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Activation of astrocytes by advanced glycation end products: cytokines induction and nitric oxide release[1]

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ABSTRACT

AIM: To investigate whether two kinds of *in vitro* prepared advanced glycation end products (AGE), Glu-BSA and Gal-BSA, could induce proinflammatory mediators IL-1β and TNF-α, as well as oxidative stress and nitric oxide (NO), in astrocytes, thus contributing to brain injury. **METHODS:** Radioimmunoassay and RT-PCR technique were used to detect two cytokines' level and existence of receptor for AGE (RAGE). DTNB reaction was used to measure reduced glutathione (GSH) level. NO content was assayed using Griess reagent provided by Promega. **RESULTS:** Enhanced protein levels of both cytokines in supernatants and cell lysates of astroglia cultures were detected after treated with AGE-BSA 1 g/L, especially Gal-BSA, for 72 h. The increases were also in a concentration-dependent manner. Changes in protein levels might be attributed to changes in transcriptional levels documented by semi-quantitative RT-PCR. Both AGE-BSA could also reduce astrocytic GSH and induce NO release. RAGE was detected in astrocytes. **CONCLUSION:** Enhanced levels of astrocytic proinflammatory mediators IL-1β and TNF-α, and oxidative stress caused by AGE might contribute to, at least partially, the detrimental effects of AGE in neuronal disorders and aging brain.

INTRODUCTION

Advanced glycation end products (AGE) are a heterogeneous group of irreversible adducts formed by amino groups of proteins reacting non-enzymatically with monosaccharides, including glucose, fructose, hexose-phosphates, *etc*. AGE accumulate in many tissues during aging and at accelerated rate during the course of diabetes. In the early 80's, Monnier and Cerami proposed that AGE-mediated crosslinking of long-lived proteins contributed to the age-related decline in the function of cells and tissues in normal aging[1]. Our study also provided proofs showing that AGE *in vivo* might contribute to *D*-galactose induced aging[2]. The detrimental effects of AGE were originally attributed to their physiochemical properties including protein crosslinking. However, recent studies increasingly emphasize the role of AGE in cellular processes and signaling events in many diseases such as

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neuropathy. Although AGE accumulated in neurons of aged humans and in amyloid plaques in Alzheimer's disease (AD)[3] and Lewy bodies in Parkinson's disease (PD)[4], the role of increased cerebral AGE level during aging and these neurodegenerative diseases has not been fully understood.

One common feature of a variety of neurodegenerative disorders is the presence of large number of activated astrocytes and microglia (gliosis). Glial activation involves changes both in morphology and in expression of proinflammatory cytokines like interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), whose expression are enhanced in aging process, AD and PD, thus exacerbating the progression of neuropathology[5]. The factors responsible for promoting glial activation in aging and AD are not exactly known. Although amyloid-β (Aβ) was involved in this process[6], this seems not to be the reason for cytokines changes in aging process and other neurodegenerative conditions where Aβ could hardly be found. Since AGE are present in all of these conditions, one possibility is that AGE could promote glial cytokine production. In present study the effects of two kinds of *in vitro* produced AGE-BSA, Glu-BSA and Gal-BSA, on cytokines induction like IL-1β and TNF-α, as well as on GSH and NO level, in cultured primary rat astrocytes were studied.

MATERIALS AND METHODS

Preparation of AGE-BSA Two kinds of AGE-BSA (termed Glu-BSA and Gal-BSA) were prepared by incubating BSA (50 g/L) (fraction 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 °C for 10 weeks in the presence of protease inhibitors (PMSF1.5 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 an identical fashion except that glucose and galactose were depleted from the incubation buffer. At the end of the incubation time, 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, 96-well ELISA plate was coated with 100 μL of AGE-BSA 3 mg/L per well in coating buffer overnight at 4 °C. 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 of rabbit anti-AGE polyclonal antiserum (1:2000) in dilution buffer (PBS, 0.02 % Tween-20, 1 % NGS) (kindly provided by Dr LI Yong-Ming in USA). The plate was incubated for 2 h at room temperature. After rinsing 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 °C for 1 h. After rinsing six times, 100 μL *p*-nitrophenyl phosphate substrate was added to each well. Optical density at 405 nm was determined by microplate reader after 30-60 min incubation at 37 °C. AGE contents were determined using standard AGE-BSA (provided by Dr LI Yong-Ming). 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 removing the menings, the cerebral cortex was dissected out and trypsinized at 37 °C for 20 min. The resultant cell suspension was filtered through Nitex mesh (40 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 confluent. This treatment yielded a highly purified culture of astrocytes consisting of more than 95 % astroglial, as determined by glial fibrillary acidic protein (GFAP) immunostaining.

Treatment of astrocytes with AGE-BSA Confluent astrocytes were trypsinized and plated into 6-well tissue culture plates at a cell density of 2×10^5/ well (for cytokines, GSH assay and total RNA extraction) or into 96-well plates at a density of 1×10^4/ well (for NO assay). After 20 h, AGE-BSA and BSA control were added to the culture medium at various concentrations and for various time as indicated.

IL-1b and TNF-a assay After treating the cells with AGE-BSA for indicated time, supernatants were collected and adherent cells were washed twice with PBS, then 1 mL MEM medium was added and cells were scraped down using scraper. The cells were lysed by four cycles of freezing and thawing. After taking 20 μL of cell lysates for testing protein concentra-

tion, cell lysates and incubation supernatants were assayed for IL-1α and TNF-β using the radioimmunoassay kit provided by the Institute of Radio-Immunity of the General Hospital of PLA, Beijing, China. The level of both cytokines was determined by the competitive binding of the cytokines in the samples with ^{125}I-radiolabled IL-1β and TNF-α standards respectively. Data were shown as the actual content of both cytokines secreted to the supernatants or in the lysates per mg cell lysates.

Determination of mRNA levels by semi-quantitative RT-PCR After treated with AGE-BSA 1 g/L for 72 h, cells were washed twice with cold PBS free of RNase. Total RNA of treated or control astrocytes was isolated by SV total RNA isolation system (Promega, USA) as recommended by the manufacturer. The quality of extracted RNA was excellent identified by an A_{260}/A_{280} ratio of 1.8 or higher. RT-PCR was performed on PTC-100 programmable thermal controller (MJ Research Inc, USA) using Superscript™ one-step RT-PCR system (Gibco-BRL, USA), with the final 50 μL RT-PCR mixture containing 0.5 mg RNA, 1 μL Superscript II RT/*Taq* mix, primers 1 mmol/L, dNTP 0.2 mmol/L, and $MgSO_4$ 1.2 mmol/L. The reactions were as follows: 50 °C for 30 min; 94 °C for 2 min; followed by 40 cycles of 94 °C for 30 s; 60 °C (for TNF-α), 64 °C (for IL-1β), 52 °C (for GAPDH housekeeping gene) or 65 °C (for RAGE) for 30 s; 70 °C for 1 min; and at the end of this cycles, 72 °C 10 min for extension. Primer pairs used to amplify rat IL-1β (816 bp), TNF-α (453 bp), GAPDH (600 bp), and RAGE fragment (606 bp) were respectively as follows: Upper 5' CTCCAGTCAGGCTTCCTTGTGC3', Lower 5' ATGTCCCGACCATTGCTGTTTC3'; Upper 5' GCCACCACGCTCTTCTGTCTACT3', Lower 5' GAGGCTGACTTTCTCCTGGTATG 3'; Upper 5' CCACCCATGGCAAATTCCATGGCA 3', Lower 5' TCTAGACGGCAGGTCAGGTCCACC 3'; Upper 5' CCTGAGACGGGACTCTTCACGCTTCGG 3', Lower 5' CTCCTCGTCCTCCTGGCTTTCTGGGGC 3'. RT-PCR products of IL-1β and GAPDH 5 μL or RT-PCR products of TNF-α and GAPDH 5 μL together with 6×PCR loading buffer 2 μL were then loaded into a 2 % agarose gel to seperate the amplified DNA species. The bands corresponding to the amplified DNA were then visualized by 0.5 % ethidium bromide staining, and resultant gels were photographed with UVP ImageStore 7500 (Life Sciences).

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 modification. Briefly, cells were washed twice with cold PBS and scraped down by 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 °C. After centrifugation at 10 000×*g* for 15 min, protein-free lysates were obtained. The reaction mixture for determination of GSH content consisted of lysates and 5,5'-dithiobis-(2-nitrobenzoic 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.

Nitrite assay Nitrite concentration was measured in a standard Griess reagents provided by Promega. 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 the 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 nitrite ($NaNO_2$ 1.26-100 mmol/L), with control baseline supernatant as the blank.

Data analysis Values were expressed as mean±SD. The statistical significance of differences among experimental groups was evaluated by Student's *t*-test.

RESULTS

AGE content in incubated samples We selected two kinds of monosaccharides-glucose and galactose for incubation because these two sugars have close relationship with diabetes and aging[3]. The results 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 incubation proteins showed no degradation identified by SDS-10 % PAGE electrophoresis (data not shown). Notably, the following AGE concentration was always selected as 1 g/L, because AGE contents per litre in both samples were comparable to human diabetic serum (20.3 kU/L±3.8 kU/L)[8].

Induction of IL-1b by AGE-BSA We tested the IL-1β level in cultured supernatants treated with AGE-

BSA 1 g/L for 24 h and 72 h, and found that after 24 h, Glu-BSA and Gal-BSA slightly increased the IL-1β production by 15 % and 25.4 % respectively, while after 72 h, IL-1β was increased by 43 % and 55.5 % respectively, when compared with control (P<0.01) (Fig 1A). It should be noted that both IL-1β and TNF-α produced by the BSA control were very similar to the baseline cytokines level, ie, the level without AGE-BSA or BSA stimulation measured by this radioimmunoassay (for IL-1β, 855 ng/g protein and for TNF-α, 2.70 μg/g protein). The increase trend of IL-1β in cell lysates was consistent with secreted ones. Furthermore, the induction of secreted IL-1β in the cultures treated with Gal-BSA for 72 h was concentration-dependent (Fig 1B). Semi-quantitative RT-PCR detected the increased mRNA levels of IL-1β (Fig 2A), which might contribute to the increased protein levels of IL-1β caused by AGE-BSA.

Induction of TNF-a by AGE-BSA Treatment with Glu-BSA and Gal-BSA (1 g/L) for 24 h just slightly

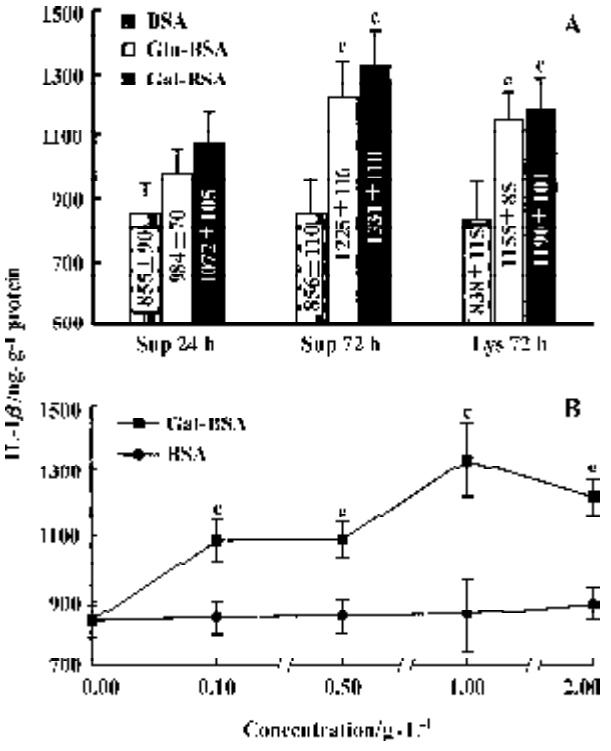

Fig 1. IL-1b levels in cultured supernatants and lysates of astrocytes treated with BSA control or AGE-BSA 1 g/L (where AGE contents in Glu-BSA and Gal-BSA were 16.9 kU/g and 17.0 kU/g respectively), for 24 h and 72 h (A), and secreted IL-1b in culture supernatants in a concentration-dependent manner after 72 h treatment with Gal-BSA (B). n=3 independent experiments. Mean±SD. ^cP<0.01 *vs* BSA.

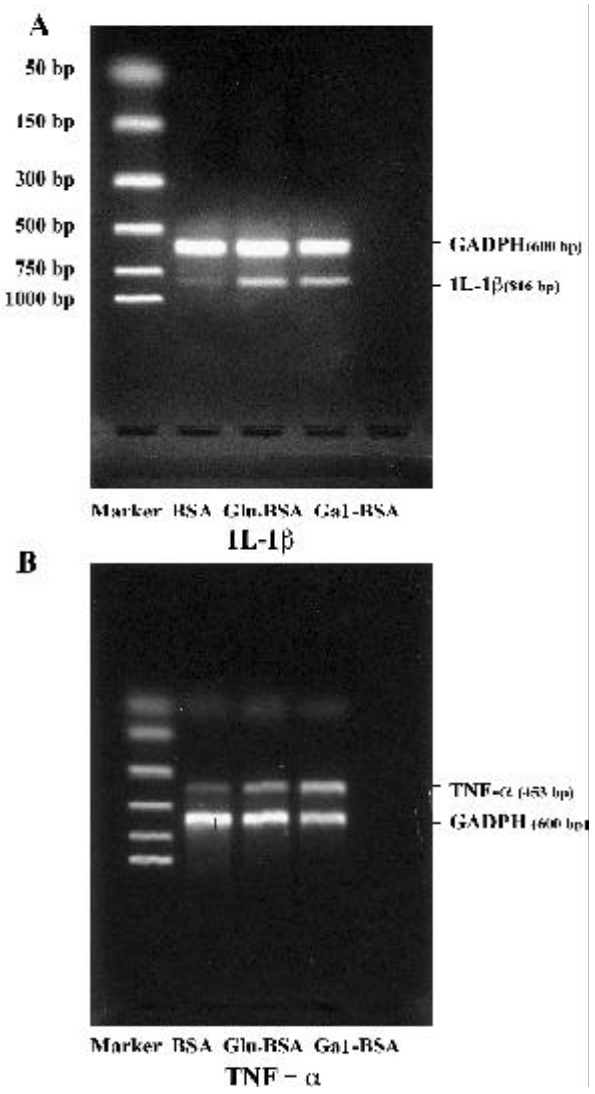

Fig 2. mRNA levels of IL-1b (A) and TNF-a (B) detected by RT-PCR in astrocytes treated by BSA control and AGE-BSA (1 g/L) for 72 h. Figures are representative results from two experiments.

increased the levels of secreted TNF-α protein by 6.3 % and 27.7 % respectively, compared with control. After 72 h, TNF-α levels in supernatants and cell lysates were raised respectively by 69.2 % and 44.8 % by Glu-BSA, and 107.9 % and 50 % by Gal-BSA, as shown in Fig 3A. Also the level of secreted TNF-α was concentration-dependent on exogenous Gal-BSA (Fig 3B). RT-PCR further confirmed the induction of TNF-α mRNA by AGE-BSA (Fig 2B).

Reduced GSH level by AGE-BSA After 18 h treatment, AGE-BSA 0.1 g/L slightly decreased GSH level, while AGE-BSA 1 g/L significantly decreased GSH level by 35 % and 43 % when compared to BSA control (Fig 4). This indicated that AGE-BSA might be

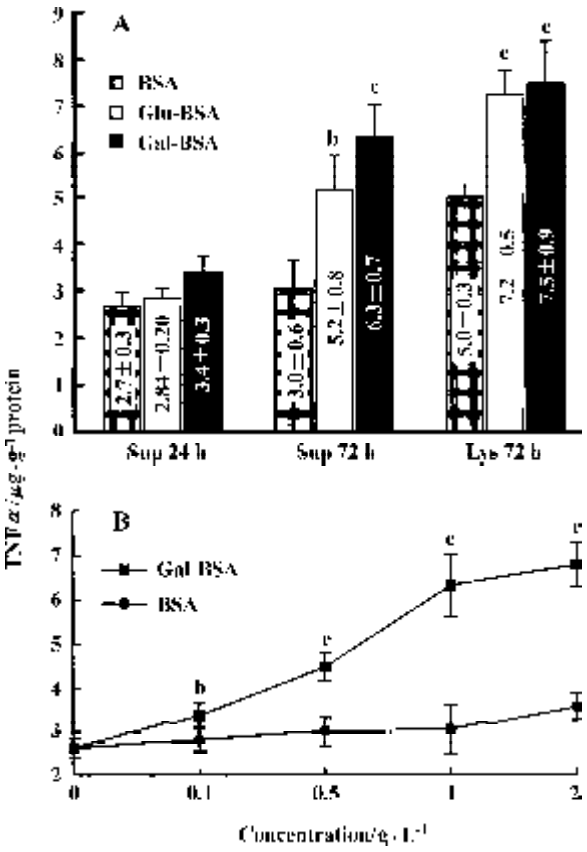

Fig 3. TNF-a levels in cultured supernatants and lysates of astrocytes treated with BSA control or AGE-BSA 1 g/L (where AGE contents in Glu-BSA and Gal-BSA were 16.9 kU/g and 17.0 kU/g respectively) for 24 h and 72 h (A), and secreted TNF-a production in culture supernatants in a concentration-dependent manner after 72 h treatment with Gal-BSA (B). *n*=3 independent experiments. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* BSA.

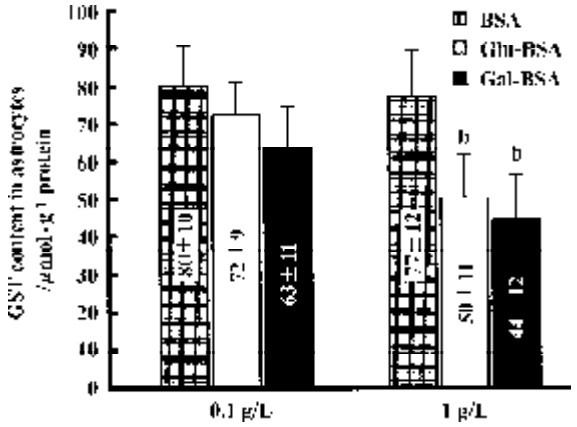

Fig 4. Reduced GSH content after AGE-BSA treatment for 18 h. *n*=3 independent experiments. Mean±SD. $^{b}P<0.05$ *vs* BSA.

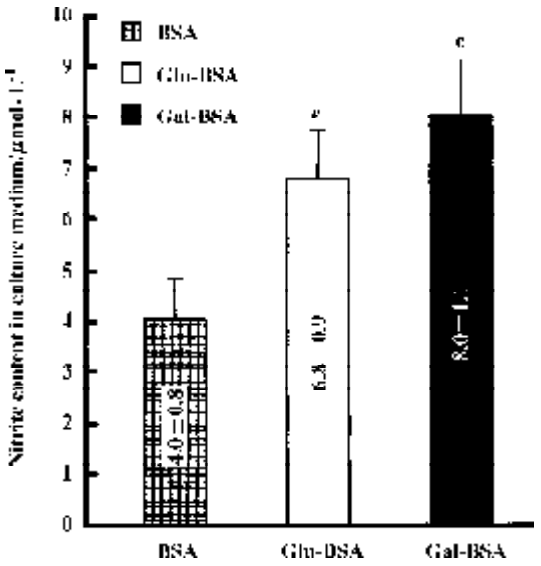

Fig 5. NO levels released by AGE-BSA (1 g/L) stimulation for 18 h. *n*=3 independent experiments. Mean±SD. $^{c}P<0.01$ *vs* BSA.

the inducers of oxidative stress that reduced GSH.

NO release by AGE-BSA As shown in Fig 5, both kinds of AGE-BSA could stimulate NO release after 18 h incubation. Specifically, Glu-BSA and Gal-BSA 1 g/L stimulation could produce 1.7 and 2 folds of NO level, respectively.

Expression of receptor of AGE in astrocytes RAGE is a kind of receptor for AGE. The existence of RAGE in astrocytes was detected (Fig 6). It seems that both kinds of AGE could decrease the expression of RAGE in astrocytes.

DISCUSSION

Although our previous study[2] showed that AGE *in vivo* contributed to *D*-galactose-induced mouse aging, the direct evidence between AGE and brain aging is not well shown. Some studies suggested that AGE might be a driving force in the acceleration of β-amyloid deposition and plaque formation in AD[3]. The direct role of AGE in dementia disorders has only been demonstrated by studies showing that AGE-modified *tau* induced neuronal oxidative stress and NF-κB regulated gene products like IL-1β, IL-6, TGF, and TNF-α. However, microglial and astrocytes other than neurons have been identified as major brain-derived sources of inflammatory mediator. Our results demonstrated that two kinds of AGE-BSA enhanced both astrocytic expression and secretion of IL-1β and TNF-α. AGE have been shown to induce inflammatory processes in

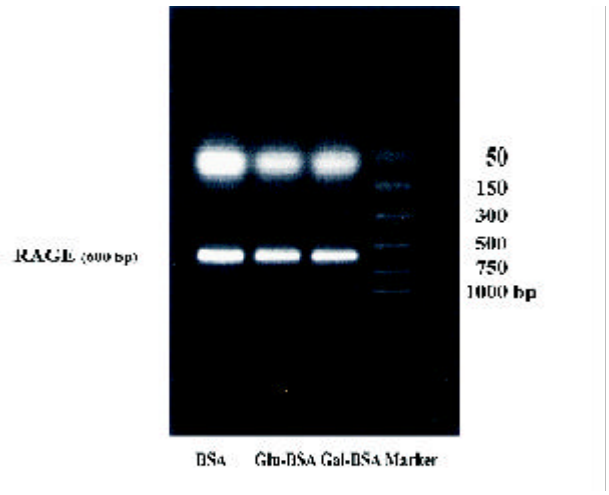

Fig 6. mRNA levels of RAGE detected by RT-PCR in astrocytes treated by BSA control and AGE-BSA (1 g/L) for 72 h. RT-PCR products of RAGE 10 μL were loaded into a 2 % agarose gel and observed by 0.5 % ethidium bromide staining.

regions other than the brain such as peripheral macrophages and mononuclear phagocytes, and the role of the inflammatory cytokines in these regions focuses on normal tissue remodeling. However, the induction of astrocytic IL-1β and TNF-α by AGE-BSA might contribute to the progress of neuropathy in aging process and age-related dementia diseases. For example, IL-1β administration and agents that induce central IL-1β activity can impair the consolidation of memories that depend on the hippocampal formation[11]. As far as AD is concerned, IL-1β can interfere with neuronal acetylcholinesterase activity and induce neuronal synthesis and processing of Aβ[5]. Furthermore, IL-1β acts on astrocytes (autocrine) to promote themselves activation and promote increased astrocytic expression of S100 and β-antichymotrpsin associated with neuritic plaque. It also acts on microglial (paracrine) to promote activation and further stimulation of IL-1β expression[5]. So once up-regulated, IL-1β can initiate a self-propagating cascade of detrimental cellular and molecular events known as cytokine cycle. Our results demonstrated that AGE played an important role as an "activator" of this vicious cycle.

It is important to point out that cytokines rarely work in isolation. For example, IL-1β release is normally associated with the release of the other cytokines like TNF-α. TNF-α is also closely related to brain aging. Some studies have documented neurotoxicity of TNF-α, and high levels of TNF-α are directly related to dementia[12]. However, some reports suggested contrasting role for TNF-α in injured brain. For example, TNF-α facilitates exon elongation through injured nerve and protects neurons from damage induced by glutamate, suggesting that TNF-α might play a role in brain's defense against neuronal damage. One explanation for this apparent discrepancies is that TNF-α is directly beneficial to neuronal viability but that TNF-α can evoke responses from glia that are harmful to neurons[13]. Apparently the effect of TNF-α promoted by AGE-BSA in our report here belongs to the latter one.

A number of studies confirmed that AGE were inducers of oxidative stress[14]. We also found that AGE acted on astrocytes resulting in depletion of one of cellular antioxidant defense mechanisms, reduced GSH, indicating the increase of cellular oxidative stress. This may explain in part why cellular GSH tended to decrease with age. 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 neurons[15]. Although TNF-α and IL-1β can both contribute to NO production, in our experiments these two cytokines might have little relation with NO production, because significant induction of NO was earlier than that of both cytokines. Thus NO production could be the direct effect of AGE on astroglia.

Therefore we conclude that accumulation of AGE in aging brain and other neurodegenerative conditions helps to trigger astrocytes to become more reactive and to synthesize and secrete toxic molecules which contribute to neurodegeneration.

How could AGE exert toxic effects to cells? As one of their cell-surface receptors, RAGE might be a possible mediator[16], since ligation of RAGE enhanced NF-κB-dependent transcription, the upstream signal of cytokines activation[16]. This also may explain why AGE and Aβ always have similar effects[6]. Using RT-PCR we detected existence of RAGE in primary astrocytes (Fig 6), which seemed down-regulated by AGE. This might be a self-protecting reaction in astrocytes against AGE. But others[17] reported that AGE enhanced RAGE expression. Further evidence needs to be shown to confirm this trend.

Taken all together, understanding more of AGE's effects on nervous system will help to develop new therapies to retard aging process or improve dementic symptom.

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