

Inhibition of Macrophage Oxidative Stress Prevents the Reduction of ABCA-1 Transporter Induced by Advanced Glycated Albumin

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Abstract We investigated the role of aminoguanidine and benfotiamine on the inhibition of reactive oxygen species (ROS) generation in macrophages induced by advanced glycated albumin (AGE-albumin) and its relationship with cell cholesterol homeostasis, emphasizing the expression of the ATP binding cassette transporter A-1 (ABCA-1). AGE-albumin was made by incubating fatty acid-free albumin with 10 mM glycolaldehyde. ROS production and ABCA-1 protein level were determined by flow cytometry in J774 macrophages treated along time with control (C) or AGE-albumin alone or in the presence of aminoguanidine or benfotiamine. Mitochondrial function was evaluated by oxygraphy. Compared to C-albumin, AGE-albumin increased ROS production in macrophages, which was ascribed to the activities of NADPH oxidase and of the mitochondrial system. Mitochondrial respiratory chain activity was reduced in cells incubated with AGE-albumin. ROS generation along time was associated with the reduction in macrophage ABCA-1 protein level. Aminoguanidine prevented ROS elevation and restored the ABCA-1 content in macrophages; on the other hand, benfotiamine that promoted a lesser reduction in ROS generation was not able to restore ABCA-1 levels. Inhibition of oxidative stress

induced by AGE-albumin prevents disturbances in reverse cholesterol transport by curbing the reduction of ABCA-1 elicited by advanced glycation in macrophages and therefore may contribute to the prevention of atherosclerosis in diabetes mellitus.

Keywords Advanced glycation end products · Cholesterol efflux · ABCA-1 · Oxidative stress · Aminoguanidine · Benfotiamine

Abbreviations

| | |
|-------------|--|
| ABCA-1 | ATP binding cassette transporter A-1 |
| ABCG-1 | ATP binding cassette transporter G-1 |
| ACAT | Acyl cholesterol acyltransferase |
| AGE | Advanced glycation end products |
| AGE-albumin | Advanced glycated albumin |
| AMG | Aminoguanidine |
| Apo A-I | Apolipoprotein A-I |
| BF | Benfotiamine |
| CML | Carboxymethyllysine |
| DHE | Dihydroethidium |
| DM | Diabetes mellitus |
| DPI | Diphenyleneiodonium |
| FAFA | Fatty acid free albumin |
| HDL | High density lipoprotein |
| LDL | Low density lipoprotein |
| RAGE | Advanced glycation end products receptor |
| ROS | Reactive oxygen species |

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Introduction

Advanced glycation end products (AGE) are prevalent in hyperglycemia, and together with the enhanced glucose

metabolism along the hexosamine, polyol and diacylglycerol-PKC pathways, set up a link between oxidative stress and the development of long-term complications in diabetes mellitus (DM) [1, 2]. By reacting with oxoaldehydes, lipoproteins and albumin can be modified by AGE especially in the postprandial phase in DM as well as in inflammation and in renal chronic disease [3–5]. Atherosclerosis that prevails in DM is associated with serum levels of AGE [6].

Advanced glycated albumin (AGE-albumin) impairs the ATP binding cassette transporter A-1 (ABCA-1) and ATP binding cassette transporter G-1 (ABCG-1) expressions and consequently also the apo A-I and HDL-mediated cholesterol efflux [7–9]. ABCA-1 plays a major role in the first step of the reverse cholesterol transport system by exporting the excess of cell cholesterol to lipid poor apo A-I which ultimately is converted into mature HDL that delivers cholesterol to the liver for bile excretion and to steroidogenic organs [10]. Methylglyoxal, glyoxal and 3-deoxyglucosone can mediate the generation of AGE, although previous reports have demonstrated that glycolaldehyde plays a major role in protein derivatization and in cell lipid efflux damage [7, 8].

Intracellular production of ROS elicited by AGE has mainly been ascribed to NADPH oxidase and to the mitochondrial respiratory chain, leading to the production of vascular adhesion molecules and inflammatory mediators, as well as to the expression of the advanced glycation end products receptor (RAGE) [11–13]. Together, these events are related to endothelial, retinal, renal and neuronal injury in DM [1, 11, 14–16].

To date, there are no data linking intracellular redox imbalance with changes in cholesterol homeostasis elicited by alterations in the expression of ABCA-1. We then investigated whether the AGE-induced ROS generation in macrophages relates to the reduction in ABCA-1 transporter level as well as the role played by anti-AGE and antioxidant compounds, such as aminoguanidine and benfotiamine.

Materials and Methods

This study was approved by The Ethical Committee of the Hospital das Clínicas of the Faculty of Medical Sciences of the University of São Paulo, Brazil (#0807/07). All protocols with humans conformed to the Declaration of Helsinki and animal experiments were done according to the US National Institutes of Health guidelines.

Preparation of AGE-Albumin

AGE-albumin was prepared by incubating bovine fatty acid free albumin (FAFA, 40 mg/mL) with 10 mM

glycolaldehyde (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-albumin) was incubated with PBS only. After extensive dialysis, samples were sterilized. Samples contained <50 pg endotoxin/mL as determined by the chromogenic *Limulus amoebocyte* assay (Cape Cod, Falmouth, MA).

Cell Culture

J774 macrophages were cultured in RPMI 1640 containing 10% fetal calf serum (FCS), 1% penicillin–streptomycin and 4 mM L-glutamine and maintained in a 5% CO₂ incubator at 37 °C.

After reaching confluence, cells were treated for different intervals of time with C or AGE-albumin alone (2 mg/mL DMEM) or in the presence of aminoguanidine (AMG) or benfotiamine (BF) to evaluate ABCA-1 protein content, ROS generation and mitochondrial respiration. In all cell culture experiments, cell viability controlled by exclusion with trypan blue was superior to 98%. Apoptosis, assessed by the number of annexin V-FITC positive cells, did not change after exposure to AGE-albumin (data not shown).

Assessment of Carbonyl Content in Macrophages

Lysates from C or AGE-albumin-treated cells were incubated with 10 mM of dinitrophenylhydrazine in 2.5 M HCl for 1 h at room temperature. The reaction was blocked by the addition of 20% trichloroacetic acid (TCA). The pellets were washed twice with absolute ethanol/ethyl acetate (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.

Immunoblot

J774 macrophages were scraped into Tris buffer saline containing protease inhibitors. Equal amounts of cell protein were applied to a polyacrylamide gel (SDS-PAGE) and CD-36 and RAGE expressions determined using anti-RAGE and anti-CD-36 antibodies (Fitzgerald Industries International, Inc., Concord, MA). The difference between the bands was analyzed in pixels, using the JX-330 Color Image Scanner (Sharp®) and ImageMaster software (Pharmacia Biotech). The results are expressed as arbitrary units, analyzing AGE-albumin versus C-albumin. Ponceau staining was utilized to assure equal protein loading.

Assessment of Carboxymethyllysine Adducts in Control and AGE-Albumin

Equal amounts of C and AGE-albumin were loaded into 10% SDS-PAGE and the content of carboxymethyllysine was determined with an anti-CML antibody (Novus Biologicals, Inc., Littleton, CO). Ponceau staining was also utilized to assure equal protein loading.

Agarose Gel Electrophoresis

Ten micrograms of C and AGE-albumin were subjected to 1% agarose gel electrophoresis and the samples' mobility was determined after 1 h run and staining with Coomassie blue.

Determination of ABCA-1 Protein Level by Flow Cytometry

ABCA-1 content by flow cytometry was analyzed due to the difficulty in attaining a good signal in SDS-PAGE. For this experiment, J774 macrophages previously treated for 24 h with 8-Br cyclic AMP (0.5 mM), were incubated for different intervals of time with C or AGE-albumin in the presence or absence of AMG (5 and 10 mM) or BF (350 μ M). After that, a 10^6 cells concentration was fixed in paraformaldehyde (PFA—4%), incubated with anti-ABCA-1 antibody (Novus Biologicals, Inc., Littleton, CO – 1:250 dilution) for 1 h at room temperature and incubated afterwards with 4 μ g/mL Alexa Fluor 488 antibody (Invitrogen, USA). Cellular fluorescence intensity was evaluated by flow cytometry using a FACS Calibur and the Cellquest software (B. D., San Jose, CA). Under all of the conditions, we corrected for basal cellular fluorescence (without Alexa Fluor 488 antibody).

Reactive Oxygen Species Production

After treatment with C or AGE-albumin, J774 macrophages (1×10^6) were incubated with 5 μ M dihydroethidium (DHE) probe (Molecular Probes, OR) for 45 min. ROS production was evaluated by flow cytometry using a FACS Calibur and the Cellquest software (B.D., San Jose, CA). In order to evaluate the contribution of the NADPH oxidase system to the AGE-induced oxidative stress, cells were incubated for 50 min with DHE in the absence or in the presence of the 10 μ M NADPH oxidase inhibitor, diphenylene iodonium (DPI) (Sigma) and the ROS production was determined.

High-Resolution Respirometry

Respiration rates were recorded with the high-resolution OROBOROS oxygraph (Paar, Innsbruck, Austria) in C and

AGE-albumin-treated macrophages (24 h treatment). The culture medium was maintained in an open chamber at 37 °C (temperature controlled by Pelletier effect), homogenized with a magnetic stirrer (600 rpm), and left to equilibrate with the atmosphere before signal calibration. Oxygen concentration was computed from the partial pressure measured by the electrode. During respiration measurements, each chamber (2-mL working volume) was closed by a piston to prevent oxygen exchange with the atmosphere. Data were recorded at 0.4 s intervals and analyzed by the Datlab Acquisition and Analysis software (Paar). As indicated, 2 μ g/mL of an ATPase inhibitor (oligomycin) was added.

14 C- Acetate Incorporation into Cholesterol and Cholesterol Esterification Rates

After 4 h of treatment of the J774 macrophages with 0.25 mM glycolaldehyde (GAD), the cells were washed twice with PBS containing 1 mg/mL FAFA and incubated for 5 h with 8 μ Ci/mL ($2\text{-}^{14}\text{C}$) sodium acetate (GE-Amersham Biosciences, USA) in DMEM containing 1 mg/mL of FAFA. Cell lipids were extracted with a hexane/isopropanol (3:2, v:v) mixture. The solvent was evaporated under N_2 flow and, after dilution in chloroform, samples were spotted on silica G plates by an automated system (Analtech Inc., USA) for thin layer chromatography development. Lipid standards (Sigma) were utilized and the unesterified and esterified cholesterol bands were isolated in order to measure the associated radioactivity. Cholesterol biosynthesis was expressed as total counts per microgram of cell protein.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 4.0 software (GraphPad Prism, Inc., San Diego, CA). One-way analysis of variance (ANOVA) and the Newman Keuls post test were utilized to compare differences among groups. Summary data are reported as mean values \pm standard error. A p -value <0.05 was considered statistically significant.

Results

Glycolaldehyde-modified albumin was characterized by the determination of CML content. As shown in Fig. 1a CML was greatly superior in AGE-albumin as compared to C-albumin. In addition, the mobility of AGE-albumin was faster than that of C-albumin on agarose gel electrophoresis (Fig. 1b).

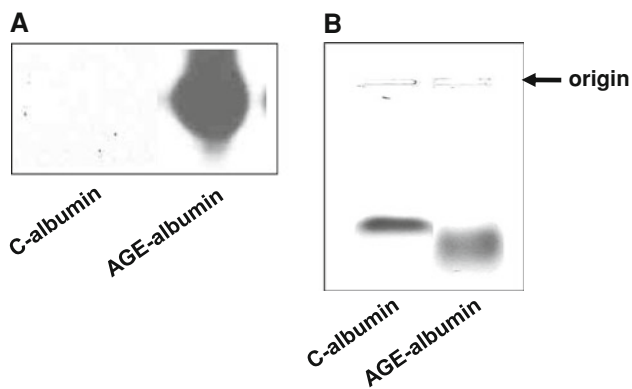


Fig. 1 Carboxymethyllysine content and electrophoretic mobility is increased in AGE-albumin. **a** CML content in albumin modified by 10 mM glycolaldehyde (AGE-albumin) and control albumin (C-albumin) was determined by immunoblot using an anti-CML antibody, **b** electrophoresis mobility was determined in 1% agarose gel and staining with Coomassie blue. *C-albumin* control albumin, *AGE-albumin* advanced glycated albumin

Further, we addressed ROS generation in AGE-albumin-treated cells and its association with the reduction in the ABCA-1 protein level. In macrophages, AGE-albumin induced a higher content of carbonyl derivatives in comparison to C-albumin (Fig. 2a). ROS generation was increased by 24% in J774 macrophages treated with AGE-albumin in comparison to C-albumin and was prevented by incubating cells with a flavoprotein inhibitor, DPI, which is consistent with the role of NADPH oxidase in ROS production in these cells (Fig. 2b).

In an attempt to investigate the oxygen flow along the mitochondrial respiratory chain, C- and AGE-albumin-treated macrophages were evaluated under two different conditions: basal and following the addition of oligomycin. In comparison to C-albumin-treated cells, oxygen flow (pmol/s/mL) was significantly reduced (46%) in cells treated with AGE-albumin (Fig. 2c). In comparison to the basal condition, in the presence of oligomycin, oxygen

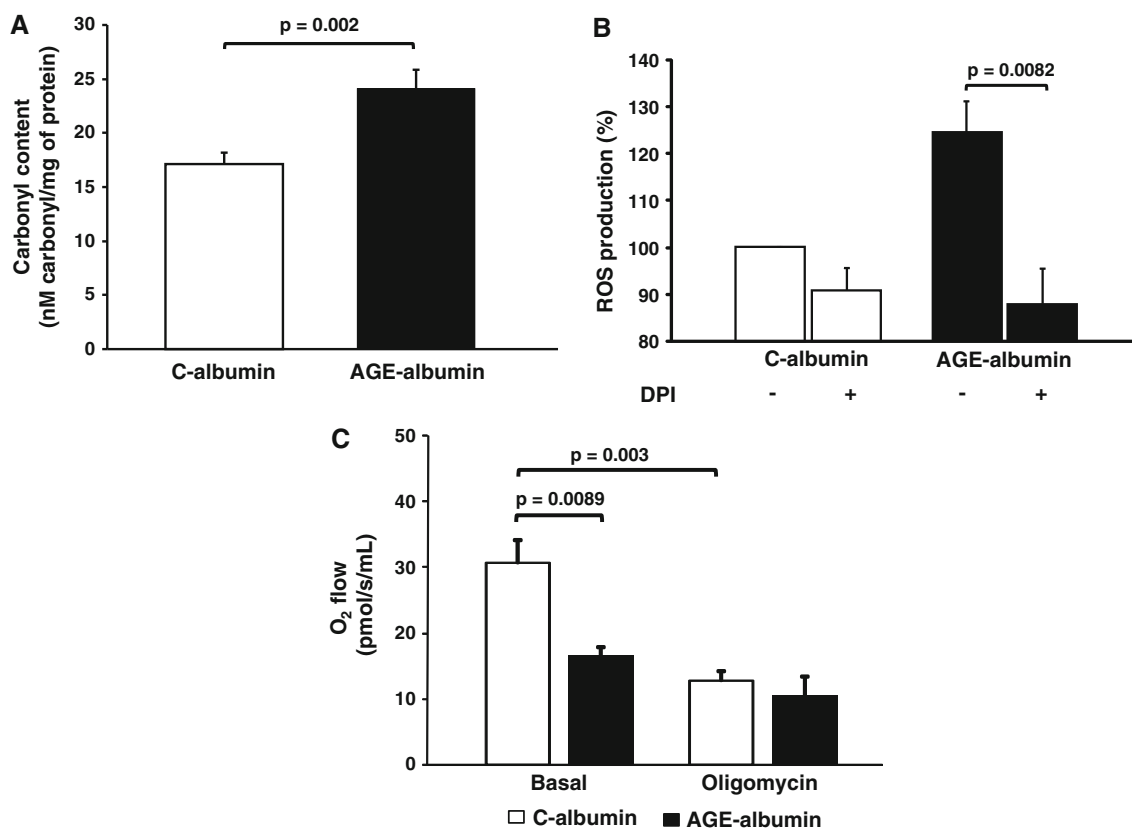


Fig. 2 AGE-albumin enhances carbonyl content and induces ROS production in macrophages: contribution of NADPH oxidase and mitochondria. **a** Carbonyl content was determined in macrophages treated for 24 h with C or AGE-albumin by spectrophotometry, **b** ROS production (mean \pm SE) was determined by flow cytometry in J774 cells treated for 24 h with C or AGE-albumin with the NADPH oxidase inhibitor, DPI ($n = 8$), **c** oxygen consumption rates

(mean \pm SE) were determined in J774 cells after treatment with C or AGE-albumin under basal conditions and after addition of oligomycin (inhibitor of ATP synthase) as indicated ($n = 4$). Results are representative of at least four independent experiments. *C-albumin* control albumin, *AGE-albumin* advanced glycated albumin, *DPI* diphenylene iodonium

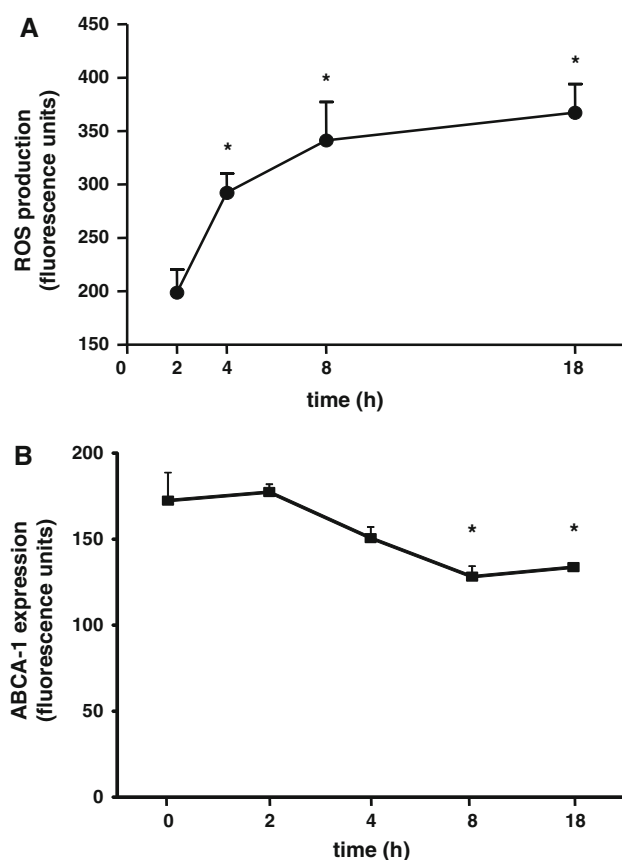


Fig. 3 AGE-albumin increases ROS and decreases ABCA-1 in a time dependent manner. J774 macrophages were treated for different periods of time with C or AGE-albumin. **a** ROS production (mean \pm SE) was determined by flow cytometry (* $p < 0.05$ compared to 2 h incubation; $n = 6$, two independent experiments), **b** ABCA-1 protein level was determined by flow cytometry (* $p < 0.05$ compared to time 0)

flow decreased in C-albumin-treated cells only, with no changes in the cells treated with AGE-albumin.

ROS production was significantly increased along time in AGE-albumin treated macrophages and approached to plateau at the 18th hour (Fig. 3a). ROS generation was accompanied by significant reductions in ABCA-1 protein level after 8 h and 18 h of treatment (Fig. 3b). The association between ROS generation and ABCA-1 reduction was further confirmed by incubating cells with AMG or BF, compounds that function as antioxidants and AGE inhibitors. ROS generation observed in macrophages treated with AGE-albumin alone was reduced 45% by cell incubation with AGE-albumin plus 10 mM AMG (Fig. 4a, inner panel). At this concentration AMG was able to restore the ABCA-1 protein level in macrophages treated with AGE-albumin (Fig. 4a). ROS generation was less inhibited by BF (26%) in cells treated with AGE-albumin (Fig. 4b, inner panel) and, accordingly, BF was not able to restore the ABCA-1 level (Fig. 4b).

It is well-known that reactive oxoaldehydes can be directly formed in the arterial wall. We then measured cholesterol biosynthesis and esterification as well as ROS generation in macrophages directly exposed to glycolaldehyde. Cells subjected to glycoxidation showed a 42% reduction in cholesterol biosynthesis (dpm/ng of cell protein \pm SD) in comparison to control cells (Fig. 5a). In contrast, the cholesterol esterification rate was increased 2.3 fold in macrophages incubated with GAD as compared to control cells (Fig. 5b). In addition, ROS generation was 28% higher in GAD-treated macrophages in comparison to control cells (Fig. 5b, inner panel). These results are in agreement with the observed impairment in the cholesterol efflux elicited by intracellular glycoxidation that subsequently leads to reductions in ABCA-1 protein and intracellular lipid accumulation [8].

Compared to C-albumin, the expression of RAGE and CD-36 were increased by 60 and 35%, respectively, after treatment with AGE-albumin (data not shown).

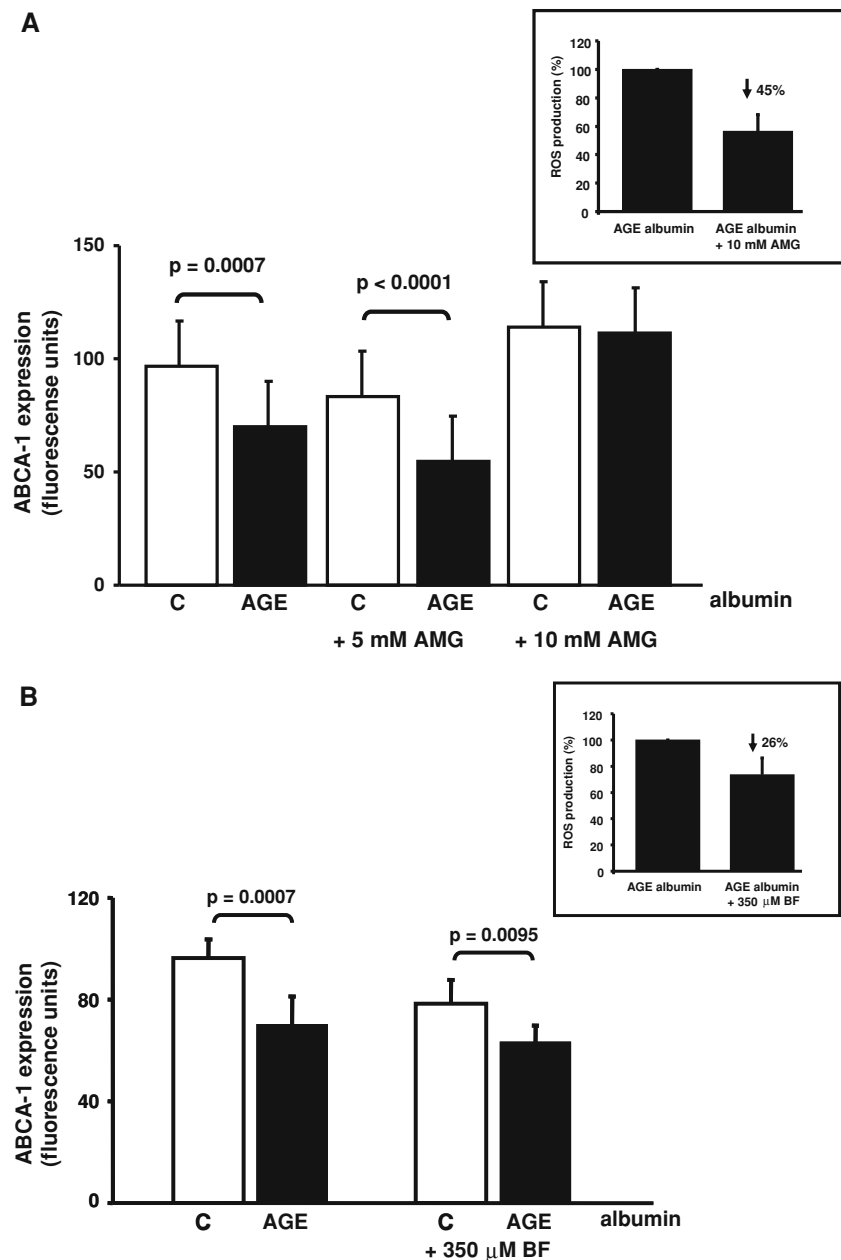
Discussion

Glycated albumin constitutes the overwhelming majority of circulating glycated proteins. AGE-modified albumin is prevalent in DM and disturbs macrophage cholesterol efflux [7]. In the present study, we analyzed the role of control and AGE-albumin on macrophage oxidative stress and its relationship to disturbances in cell cholesterol homeostasis.

Reactive oxygen species were significantly increased in cells treated with AGE-albumin. Our results indicate a combined role of the NADPH oxidase and the mitochondrial respiratory chain in the oxidative stress, although a lower magnitude of ROS levels was detected in our study when compared to data from other authors dealing with different cell types [11, 17].

A diminished mitochondrial respiration contributes to the generation of ROS by mitochondria in consequence of the high reduction state of respiratory chain components [18]. The bulk of mitochondrial ROS generation occurs at the electron transport chain, as a byproduct of respiration. Enhanced electron transport that reflects increased mitochondrial respiratory rates may prevent ROS formation by decreasing oxygen tension in the mitochondrial microenvironment. On the other hand, conditions that lead to lower respiratory rates are frequently accompanied by enhanced ROS release [19]. Then, the contribution of mitochondria to ROS generation and oxidative stress in macrophages exposed to AGE-albumin can be confirmed by the reduction in mitochondrial oxygen flux, increasing both cellular oxygen tension and the reduced state of monoelectronic donors of the respiratory chain. Oligomycin did not show

Fig. 4 Aminoguanidine but not benfotiamine greatly diminishes ROS production in AGE-albumin-treated macrophages and restores ABCA-1 protein level. J774 macrophages were treated with AGE-albumin for 8 h in the presence or absence of **a** 5 and 10 mM aminoguanidine (AMG) or **b** 350 μ M of benfotiamine (BF). ROS production (*inner panels*) and ABCA-1 protein levels were determined by flow cytometry (mean \pm SE; $n = 4$)



an effect in AGE-albumin-treated cells likely because of the already low oxygen consumption rate observed under basal conditions.

The increase in ROS production in macrophages treated with AGE-albumin was accompanied by a severe reduction in ABCA-1 expression. Moreover, the ABCA-1 protein level reduction was consistently observed in several independent experiments. Elevations in ROS were prevented by 10 mM of AMG, a substance that has been shown in identical concentrations to prevent disturbances in cholesterol efflux elicited by AGE by diminishing CML formation [7]. Since no changes in ABCA-1 mRNA levels have been ascribed to cell glycoxidation [8], it is

reasonable to consider that post-transcriptional degradation of ABCA-1 could contribute to its reduction in the AGE-albumin-treated macrophages [20]. In fact, ABCA-1 gene transcription is unaltered by glycoxidative stress [8, 9].

Lipid accumulation can also be worsened in the presence of AGE-albumin due to the enhanced expression of scavenger receptors, LOX-1, CD-36 and RAGE [21, 22] and also by the increased rate of cholesterol esterification by ACAT shown by us. In other words, increased cholesterol flow into cells aggravates atherosclerosis ascribed to an AGE-mediated impairment in the reverse cholesterol transport.

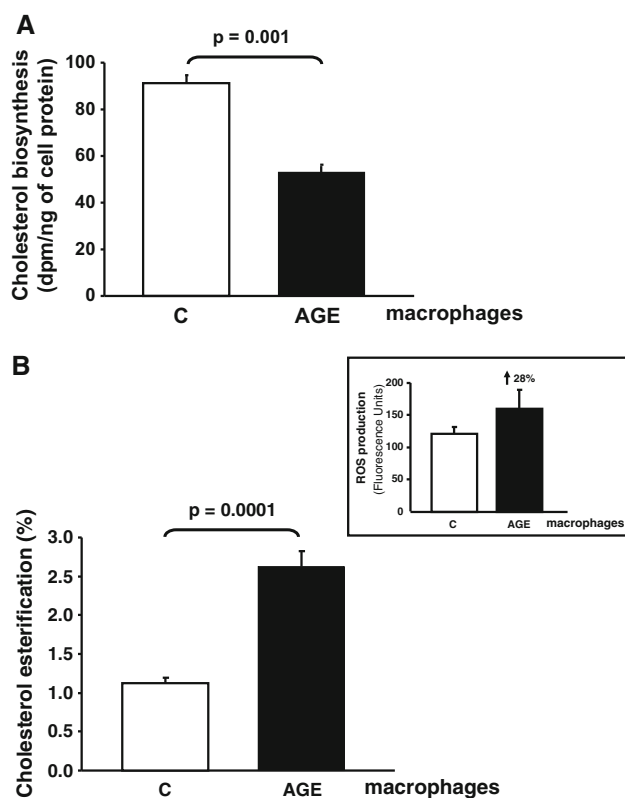


Fig. 5 Glycoxidation reduces cholesterol biosynthesis and enhances cholesterol esterification in macrophages. J774 macrophages were treated with 0.25 mM glycolaldehyde. Control (C) cells were incubated with DMEM only. Cells were treated for 5 h with 8 $\mu\text{Ci}/\text{mL}$ ($2\text{-}^{14}\text{C}$) sodium acetate in order to determine **a** the cholesterol biosynthesis and **b** the percentage of cholesterol esterification (mean \pm SD; $n = 6$). ROS production (*inner panel*) was determined by flow cytometry (mean \pm SE; $n = 5$)

AGE-albumin interacts with RAGE and as consequence of intracellular signal transduction ROS is produced leading to activation of NF- κB that ultimately leads to RAGE expression [22, 23]. Therefore, AGE-albumin is able to generate and propagate oxidative stress in cells, which was also reflected by the increase in cellular carbonyl content.

Our findings are strengthened by the demonstration that serum albumin isolated from uncontrolled type 1 and 2 DM patients reduces the apo A-I and HDL-mediated cholesterol efflux, inducing macrophage lipid accumulation in a similar pattern to that observed in macrophages treated with AGE-albumin (the same in-vitro modified albumin utilized in the present study) [24]. In addition, albumin isolated from DM patients presented a higher amount of CML as compared to albumin isolated from healthy individuals [24]. AGE-albumin utilized in the present study also showed a large amount of CML in comparison to C albumin, although we cannot exclude that other AGE adducts may be present. Besides, taking into account that oxoaldehydes can be generated in the arterial wall, produced in

non-hyperglycemic conditions and are also acquired from food sources, the contribution of AGE-albumin to disturbances in cholesterol homeostasis may be important in a broad spectrum of metabolic conditions. From this point of view, cell lipid accumulation can be minimized by drugs that reduce ROS generation induced by AGE, which should be addressed in future experiments.

Conclusion

AGE-albumin disturbs macrophage lipid homeostasis by increasing the expression of scavenger receptors and diminishing ABCA-1. The reduction in the ABCA-1 protein level elicited by AGE-albumin in macrophages is associated with the oxidative stress via NADPH oxidase system and mitochondria. Anti-AGE and antioxidant compounds known to mitigate greatly the ROS production might hinder the disturbances in the reverse cholesterol transport thus contributing to the prevention of atherosclerosis in DM and other conditions of carbonyl stress such as inflammation and chronic kidney disease.

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Conflict of interest We declare that no conflict of interest was involved in this study.

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