaldehydes in the intracellular accumulation of misfolded and glycated proteins in diabetes mellitus. Nonetheless, the concentration of albumin utilized per milliliter of cell medium was highly superior when compared to that utilized by us (4 mg/mL vs. 2 mg/mL, respectively). Also, AGE-alb increases generation of ROS due to mitochondrial dysfunction (de Souza Pinto et al., 2012) and considering that the ubiquitin–proteasome complex is a high energy requiring system, the damage in mitochondrial function, induced by AGE, may contribute to the impairment in proteasomal activity.

Together with the observation that MG132 does not recover ABCA-1 levels, we assume that AGE-alb already damages the proteasomal system, which excludes the role of ERAD in ABCA-1 reduction in AGE-treated cells.

Zelcer et al. (2009) previously reported a proteasome-independent pathway for LDL-receptor (B-E receptor) degradation. In agreement with their findings, our experimental conditions showed that ABCA-1 levels do not appear to be regulated by ERAD/ubiquitin-proteasomal degradation. ABCA-1 is a large and complex polypeptide, and a wide range of posttranslational modifications carried out by ER machinery are required to reach a final mature conformation. Alteration of one or more of these mechanisms could impair the ABCA-1 structure and functionality (Kang et al., 2010).

Our study describes, for the first time, the effect of AGE-alb on macrophage ER stress, which is indeed associated with ABCA-1 content and function impairment in diabetes mellitus. The exact mechanism by which ER stress affects ABCA-1 content remains elusive and requires further investigation. ER dysfunction appears to play an important role in a wide range of diseases, including obesity, hepatic steatosis and DM (Hotamisgil, 2010). In this regard, chemical chaperones, such as 4-PBA, represent an exciting area of research for clinical application and may contribute to the prevention of atherosclerosis in DM.

5. Conclusion

In conclusion, diabetes mellitus and other cardiovascular stress conditions, despite modifications in the lipoprotein chemical composition and concentration, advanced glycated albumin contributes to atherosclerosis by inducing ER dysfunction, which relates to the reduction in ABCA-1-mediated macrophage reverse cholesterol transport.

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References


Machado AP, Pinto RS, Moyes ZP, Nakandakare ER, Quintão EC, Passarelli M. Aminoguanidine and metformin prevent the reduced rate of HDL-mediated cell cholesterol efflux induced by formation of advanced glycation end products. The International Journal of Biochemistry & Cell Biology 2006;38:392–3.


