Effects of AGEs on Oxidation Stress and Antioxidation Abilities in Cultured Astrocytes[1]

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Objective   To investigate whether two kinds of *in vitro* prepared advanced glycation end products (AGEs), Glu-BSA and Gal-BSA, could change oxidation stress and anti-oxidation abilities in astrocytes, and thus might contribute to brain injury.   **Methods**   Changes of GSH, MDA, SOD, MAO-B, nitric oxide were measured after AGEs treatment.   **Results**   Both 0.1 g/L Glu-BSA and Gal-BSA could slightly decrease GSH level, while 1 g/L of them significantly decreased GSH level by 35% and 43% respectively. The MDA levels of both 1 g/L AGEs treated groups (306±13 and 346±22) were higher than that of the normal group (189±18), which could be inhibited by free radical scavenger NAC. The SOD activities of both 1 g/L AGEs treated groups (67.0±5.2 and 74.0±11.0) were lower than that of the normal group (85.2±8.0). Both 0.1 g/L AGEs could slightly increase the activity of MAO-B, while 1 g/L of them could increase MAO-B activity by 1.5 and 1.7 folds respectively. Both AGEs stimulation could produce NO level by 1.7 and 2 folds respectively.   **Conclusion**   Enhanced levels of astrocytic oxidation stress and decrease of antioxidation abilities may contribute to, at least partially, the detrimental effects of AGEs in neuronal disorders and aging brain.

Key words: Advanced glycation end products; Astrocyte; Superoxide dismutase; Glutathione; Malondialdehyde; MAO-B; Nitric oxide

INTRODUCTION

Noenzymatic glycation of proteins, leading to the formation of irreversible AGEs, has been found to occur during aging and at accelerated rate in cases of diabetes[1,2]. When proteins are exposed to glucose or other carbohydrates, the Maillaird reaction, a nonenzymatic process, is initiated. It generates first reversible Schiff base adducts and subsequently more stable Amadori rearrangement products. Through a series of oxidative and nonoxidative reactions, it eventually yields the irreversible advanced glycation end products (AGEs) linked with amino groups, e.g., lysine residues, of several proteins.

AGEs are accumulated in many tissues during aging and at an accelerated rate during the course of diabetes. In the early 80s, Monnier and Cerami proposed that AGEs-mediated cross-linking of long-lived proteins contribute to the age-related decline in the function of cells and tissues in normal aging[3]. Our previous study also provided evidences showing

that AGEs *in vivo* might contribute to *D*-galactose induced aging model[4]. The detrimental effects were originally attributed to their physiochemical properties including protein cross-linking. However, recent studies increasingly emphasized the role of AGEs in cellular processes and signaling event in many diseases such as neuropathy. Although AGEs accumulated in neuron of aged humans and amyloid plaques in Alzheimer's disease (AD) and Lewy bodies in Parkinson's disease (PD), the role of increased cerebral AGEs level during aging and in these neurodegenerative diseases has not been fully understood.

Oxidative stress is possibly involved in the aging process and is one of the pathogenic mechanisms of a variety of neurodegenerative diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease. Reactive oxygen species produced continuously during oxidative metabolism, are generated at high rates within the brain. The brain is particularly susceptible to oxidative damage, and astrocytes are chiefly responsible for its antioxidant defense[5]. AGEs are co-localized with astrocytes and microglial cells in Alzheimer's disease brain, which might activate astrocyte and microglia, and play a role in the pathogenesis of Alzheimer's disease[6]. In the present study, the effects，of two kinds of *in vitro* produced AGE-BSA, Glu-BSA and Gal-BSA on some important molecules and enzymes involving the oxidative stress in cultured primary rat astrocytes were studied.

MATERIALS AND METHODS

Preparation of AGE-BSA

Two kinds of AGE-BSA (Glu-BSA and Gal-BSA) were prepared by incubating BSA (50 g/L) (fracton V, Boehringer-Mannheim Biochemicals) in phosphate-buffered saline (PBS) 10 mmol/L, pH 7.4, with *D*-glucose 0.5 mol/L or *D*-galactose 0.5 mol/L at 37℃ for 10 weeks in the presence of protease inhibitors (PMSF 1.5 mmol/L and edetic acid 0.5 mmol/L) and antibiotics (penicillin 100 kU/L and streptomycin 100 mg/L) under sterile conditions as described previously[7]. Nonglycated albumin, used as control, was prepared in identical fashion except that glucose and galactose were depleted from the incubation buffer. At the end of the incubation period, reactants with low molecular weight and monosaccharides were removed by dialysis. The degree of AGE modification of these proteins was determined by competitive AGE-ELISA as described before[8]. Briefly, a 96-well ELISA plate was coated with 100 μL of AGE-BSA 3 mg/L per well in coating buffer overnight at 4℃. Wells were washed three times with 150 μL washing buffer (PBS, 0.05% Tween-20), then blocked with 100 μL of blocking buffer [PBS, 1% normal goat serum (NGS)] at room temperature for 1 h. After three rinses, 50 μL of competing antigen was added, followed by 50 μL rabbit anti-AGE polyclonal antiserum (1:2000) in dilution buffer (PBS, 0.02% Tween-20, 1% NGS) (kindly provided by Dr. Yong-Ming LI in USA). The plate was incubated for 2 h at room temperature. After rinsed three times, secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG) in dilution buffer (1:2000) was then added to each well and the plate was incubated at 37℃ for 1 h. After rinsed six times, 100 μL *p*-nitrophenyl phosphate substrate was added to each well. Optical density at 405 nm was determined by a microplate reader after 30-60 min incubation at 37℃. AGE contents were determined using standard AGE-BSA (provided by Dr. Yong-Ming LI). All protein concentrations were assayed by Bradford method.

Cell Culture

Cortical astrocyte cultures were prepared from neonatal (1-2 d old) Wistar rat pups by the method of McCarthy and Vellis[9]. Briefly, after the menings were removed, the cerebral cortex was dissected out and trypsinized at 37℃ for 20 min. The resultant cell suspension was filtered through Nitex mesh (30 μm), planted (one brain in one 75 cm^2 flask) and cultured in MEM (Gibco-BRL, USA) medium containing 10 % fetal bovine serum (Hyclone Laboratories, USA) and antibiotics (penicillin 100 kU/L and streptomycin 100 mg/L). After 11 d in culture, cells were trypsinized and replated into the flasks at a density of 6×10^5/flask and grown until confluence. This treatment yielded a highly purified culture of astroytes consisting of more than 95% astroglial, as determined by glial fibrillary acidic protein (GFAP) immunostaining. The senescent astrocytes were termed as the cultured cells after 40 days, which grew slowly and showed senescence-like phenomenon.

GSH Assay

Reduced GSH content in each group treated with various amounts of AGE-BSA and BSA control for 18 h was measured by the method of Tietze[10], with minor modifications. Briefly, cells were washed twice in cold PBS and scraped down by a scraper in sodium phosphate buffer 125 mmol/L containing edetic acid 6.3 mmol/L (pH 7.5). After sonication, 20 μL was taken for protein assay, then 1% trichloroacetic acid was added to the lysates, and the mixture was allowed to precipitate for 2 h at 4℃. After centrifugation at $10\,000\times g$ for 15 min, protein-free lysates were obtained. The reaction mixture for the determination of GSH content consisted of lysates and 5, 5′-dithiobis-(2-nitrobenzic acid) (DTNB) 6 mmol/L. The absorbance at 405 nm was monitored for 6 min using a microtiter plate reader (Bio-Rad Ltd, Japan). The content of GSH was calculated from the change in the rate of absorbance on the basis of a standard curve.

MDA Assay

Confluent astrocytes were plated into 24-well tissue culture plates at a density of 5×10^4 cells/well. After 24 h, AGE-BSA and BSA control were added to the culture medium. After 72 h, astrocytes were rinsed one time in PBS, and then added 0.5 mL PBS to per well. The cells were lysed by freezing and thawing at 37℃ and at -70℃ three times. The inhibition group was added pre-incubated 10 mmol/L N-acetylcysteine for 30 min. Then all groups were added the same volume solution so that the rate of 0.67% thiobarbituric acid and acetate was the same. All samples were incubated in boiling water for 15-30 min. The absorbance was monitored at 532 nm using a microtiter plate reader (Bio-Rad).

SOD Assay

Total SOD activity was measured using SOD assay kit (Nanjing Jiancheng Biotechnology Institute, China). Briefly, astrocytes were plated into 6-well tissue plates at a density of 1.55×10^4 cells/well. After 24 h, AGE-BSA, BSA and PBS were added to the culture medium. After 24 h, cells were washed twice in cold PBS and then lysed by freezing and thawing at 37℃ and at -70℃ three times. After treatment according to the manual, sample absorbance was monitored at 550 nm (Bio-Rad).

MAO-B Assay

Confluent astrocytes were plated into 6-well tissue culture plates at a density of 5×10^4 cells/well. After 24 h, AGE-BSA and BSA control were added to the culture medium. After 72 h, astrocytes were added with PBS and lysed by freezing and thawing at 37℃ and at

-70℃ three times, then 8 mmol/L benzyl amine was added for 2 h at 37℃. 60% perchloric acid was added to stop reaction and cyclohexane was used to extract. Then contents were centrifugated for 10 min at 3 000 rpm. Supernatant absorbance was monitored at 242 nm using a microtiter plate reader (Bio-Rad).

Nitrite Assay

Nitrite concentration was measured in a standard Griess reagent provided by Pormega. Briefly, 50 μL of supernatant was incubated with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid). After 10-min incubation at room temperature, the absorbance of cromophore so-formed was measured at 560 nm using a microtiter plate reader (Bio-Rad Ltd, Japan). Nitrite concentrations were calculated by comparison with a standard calibration curve with sodium nitric ($NaNO_2$: 1.26-100 mmol/L), with control baseline supernatant as the blank.

Data Analysis

Values were expressed as $\bar{x} \pm s$ from three independent experiments performed in triplicate. The statistical significance of differences among experimental groups was evaluated by Student's t-test.

RESULTS

AGEs Content of Incubated Samples

We selected two kinds of monosaccharides-glucose and galactose for incubation because these two sugars had close relationship with diabetes and aging[11]. The result showed that AGE contents were 16.9 kU/g and 17.0 kU/g protein in Glu-BSA and Gal-BSA respectively, while 0.29 kU/g protein in BSA control. All of the incubated proteins showed no degradation identified by 10% SDS-PAGE electrophorsis. Notably, the following AGE concentration was always selected as 1 g/L, because AGEs contents per liter in both samples were comparable to human serum[8] (20.3 kU/L $\pm$ 3.8 kU/L).

Reduced GSH Level by AGE-BSA

After 18 h treatment, AGE-BSA 0.1 g/L slightly decreased GSH level, while 1 g/L AGE-BSA significantly decreased GSH level by 35% and 43% when compared to BSA control (Fig. 1). This indicated that AGE-BSA might be the inducers of oxidative stress.

Increased MDA Levels by AGE-BSA

After 72 h treatment, both kinds of AGE-BSA could increase the levels of MDA in the astrocytes. The increase of MDA could be inhibited by 10 mmol/L N-acetylcysteine (Fig. 2).

Decreased SOD Activity by AGE-BSA

After treated with AGEs 1 g/L for 24 h, decreased SOD activity was detected in rat astroctyes. The senescent astrocytes had the same phenomenon as the treated cells (Table 1).
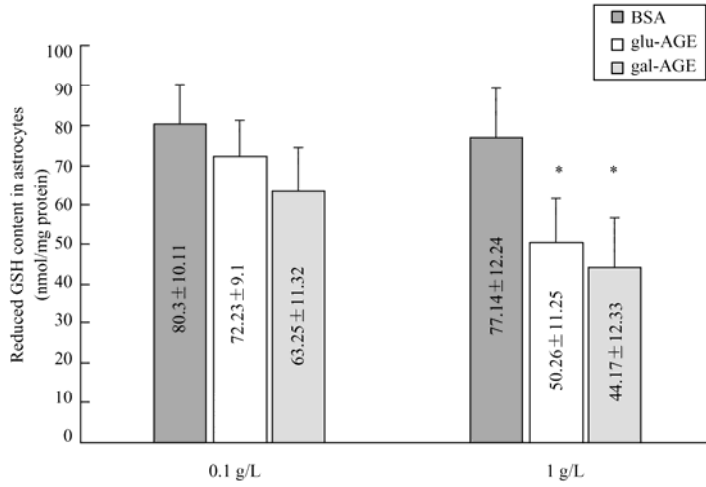
FIG. 1. rGSH content after treatment with both kinds of AGEs for 18 h. *$P<0.05$ vs BSA.
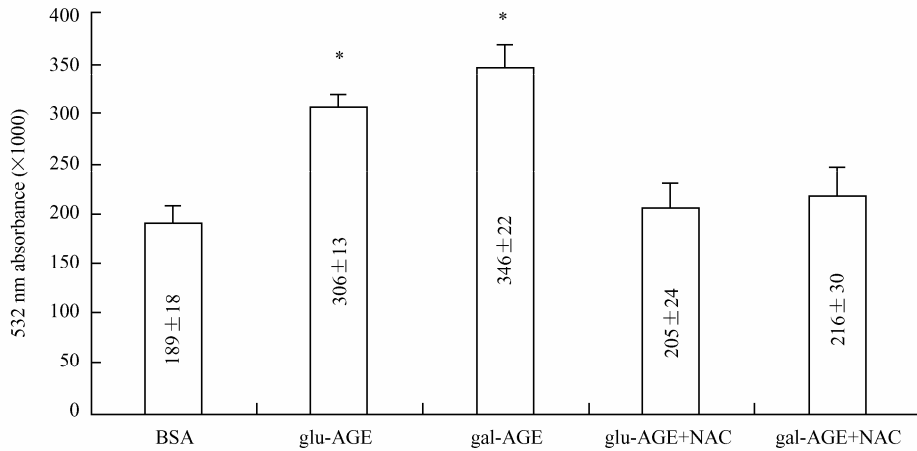
FIG. 2. Level of lipid peroxidation after 72 h treatment with both kinds of AGEs (1 g/L). 10 mmol/L N-acetylcysteine (NAC) was pretreated for 30 mins. *$P<0.05$ vs BSA.

TABLE 1

SOD Activity in Rat Astroglial After Treated with AGEs 1 g/L for 24 h

Sample	SOD Activity (NU/mg Protein)
PBS	88.0 ± 9.2
Senescent Cells	61.4 ± 7.6[a]
BSA Control	85.2 ± 8.0
glu-AGE	67.0 ± 5.2[b]
gal-AGE	74.0 ± 11.0[b]

Note. [a]$P<0.05$ vs PBS, [b]$P<0.05$ vs BSA control.

Increased MAO-B Activity by AGE-BSA

After treated with AGEs 1 g/L for 72 h, both kinds of AGE-BSA could increase the activities of MAO-B (Fig. 3).

NO Release by AGE-BSA

After 18 h incubation, Glu-BSA and Gal-BSA 1 g/L stimulation could produce NO level by 1.7 and 2 folds, respectively (Fig. 4).
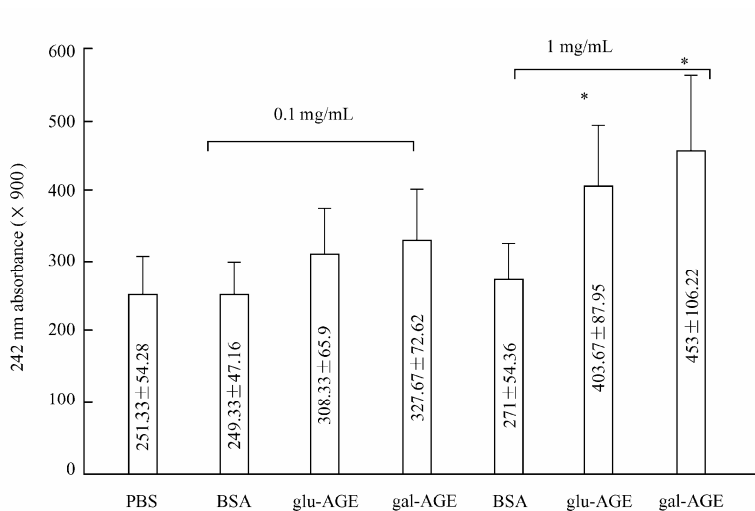

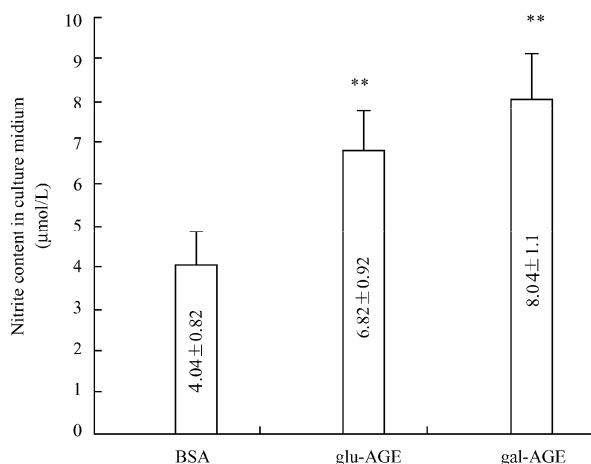FIG. 3. MAO-B activity after treated with AGEs for 72 h. *$P<0.05$ vs BSA.

FIG. 4.   NO levels by both AGEs (1 g/L) stimulation for 18 h. **$P<0.01$ vs BSA.

DISCUSSION

Glycation and oxidative stress are closely linked and often referred to as "glycoxidation"

processes[12]. All glycation steps generate oxygen free radicals, some of these steps are common with these of lipid peroxidation. AGEs bind to membrane receptors such as RAGE, and induce an oxidative stress and a pro-inflammatory status. Products of lipid peroxidation (MDA) bind to proteins and amplify glycoxidation-induced damages.

The brain is especially sensitive to oxidative damage because of its high content of oxidative fatty acids, high use of oxygen, and low levels of antioxidants[13]. We found that AGEs acted on astrocytes resulting in not only the increase of MDA levels, but depletion of an important cellar antioxidant, reduced GSH, indicating the increase of cellular oxidative stress and the decrease of anti-oxidation defense systems. The increase of MDA also could be inhibited by free radical scavenger NAC. The cellular distribution of malondialdehyde (MDA) was assessed immunohistochemically in brain specimens from young and normal elderly subjects as well as patients with Alzheimer's disease (AD). MDA was increased in the cytoplasm of neurons and astrocytes in both normal aging and AD, but was rarely detected in normal young subjects[14].

The GSH level was higher in astrocytes than in neurons, and its depletion in astrocytes increased neuronal susceptibility to H_2O_2 and paraquat[15]. GSH had important functions as an antioxidant, and as a transport and storage form of cysteine, it was a reaction partner for the detoxification xenobiotica and was a cofactor in isomerization reactions. In addition, GSH maintained the thiol redox potential in cells keeping sulfhydryl groups of cytosolic proteins in the reduced form. Some results suggested that GSH also played a role in the regulation of apoptosis[16]. Astrocytes appeared to contain higher GSH levels than neurons both *in vivo* and in culture. *In vivo* different types of brain cell were in close contact with each other. Evidence is growing that, especially between astrocytes and neurons, an intensive metabolic exchange occurred. Such interactions also appeared to be important regarding cerebral glutathione homeostasis and protection of the brain against oxidative stress[17].

Superoxide radicals are normally produced during mitochondrial respiration but also as part of the inflammatory response. To protect themselves from damage caused by free radicals, mammalian cells have developed chemical and enzymatic antioxidant defense systems. Superoxide dismutase (SOD) which catalyzes the conversion of superoxide radicals to hydrogen peroxide is one of the most important antioxidant enzymes. Some reports demonstrated that the SOD activity decreased with aging, but there were other reports with conflicting results[18]. In our study, AGEs could decrease the normal astrocyte SOD activity to the same level as the senescent cell.

Monoamine oxidase (MAO) metabolizes biogenic and dietary amines in the central nervous system and peripheral tissues, and yields hydrogen peroxide (H_2O_2). Two distinct MAOs have been described. MAO A has a higher affinity for serotonin, norepinephrine, and inhibitor clorgyline. MAO B exhibits a higher affinity for phenylethylamine, benzylamine, and inhibitor deprenyl. In addition, MAO B converts 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine to its toxic metabolite 1-methyl-4-phenylpyridine, which selectively destroys nigrostriatal neurons. The neurodegeneration induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine is similar to the neuronal damage in Parkinson's disease and could be prevented by the MAO B inhibitor deprenyl[19]. Moreover, MAO B activity, rather than MAO A activity, increases progressively in the brain throughout adult life. Aberrant increase of MAO B activity in the elderly has been implicated in neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Huntington's disease[20].

As a part of oxidative stress, increased level of NO may also play a neurotoxic role, since NO produced from glial can be toxic to neuron. Our previous study proved that AGE could induce the production of cytokine, like IL-1βand TNF-α. Although both IL-1β and

TNF-α can contribute to NO production, these two cytokines might not the direct inducer of the production, because significant induction of NO was earlier than that of both cytokines[21]. Thus NO production could be the direct effect of AGE on astrocytes.

In our study, AGEs could not only increase the oxidation of cultured astrocytes and destroy their defense system, but also increase the activity of enzyme associated toxic free radical and release toxic molecule. This may help us further understand the detrimental effect of AGEs on nervous system.

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