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Differential effects of α -tocopherol and N-acetyl-cysteine on advanced glycation end product-induced oxidative damage and neurite degeneration in SH-SY5Y cells

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ABSTRACT

Advanced glycation end products (AGEs) result from non-enzymatic glycation of proteins and cause cellular oxidative stress in a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent manner. Due to these effects, AGEs are implicated as a causal factor in diabetic complications. Several antioxidants, including vitamin E, improve cell viability and diminish markers of oxidative damage in cells exposed to AGEs. However, vitamin E has been studied in cell culture systems with primary focus on apoptosis and lipid peroxidation, while its influences on AGE-induced protein and DNA oxidation, intracellular antioxidant status and cell morphology remain largely unknown. Here, we verify the suppression of AGE-induced cell death and lipid peroxidation by 200 μ M α -tocopherol in SH-SY5Y cells. We report the partial inhibition of DNA oxidation and a decrease in protein carbonyl formation by α -tocopherol with no effects on intracellular GSH concentrations. We observed that 2 mM N-acetyl cysteine (NAC) also had a suppressive effect on DNA and protein oxidation, but unlike α -tocopherol, it caused a marked increase in intracellular GSH. Finally, we compared the ability of both antioxidants to maintain neurites in SH-SY5Y cells and found that α -tocopherol had no effect on neurite loss due to AGEs, while NAC fully maintained cell morphology. Thus, while α -tocopherol suppressed AGE-induced macromolecule damage, it was ineffective against neurite degeneration. These results may implicate thiol oxidation and maintenance as a major regulator of neurite degeneration in this model.

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1. Introduction

Diabetes is a prevalent disease hallmarked by dysregulation of blood glucose concentrations and subsequent damage to tissues like the eyes, nerves, and kidneys. Cellular damage is in large part mediated by an enhanced generation of reactive oxygen species (ROS) [1]. When concentrations of ROS exceed that which can be effectively neutralized by endogenous antioxidant defenses, a state of oxidative stress arises. The mechanisms by which hyperglycemia causes intracellular stress include an increase in mitochondria-generated superoxide radicals [2,3]. High glucose further potentiates oxidative damage in diabetes by compromising cellular redox status via depletion of NADPH and thus GSH through the polyol pathway [2].

Oxidative damage is also created from the products of nonenzymatic protein glycation termed as advanced glycation end products (AGEs). AGEs induce a range of cellular effects, mostly through protein cross-link formation and through binding to the receptor for advanced glycation end products (RAGE) [4]. RAGE is a transmembrane

receptor of the immunoglobulin superfamily that has several potential ligands, each of which can induce different downstream effects ranging from neurite outgrowth and cell survival [5,6] to cell proliferation to apoptosis, depending on the specific ligand and the RAGE domain with which it interacts [7]. In diabetes, the binding of AGEs to RAGE initiates many of the downstream effects observed in models of the disease, including the activations of NADPH oxidase, p21, RAS, p38 MAPK, and NF- κ B [8].

Experimental results suggest that AGEs have a causative role in several outcomes of tissue damage in diabetes, including progression of diabetes-associated nephropathy [9,10], retinal degeneration [11,12], and neuropathy [13,14]. In vitro studies and cell culture models of diabetic neuropathy using dorsal root ganglia neurons [15], human NT2 neurons [16], and SH-SY5Y cells [17] confirm that AGEs induce superoxide radical generation in neuronal-derived cells through activation of NADPH oxidase in a PKC δ -dependent manner [16,17]. These actions result in an accumulation of oxidative damage markers like malondialdehyde (MDA) and outcomes like RAGE upregulation, DNA fragmentation, and apoptosis [15,18]. Antioxidants such as EGCG [18], ginkgo biloba extract [16], α -lipoic acid [15,19], and N-acetyl-cysteine (NAC) [19] suppress AGE-induced macromolecule oxidation and its downstream effects.

Vitamin E is a potent chain-breaking antioxidant that resides in cellular membranes and maintains their integrity through inhibition of lipid peroxidation. Vitamin E also slows protein glycation in vitro

Abbreviations: NAC, N-acetyl cysteine; AGEs, advanced glycation end products; MDA, malondialdehyde; PUFA, polyunsaturated fatty acids; RA, retinoic acid; α -T, α -tocopherol

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by abating the formation of the lipid peroxidation product MDA [20], a contributor to AGE synthesis [21]. However, the relevance of these findings to human diabetics is unclear, as some evidence supports no effect of vitamin E supplementation on protein glycation *in vivo* [22]. Despite this conflict, the data suggest that a protective role exists for vitamin E against AGEs and their downstream damage. Vitamin E partially prevents endothelial cell proliferation due to AGE-BSA [23] and prevents diabetes-associated PKC- β 2 upregulation, resulting in improved mesenteric artery vasodilation [22]. Vitamin E suppresses AGE-induced NF- κ B activation [24] and cytokine expression in mesangial cells [24,25]. In human NT2 neurons, vitamin E decreases ROS production, MDA generation, RAGE upregulation, and apoptosis caused by incubation with ribose-glycated FBS [16].

The role of vitamin E has been evaluated most frequently in AGE-induced changes in cell proliferation, apoptosis, ROS formation, and in the formation of lipid peroxidation markers like MDA. However, the role of vitamin E is less clear with regard to changes in intracellular antioxidant status, cell morphology, and the oxidation of protein and DNA that result from AGE exposure. Differences in these effects compared to those of a prototypical thiol antioxidant would provide insight into the mechanisms of protection against AGEs. Contrasting effects of these antioxidants on cellular damage may highlight the optimum chemical properties for protection against AGEs. In this study, we investigated the effects of vitamin E and NAC on AGE-induced oxidative damage to determine their separate protective capabilities in SH-SY5Y cells.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM), Ham's F12 medium, penicillin-streptomycin, and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Fatty acid-free bovine serum albumin (BSA) and glycolaldehyde were obtained from Sigma (St. Louis, MO). SH-SY5Y cells were obtained from ATCC (Manassas, VA).

2.2. Preparation of AGE-BSA

AGE-BSA was prepared as previously reported [26] with modifications. 5 mg/ml fatty acid-free BSA was incubated with 33 mM glycolaldehyde in PBS for 20 h at 37 °C. Control BSA was incubated under the same conditions but without glycolaldehyde. We found that excessive cross-linking occurred when BSA was incubated with glycolaldehyde for any longer than 36 h, resulting in a non-cytotoxic protein product (data not shown). BSA and AGE-BSA were dialyzed against PBS for 3 days at 4 °C and sterilized by vacuum filtration through a polyethersulfone filter (VWR, West Chester, PA). AGE-modification was confirmed by an approximately 40-fold increase in absorbance at 340 nm, a 5.2-fold increase in fluorescence at 335 nm (excitation) and 420 nm (emission), and further by western blot with an anti-AGE antibody (Millipore) (data not shown). Protein concentrations were verified using a Pierce BCA protein assay kit. Aliquots were frozen at -20 °C.

2.3. SH-SY5Y cell culture and treatments

SH-SY5Y cells were grown and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were maintained at 37 °C and in 7% CO₂. To encourage neurite outgrowth, SH-SY5Y cells were treated with 10 μ M RA in DMEM:F12 (1:1) supplemented with 1% FBS for 3–5 days. Cells were then exposed to 1–1.25 mg/ml BSA, AGE-BSA, or an equivalent dilution of PBS for 72 h. Cells receiving α -tocopherol (diluted in ethanol) or NAC (diluted in PBS) treatments were pretreated for 24 h before the addition of AGE-BSA, BSA, or PBS. The antioxidant treatments were then refreshed with

AGE-BSA, BSA, or PBS. In experiments directly comparing AGE-BSA \pm α -tocopherol or NAC, all treatments included 0.1% ethanol and PBS in an effort to control for vehicle effects.

2.4. Effect of AGE-BSA on cell viability

The viability of SH-SY5Y cells following treatments was measured using the MTT assay [27]. MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide, Sigma Aldrich) is a yellow compound that when reduced by functioning mitochondria, produces purple formazan crystals that can be measured spectrophotometrically. MTT was dissolved in PBS for a final concentration of 5 mg/ml and then diluted in cell culture media for a final concentration of 0.5 mg/ml. Cells were incubated with MTT for 3 h, after which the resulting purple formazan crystals were solubilized by 20% SDS in 50% dimethylformamide (DMF). The absorbance of each sample was then read at 570 nm as a test wavelength and 690 nm as a reference wavelength. Data are expressed as percent of PBS controls.

2.5. Effect of AGE-BSA on cell morphology

SH-SY5Y cells were plated in a 6-well plate and treated with PBS, BSA, or AGE-BSA with and without NAC and α -tocopherol. Changes in cell morphology were documented using differential interference contrast (DIC) microscopy on an Olympus 1X70 inverted microscope with an attached Olympus DP70 digital camera system. Separate images were recorded for 10, 20, and 40 \times objectives. At least 4 random fields of observation were recorded per treatment. Images taken at 40 \times were then opened using the Image J program, and neurites were quantified in a blind manner for at least 4 images from 3 independent samples. Data are expressed as number of neurites per cell.

2.6. Vitamin E measurement

To quantify cellular vitamin E status, cells were washed with PBS and scraped into PBS: ethanol + 1 mg/ml BHT (1:1) with δ -tocopherol as an internal standard. Tocopherols were extracted twice with hexane. The hexane layers were combined in a new tube and evaporated to dryness, and tocopherols were resuspended in 250 μ l ethanol + 1 mg/ml BHT. Tocopherol content was analyzed by high performance liquid chromatography (HPLC) with electrochemical detection using an ESA Coullarray system (Chelmsford, MA). A flow rate of 1 ml/min was used with 50% mobile phase A (methanol:isopropanol:ammonium acetate buffer 1.0 M, pH 4.36, 78:20:2) and 50% mobile phase B (methanol:isopropanol:ammonium acetate buffer 1.0 M, pH 4.36, 59:40:1). The α -tocopherol eluted at approximately 7.6 min and had a predominant peak at 200 mV, and δ -tocopherol eluted at 6.1 min with its predominant peak at 600 mV.

2.7. GSH measurement

To measure intracellular GSH concentrations, cells were scraped into PBS, centrifuged, and the pellet resuspended in 5% metaphosphoric acid with 100 μ M EDTA. Cells were lysed by several freeze/thaw cycles. Protein was precipitated by centrifugation and later quantified using the Pierce BCA protein assay kit. The supernatant was filtered and analyzed by HPLC coupled with electrochemical detection. An ESA Coullarray system was used with 5% mobile phase B (20% methanol, 30% mobile phase A, 50% acetonitrile) in mobile phase A (50 mM sodium phosphate, pH 3). GSH eluted at approximately 4.6 min, with the predominant peak at 900 mV.

2.8. Lipid peroxidation

To measure total MDA, 6.0 \times 10⁶ SH-SY5Y cells that had been exposed to treatments with and without antioxidants for 48 h were

washed twice with ice-cold PBS and scraped into fresh PBS. BHT in ethanol was added to cells to prevent artifactual generation of lipid peroxidation products. Cells were lysed by freeze/thaw cycles and underwent acid hydrolysis by HCl at 60 °C for 80 min to free conjugated MDA. 10% trichloroacetic acid was then added and proteins were precipitated and quantified using Pierce BCA total protein assay. The supernatant was combined at a 1:1 ratio with 0.67% thiobarbituric acid and incubated at 90 °C for 30 min. After cooling, aliquots were measured for fluorescence at 530 nm (excitation) and 550 nm (emission) on a SpectraMax Gemini XS fluorescence plate reader (Molecular Devices, Sunnyvale, CA). 1,1,3,3-tetramethoxypropane was used as a standard.

Under conditions of oxidative stress, percentages of polyunsaturated fatty acids (PUFAs) in tissue often decline due to peroxidation. To measure changes in PUFA concentrations, extraction of total lipids was performed using a slightly modified method of Muller et al. [28]. SH-SY5Y cells were treated appropriately for 72 h and then washed twice with ice-cold PBS. Cells were scraped into PBS, centrifuged, and the cell pellet was resuspended in DDI water. Total cell lipids were extracted three times using chloroform/methanol (2/1, v/v + 0.001% BHT). Organic phases were combined in another tube and evaporated to dryness under nitrogen. Lipids were methylated in two steps. First, lipids were transmethylated using 5% potassium methylate solution in methanol for 30 min at 60 °C. Then, 1 N methanolic sulfuric acid was added and after vortexing, the tubes were incubated at 60 °C for 15 min. Saturated NaCl solution in water and n-hexane were added and the tubes were vortexed to bring the fatty acid methyl esters into the hexane phase. After centrifugation, the hexane layer was transferred to a new tube and the hexane extraction was repeated. Hexane layers were combined and evaporated to dryness under nitrogen. Fatty acid methyl esters were resuspended in 500 μ l n-hexane and analyzed on a Varian 3900 Gas Chromatograph.

2.9. Protein carbonyls

SH-SY5Y cells were treated for 72 h with PBS, BSA, or AGE-BSA \pm antioxidants. The cells were then washed with ice-cold PBS and scraped into RIPA buffer containing DTT. Insoluble protein fractions were separated by centrifugation and the protein concentration of the soluble portion was quantified using the Pierce BCA protein assay kit. Each sample was analyzed for protein carbonyl content using the Oxyblot Protein Oxidation Detection Kit (Chemicon, Temecula, CA) and subsequent western blot. Blots were scanned and analyzed by UN-SCAN-IT gel 6.1 software (Silk Scientific, Inc, Orem, Utah), normalized to β -actin expression, and expressed as percent of PBS control.

2.10. DNA oxidation

SH-SY5Y cells were plated on collagen-coated cover slips (BD Biosciences, San Jose, CA) and treated for 48 h with PBS, BSA, and AGE-BSA \pm antioxidants. Cells were then washed several times with PBS and fixed in 0.5% glutaraldehyde in PBS for 10 min at room temperature. Cells were rinsed with PBS 3 times and permeabilized with 3 washes in 0.5% Triton X-100 in PBS for 5 min each. Cells were once again washed 3 times in PBS and incubated with a primary antibody raised against 8-hydroxy-2'-deoxyguanosine (Trevigen, Gaithersburg, MD) and then a secondary antibody tagged with Alexa Fluor 488 (Molecular Probes, Invitrogen, Carlsbad, CA) according to instructions supplied by Trevigen. Cover slips were mounted with Prolong Gold antifade reagent with DAPI (Invitrogen) and allowed to cure overnight. Cover slips were sealed the next morning and kept at room temperature. Slides were analyzed by confocal microscopy on a Zeiss LSM 710 microscope (Zeiss, Germany) at 40 \times with Immersol W water-immersion fluid (Zeiss). During analysis, random nuclei were selected blindly, based solely on DAPI fluorescence. These regions were encircled and the mean Alexa Fluor 488 fluorescent

intensities within these boundaries were then determined. Approximately 150 cells in 3 independent experiments were analyzed per treatment and expressed as a percent of the fluorescent intensity of PBS-treated cells.

2.11. Statistical analysis

Results are expressed as mean \pm S.D, except for neurite number, which is expressed as mean \pm SEM. All experiments were repeated independently three times, or more where indicated. Statistical significance was determined by ANOVA, except for GSH analysis, which was analyzed by Student's *t* test for comparison within treatment groups. Post hoc analysis was performed using Tukey's tests for multiple comparisons of groups.

3. Results

3.1. Effect of AGE-BSA on cell viability

The goal of this study was to characterize the functions of the lipid peroxyl radical scavenger vitamin E in influencing AGE-induced oxidative damage, antioxidant status, and neurite degeneration; the antioxidant NAC served as the positive control. Treatment of SH-SY5Y cells with AGE-BSA induced a significant dose-dependent decrease in cell viability, whereas control BSA had no negative effect (Fig. 1A). To verify previous results that antioxidants – including vitamin E – partially suppress AGE-induced declines in cell viability, we incubated SH-SY5Y cells with 1.0 mg/ml AGE-BSA or control BSA or an equivalent dilution of PBS for 72 h with and without 2.0 mM NAC or 200 μ M α -tocopherol. Our preliminary studies indicated that these concentrations were appropriate, as the protective effects of α -tocopherol and NAC reached plateaus at 200 μ M and 2 mM respectively (data not shown). AGE-BSA decreased cell viability approximately 50% over a 72-hour period. Co-administration of NAC (Fig. 1B) or α -tocopherol (Fig. 1C) improved viability, approximately 40% and 20% respectively. NAC provided considerable protection against AGE-BSA, as there was no significant difference between cells treated with AGE-BSA + NAC and cells treated with control BSA. However, cellular protection provided by α -tocopherol was not complete, as there was significant difference between AGE + α -tocopherol treated cells and BSA-treated cells. Neither NAC nor α -tocopherol had any influence on the viability of PBS or BSA controls.

3.2. Effect of AGE-BSA on cell morphology

RA induces the growth and maintenance of neurites in SH-SY5Y cells. Because oxidative conditions can cause degeneration of neurites, we tested the effects of AGE-BSA on cell morphology as well as the influences that α -tocopherol and NAC have on this process. To accomplish this, SH-SY5Y cells were treated and examined by DIC microscopy. Treatment with AGE-BSA reduced the number of cellular neurites, while BSA had no effect on neurite density (Fig. 2). Co-treatment with α -tocopherol did not suppress AGE-induced neurite degeneration. However, NAC maintained neurite number when administered with AGE-BSA. Therefore, NAC and not α -tocopherol maintained the morphology and structure of neuronal cells in the presence of AGEs.

3.3. Effect of AGE-BSA and α -tocopherol and NAC on cellular antioxidant status

Under stress conditions, antioxidant pools may become depleted due to oxidation. Because of this, antioxidant status is often a useful marker of cellular stress. To test the effects of α -tocopherol and NAC on antioxidant status, cellular vitamin E and GSH concentrations were measured. The concentrations of vitamin E before supplementation were very low, and these values increased several hundred-fold

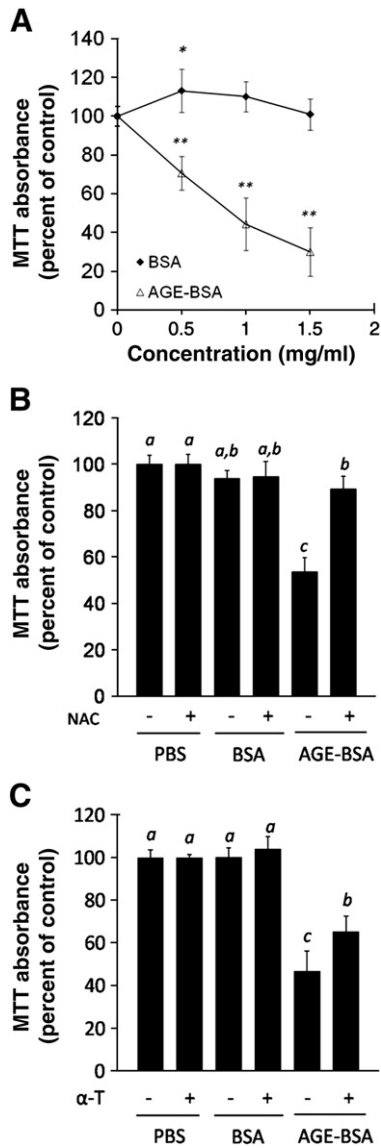


Fig. 1. Effects of AGE-BSA and antioxidants on SH-SY5Y viability. Cell viability of SH-SY5Y cells treated with 0–1.5 mg/ml BSA or AGE-BSA for 72 h (A). Cell viability of SH-SY5Y cells treated with PBS, BSA, or AGE-BSA ± 2 mM NAC (B) or 200 μM α-tocopherol (C) for 72 h. Viability is expressed as absorbance of MTT [570 nm–690 nm]. Values are expressed as mean ± S.D. (n = 9–12). Data were analyzed by ANOVA. *P < 0.05 vs. PBS control, **P < 0.0001 vs. PBS control. Means with superscript letters that are not the same are significantly different (p < 0.05).

after supplementation (Table 1). AGE-BSA did not decrease cellular vitamin E concentrations, perhaps because the extremely low concentrations of this vitamin did not provide suitable targets for oxidation and subsequent depletion. In PBS-treated cells, α-tocopherol supplementation significantly increased the concentration of this vitamin compared to controls treated with ethanol vehicle alone. Additionally, AGE-BSA and control BSA both showed significantly higher cellular vitamin E concentrations than the values for PBS treatment in α-tocopherol-supplemented cells. This finding might suggest that BSA enhances cellular vitamin E uptake by functioning in its known role as a lipid transporter in this cell culture model. It might also suggest that glycation of BSA does not diminish this function.

We also measured the intracellular changes in GSH concentration in response to AGE-BSA and the antioxidant co-treatments. We found that AGE-BSA significantly decreased intracellular GSH when compared to PBS and BSA controls (Fig. 3). The co-treatment of cells with α-tocopherol had no influence on this process as the decline in

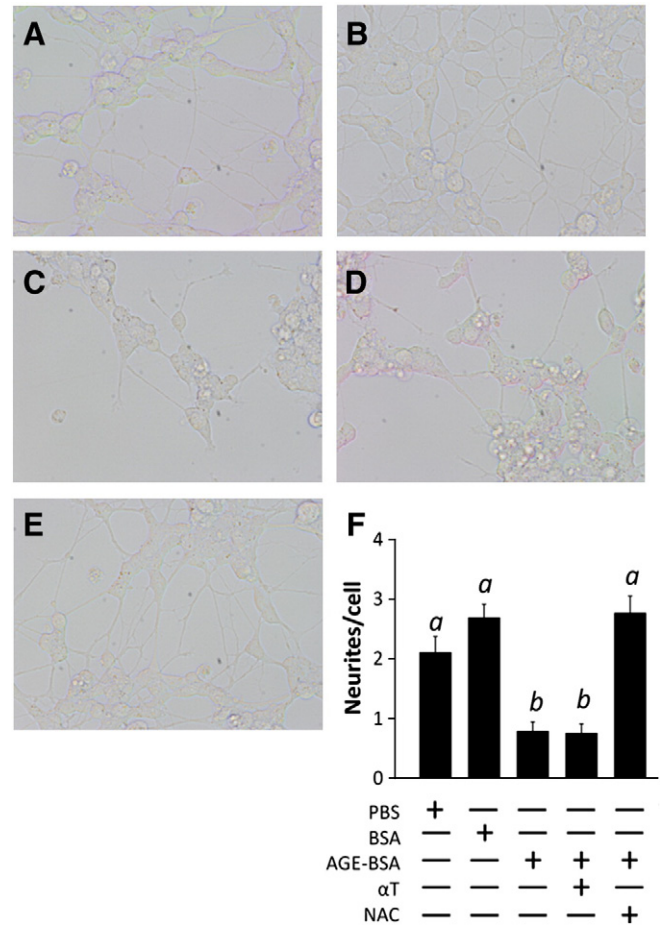


Fig. 2. Effects of AGE-BSA and antioxidants on cell morphology. Representative DIC images of AGE-treated SH-SY5Y cells. SH-SY5Y cells were treated with (A) PBS, (B) BSA, (C) AGE-BSA, (D) AGE-BSA + 200 μM α-tocopherol, and (E) AGE-BSA + 2 mM NAC for 72 h. Images of at least 4 random fields per treatment were captured by DIC microscopy. (F) Neurite number per cell for SH-SY5Y cells treated with PBS, BSA, AGE-BSA, AGE + 200 μM α-tocopherol, and AGE-BSA + 2 mM NAC; neurites were quantified using Neuron J. Bar values are expressed as mean ± SEM. Data were analyzed by ANOVA, and mean values with superscript letters that are not the same are significantly different (p < 0.05).

GSH due to AGE-BSA remained unaltered and the values with and without α-tocopherol supplementation were not significantly different. Therefore, α-tocopherol showed no effect on the intracellular GSH concentration. NAC significantly raised intracellular GSH concentrations by approximately 30 nmol/mg protein. There was no decrease in GSH due to AGE-BSA in NAC-treated cells. Thus, NAC may be protective against AGEs by increasing intracellular GSH and maintaining this pool after AGE-BSA administration.

Table 1

Influence of BSA and AGE-BSA on α-tocopherol levels. Cellular α-tocopherol concentrations were measured in SH-SY5Y cells treated with PBS, BSA, or AGE-BSA ± 200 μM α-tocopherol or 2 mM NAC for 72 h. Data were analyzed by ANOVA and bar values are expressed as mean ± S.D. (n = 3–4). Mean values with superscript letters that are not the same are significantly different (p < 0.05).

Treatment	α-tocopherol (nmol/mg protein)
PBS	0.014 ± 0.008 ^a
BSA	0.028 ± 0.020 ^a
AGE-BSA	0.032 ± 0.008 ^a
AGE-BSA + NAC	0.024 ± 0.012 ^a
PBS + αT	8.90 ± 1.62 ^b
BSA + αT	11.4 ± 1.48 ^c
AGE-BSA + αT	12.0 ± 1.82 ^c

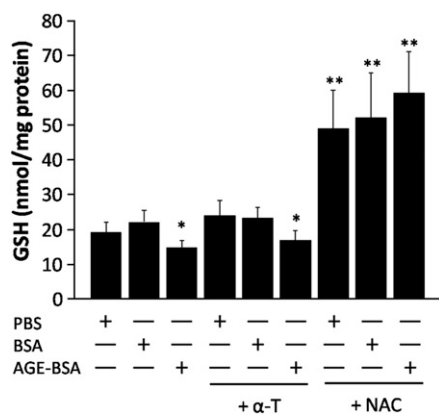


Fig. 3. Effects of AGE-BSA and antioxidants on GSH levels. Intracellular GSH concentrations for SH-SY5Y cells treated with PBS, BSA, or AGE-BSA \pm 200 μ M α -tocopherol or 2 mM NAC for 72 h. Data were analyzed by ANOVA to determine differences between antioxidant treatment groups and Student's t tests were used to compare samples within those groups. Bar values are expressed as mean \pm S.D. (n=4). * P <0.05 vs. within-treatment PBS and BSA controls when compared by Student's t test, ** P <0.0001 vs. all samples not receiving NAC treatment.

3.4. Effect of AGE-BSA and antioxidants on macromolecule oxidation

To examine the accumulation of oxidized products, we next tested the abilities of α -tocopherol and NAC to suppress AGE-induced oxidative damage to macromolecules. AGE-BSA treatment caused TBARS levels (expressed in nmol/mg total protein) to rise 17% in comparison to PBS-treated controls and 25% in comparison to unglycated BSA-treated controls (Fig. 4A). We then verified previous results that α -tocopherol decreases AGE-induced lipid peroxidation [16] by confirming that vitamin E – and not NAC – suppresses the modest increase in total intracellular MDA caused by AGE-BSA.

We next examined changes in protein oxidation as a function of AGE-BSA treatment by measuring total protein carbonyl content. We found that treatment with AGE-BSA increased total protein carbonyls in SH-SY5Y cells (Fig. 4B–C). Both α -tocopherol and NAC suppress this effect of AGEs on total protein oxidation. The level of 8-hydroxy-2'-deoxyguanosine in intact DNA was also assessed as a marker of DNA oxidation. AGE-BSA significantly increased the amount of this marker, while α -tocopherol partially and NAC completely suppressed the damage (Fig. 4D).

4. Discussion

In this study, we compared the effects that α -tocopherol and NAC have on outcomes related to oxidative damage in SH-SY5Y cells exposed to AGEs. We used SH-SY5Y cells as this cell line has been employed in cell culture models for several neurodegenerative diseases like Parkinson's disease [29] and Alzheimer's disease [30]. SH-SY5Y cells have also been utilized in models for diabetic neuropathy wherein the effects of high glucose [31] and AGEs [17,19,32,33] were tested. Additionally, we chose to use SH-SY5Y cells that had been pre-treated with RA as this causes sensitization to AGE-induced stress by increasing the activity of PKC δ and the expression of p47 phox [17]. SH-SY5Y cells are useful for the evaluation of antioxidants and their relative abilities to ameliorate AGE-induced oxidative damage [18]. Using SH-SY5Y cells, we confirmed previous findings that α -tocopherol reduces the cytotoxic effects of AGEs in neuronal-derived NT2 cells [16].

RAGE signaling induces oxidative damage and apoptosis in dorsal root ganglia neurons [15] and in cell lines using either NT2 cells [16] or SH-SY5Y cells [17]. Vitamin E provides cellular protection against RAGE-mediated stress, the mechanism of which is likely through the function of the vitamin as a chain-breaking antioxidant against

lipid peroxidation. Indeed, we confirmed that the vitamin E isoform α -tocopherol suppressed a modest AGE-induced increase in total MDA while NAC did not. A reasonable explanation for the modest increase in lipid peroxidation compared to the more pronounced effects on the other macromolecules became apparent with follow-up measurements of cellular lipid profiles. We found PUFA in these cells to be quite low (7% of total cellular fatty acids; data not shown) and reflective of FBS. We found that AGE-BSA did not decrease cell PUFA percentage. NAC also had no effect on PUFA. Treatment with α -tocopherol appeared to increase the percent of PUFA, but this effect was not statistically significant. Therefore, the limited lipid peroxidation observed may be due to lack of PUFA targets to form MDA.

Previous studies showed the benefits of hydrophilic antioxidants like NAC [34], and flavonoid antioxidants like epigallocatechin gallate (EGCG) [18] on lipid peroxidation under similar experimental conditions. Because EGCG is capable of reducing tocopheroxyl radical back to tocopherol [35] and GSH can do the same in an ascorbate-dependent mechanism [36], the observed effects of NAC or flavonoids on lipid peroxidation may involve recycling of α -tocopherol present in the cellular membranes. Here, we quantified basal α -tocopherol concentrations and those after 200 μ M α -tocopherol supplementation. We observed very low basal α -tocopherol concentrations in cellular membranes. We suspect that this explains why we did not see any effect of NAC on AGE-induced MDA formation. With low α -tocopherol concentrations present in these cells, there is very little α -tocopherol for GSH synthesized from NAC to recycle via ascorbate and thus, NAC is unlikely to aid in preventing lipid peroxidation. We also found that within cells supplemented with α -tocopherol, BSA and AGE-BSA-treated cells had significantly higher α -tocopherol concentrations than PBS-treated cells. BSA may be facilitating α -tocopherol uptake, which is consistent with the role of albumin as a transporter of lipids. Glycation of BSA does not affect this process. These findings further demonstrate the critical nature of having a BSA control with such studies and that BSA – and not PBS – may serve as the most appropriate control with which to compare the effects of AGE-BSA.

We observed that both α -tocopherol and NAC suppress protein carbonyl formation after AGE administration. NAC efficiently inhibited AGE-induced DNA oxidation, which α -tocopherol also reduced albeit not completely. Thus, both antioxidants have similar effects in suppressing protein and DNA oxidation. However, NAC significantly increased intracellular GSH while α -tocopherol did not. Therefore, we conclude that NAC influences macromolecule oxidation by sustaining the intracellular glutathione pool and potentially through maintaining the reduced state of intracellular thiols. In all, both α -tocopherol and NAC were effective against the oxidation of macromolecules but only NAC – and not α -tocopherol – is likely to be effective in maintaining the cellular redox state under AGE stress conditions.

Using DIC microscopy, we documented cell morphological changes that occur as a result of various AGE treatment combinations. SH-SY5Y cells were first treated for 3–5 days with RA as this increases expression of tyrosine hydroxylase (TH), a marker of neuronal differentiation (p <0.01, data not shown). Because RA also induces neurite outgrowth, deterioration of neurite structure due to a particular treatment is more indicative of degeneration mechanisms rather than an inhibition of outgrowth. While PBS and BSA did not significantly affect cell morphology, AGE-BSA negatively influenced overall morphology with respect to the number of neurites per cell. Additional experiments qualified that this is likely not due to loss of a differentiated state of the cells as TH expression in BSA-treated cells and AGE-BSA-treated cells is not significantly different (data not shown). We then examined if antioxidants can suppress AGE-induced neurite degeneration. Our data showed that α -tocopherol had no influence on neurite deterioration while NAC provided complete protection, which parallels the effects that these antioxidants have on intracellular GSH. There was no difference between control cells treated with PBS or BSA and cells treated with AGE-BSA + NAC, thus

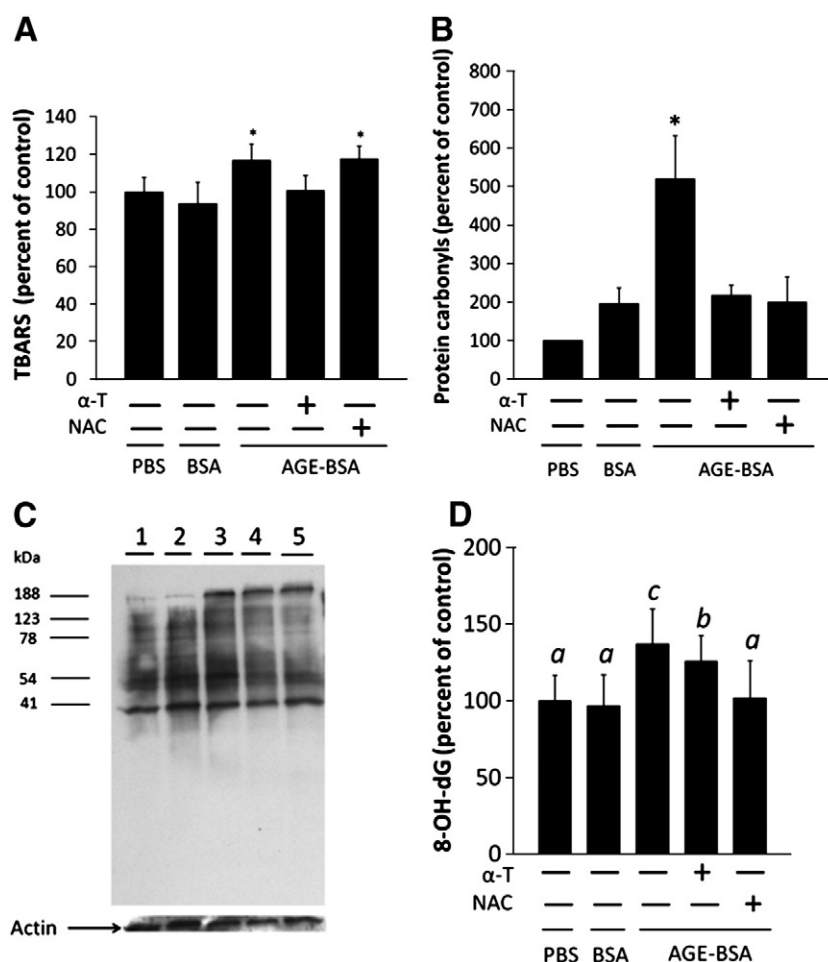


Fig. 4. Effects of AGE-BSA and antioxidants on oxidative damage. (A) TBARS for SH-SY5Y cells treated with PBS, BSA, or AGE-BSA ± antioxidants for 48 h. Data is expressed as a percentage of PBS control. Data were analyzed by ANOVA and bar values are presented as mean ± S.D. (n = 3). *P < 0.05 vs. BSA control. (B) Analysis of DNPH-derivatized proteins isolated from SH-SY5Y cells treated with PBS, BSA, or AGE-BSA ± antioxidants. Data is expressed as a percentage of PBS control. Data were analyzed by ANOVA and bar values are presented as mean ± S.D. (n = 3 from 3 independent experiments). *P < 0.001 vs. BSA control. (C) Representative blot of carbonylated proteins from cells treated with PBS (lane 1), BSA (lane 2), AGE-BSA (lane 3), AGE-BSA + 200 μM α-tocopherol (lane 4), and AGE-BSA + 2 mM NAC (lane 5). (D) Effects of AGE-BSA and antioxidants on DNA oxidation. Confocal analysis of anti-8-oxo-2'-deoxyguanosine-staining in SH-SY5Y cells treated with PBS, BSA, or AGE-BSA ± antioxidants. Data is expressed as a percentage of PBS control. Data were analyzed by ANOVA and bar values are presented as mean ± S.D. (n = approximately 150 cells from 3 independent experiments). Mean values with superscript letters that are not the same are significantly different (p < 0.05).

demonstrating an optimal protective effect of NAC against neurite degeneration in this model. Data from several models have shown that oxidants can either induce neurite degeneration or prevent neurite outgrowth and that these effects are ameliorated by antioxidants. Examples include the prevention of lead-induced neurite degeneration in PC12 cells by selenocystine [37] and the maintenance and promotion of neurite outgrowth by EGCG in serum-starved PC12 cells [38]. α-tocopherol has been studied previously with regard to effects on neurite outgrowth and maintenance. α-tocopherol supports the survival and neurite outgrowth for fetal rat brain neurons grown in culture [39]. α-tocopherol also ameliorates changes in neurite structure in a mouse model for Alzheimer's disease [40]. In contrast, α-tocopherol has no effect on α-synuclein-induced neurite retraction [41]. Therefore, it is likely that the effects of α-tocopherol on neurite alterations depend on the disease model and the specific mechanism of oxidation-induced neurite degeneration.

In this study, we found that α-tocopherol suppresses AGE-induced macromolecule damage but has no effect on AGE-BSA-induced neurite degeneration. The thiol antioxidant NAC maintained cellular morphology. This may point toward thiol oxidation as being a primary regulator of neurite degeneration. Indeed, agents that cause oxidation of tubulin cysteines induce depolymerization of preformed microtubules [42]. Neurite degeneration caused by oxidative stress is blocked by GSH

and L-cysteine, while L-methionine, an amino acid with a sulfur atom but not a thiol group, has no effect [43]. These data provide evidence that thiol-containing compounds are effective in reducing neurite degeneration caused by ROS. Our results further confirm these findings as we show here that both α-tocopherol and NAC suppressed AGE-induced macromolecule damage but only NAC was able to protect neurite structures under stress conditions. These trends are particularly important because under some conditions, neurite degeneration precedes apoptosis and is critically tied to the fate of the cell [44,45]. Therefore, the effect of α-tocopherol on AGE-induced cell death may be limited by its inability to influence neurite density.

The implications of this study reach beyond preventive measures of maintaining nerve integrity in diabetics. Outgrowth of neurite components within the context of peripheral neuron regeneration is a vital topic of study for individuals who have already lost a significant degree of nerve function. Recent work shows that AGEs contribute to a limited ability for neuronal regeneration in diabetes [46], but as we observed in this study, a thiol-containing compound like NAC may be effective in promoting neurite integrity under stress conditions. We propose that follow-up studies be performed to examine the role of particular antioxidants in neurite outgrowth under conditions of high glucose or AGE exposure to eventually optimize long-term outcomes for patients with diabetic neuropathy.

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