• Basic Research •

Effect of thioltransferase on oxidative stress induced by high glucose and advanced glycation end products in human lens epithelial cells

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Abstract

● **AIM:** To study the effect of thioltransferase (TTase) on oxidative stress in human lens epithelial cells (HLECs) induced by high glucose and advanced glycation end products (AGEs).

● **METHODS:** HLECs were treated with 35.5 mmol/L glucose or 1.5 mg/mL AGEs modified bovine serum albumin (AGEs-BSA) as the experimental groups, respectively. Cells were collected at the time point of 1, 2, 3, and 4d. The TTase activity were measured accordingly. TTase mRNA levels were detected by quantitative reverse transcription polymerase chain response (qRT-RCR) and its protein level was detected by Western blot. The siRNA was used to knock down the expression of TTase. The activity of catalase (CAT) and superoxide dismutase (SOD), the content of reactive oxygen species (ROS) and the ratio of oxidized glutathione/total glutathione (GSSG/T-GSH) were assessed in different groups, respectively.

● **RESULTS:** The level of TTase mRNA gradually increased and reached the top at 2d, then it decreased to the normal level at 4d, and the TTase activity increased from 2 to 3d in both high glucose and AGEs-BSA groups. The TTase expression elevated from 2d in high glucose group, and it began to rise from 3d in AGEs-BSA group. The activity of CAT and SOD showed a decrease and the content of ROS and the ratio of GSSG/T-GSH showed an increase in high glucose and AGEs-BSA group. These biochemical alterations were more prominent in the groups with TTase siRNA.

● **CONCLUSION:** High glucose and AGEs can increase ROS content in HLECs; therefore, it induces oxidative stress. This may result in the decreased GSH and increased GSSG content, impaired activity of SOD and CAT. The up-regulated TTase likely provides oxidation damage repair induced by high glucose and AGEs in the early stage.

● **KEYWORDS:** thioltransferase; oxidative stress; glucose; advanced glycation end products

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INTRODUCTION

Diabetes mellitus (DM) is a common endocrine disorder characterized by hyperglycemia and predisposes to chronic complications affecting the eyes, blood vessels, nerves and kidneys[1]. DM can lead to pathologies in many tissues in the eye structure, with both a systemic chronic metabolic disease and a microangiopathic character[2]. As the main cause of blindness, cataract is also considered a major cause of visual impairment in diabetic patients as the incidence and progression of cataract are higher in patients with DM[3-6]. The process of cataractogenesis in diabetic patients occurs at a much earlier age than senile cataract[6-7]. Although surgery is the only effective treatment for cataract, visual outcomes following cataract surgery among cases with DM were poorer compared to cases without DM due to higher incidence of intraoperative and postoperative complications

of DM patients[8-13]. DM is characterized by elevated levels of glucose, which starts forming covalent adducts with plasma proteins through a nonenzymatic process known as glycation. It causes accumulation of advanced glycation end products (AGEs) which are the toxic byproducts of the nonenzymatic reaction[14]. Glycation of lens protein has been considered to be one of the mechanisms responsible for diabetic cataract, which is the leading cause of blindness. It has been suggested that glycation of lens crystalline may cause conformational changes resulting in exposure of thiol groups to oxidation and cross-link formation, thus contribute to cataract formation in diabetes.

Thioltransferase (TTase), also known as glutaredoxin, is a thiol-disulfide oxidoreductase which specifically reduces glutathionylated proteins (PSSG) to protein-SH, with glutathione (GSH) as its cofactor. TTase is present ubiquitously in eukaryotic and prokaryotic cells and it has cytosolic (TTase-1 or Grx1) and mitochondrial (TTase-2 or Grx2) isoforms. TTase-1 is a 11.8 kDa, heat-stable cytosolic protein with multiple catalytic functions for many biochemical processes. TTase has been found in most ocular tissues, and it was concentrated in the anterior segment of the eye which was vulnerable to oxidative damage[15]. Raghavachari and Lou[16] first reported that TTase was present in the lens with structural and functional characteristics similar to TTase from other tissues, and lens epithelium showed about 3-4 times higher activity than cortex and nucleus which contained similar activity of TTase. Qiao *et al*[17] found that TTase showed a well tolerance to high temperature and oxidative stress induce by H_2O_2. Our previous study has shown TTase can repair the H_2O_2-damaged glyceraldehyde 3-phospho-dehydrogenase, a key glycolytic enzyme for ATP production, in human lens epithelial cells (HLECs). Along with alpha-crystallin, TTase can partially revive glutathione reductase (GR) activity in cataract or clear human aged lenses[18]. Löfgren *et al*[19] found that the antioxidative stress ability of lens epithelial cells from the TTase knockout mice decreased, while it restored to nearly normal by introducing the recombinant TTase into lens epithelial cells. TTase plays an important role in regulating redox homeostasis. Many studies have shown the powerful oxidation defense of TTase can be achieved through its up-regulated gene expression during the early stage of oxidative stress. H_2O_2 can induce TTase gene expression in both HLECs and pig lenses in culture[20-22]. This alteration was also found in mouse lens with ultraviolet radiation in our previous study[23].

Multiple mechanisms have been proposed for the pathogenesis of cataract in DM, including protein glycation[24-25], osmotic stress[26], oxidative stress[27] and more recently autoimmunity[28]. Although it has been shown that the intracellular accumulation of sorbitol converted from glucose in the presence of aldose reductase (AR) through the polyol pathway leads to osmotic changes resulting in hydropic lens fibers and apoptosis of lens epithelial cells that form cataracts[29-30], studies have demonstrated that protein glycation, in combination with oxidative stress might play major roles in hyperglycemic-induced cataracts due to human lenses have a very low activity of AR[31]. Osmotic stress, however, could act synergistically with other factors[32]. It has been suggested that glycation of lens crystallin may cause conformational changes resulting in exposure of thiol groups to oxidation and cross-link formation[25]. Furthermore, the lens crystallin have virtually no turnover and readily accumulate AGEs which in turn cause aggregation of the lens crystallin producing the high molecular weight material responsible for opacification[33]. Hence, identification of the potential role of TTase in diabetic cataract is of great importance for prevention and treatment of the blindness. In this study, we intend to examine the effects of high glucose and AGEs on expression and activity of TTase in human lens epithelial cell line (HLE-B3), and explore the possible mechanism of TTase to counteract the oxidative stress induced by them *via* TTase RNA interference. Our findings suggest that TTase might play a protective role in oxidative stress induced by high glucose and AGEs in the early stage.

MATERIALS AND METHODS

Materials Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from HyClone (USA). Trypsin 0.25% was from Gibco (USA). D-glucose, mannitol, GR, GSH, hydroxyethyldisulfide (HEDS), NADPH, were purchased from Sigma-Aldrich (USA). Bovine serum albumin (BSA, nonglycated control of AGE) and AGEs modified BSA (AGEs-BSA) were obtained from Bio Vision (USA). Rabbit polyclonal antibody for human TTase, rabbit polyclonal antibody for β-actin were purchased from Abcam (Cambrige, UK). Goat antibody for rabbit IgG was from LI-COR (IRDye650, USA). Enzyme and protein quantification kits were obtained from Beyotime Biotechnology (Shanghai, China). All other chemicals and reagents were standard commercial products of analytical grade.

Cell Culture and Treatment HLE-B3, immortalized by infecting with adenovirus 12-SV40, was generously provided by Zhongshan Ophthalmic Center, Sun Yat-sen University. The cells were grown in DMEM medium supplemented with 10% FBS in 6-well culture plates in humid atmosphere with 5% CO_2 at 37°C. The culture medium was changed every other day. Cells reached confluence within 4d. They were trypsinized and seeded in 6-well plates per well as follows: culture medium containing 5.5 mmol/L glucose (control), culture medium containing 35.5 mmol/L glucose or mannitol, culture medium containing 1.5 mg/mL BSA or 1.5 mg/mL modified bovine serum albumin (AGEs-BSA) with 5.5 mmol/L glucose.

The culture medium was renewed every day. The cells were washed and harvested everyday ranging from 1 to 4d. The cells collected were lysed by RIPA buffer and centrifuged. The supernatant was saved at -80℃ for further analysis.

siRNA Transfection The small interfering RNA (siRNA) duplexes used in this study were chemically synthesized by GenePharma (Shanghai, China). 5'-GAGUCUUUAUUGGUAAAGATT-3' and 5'-UCUUUACCAAUAAAGACUCTT-3' were used for repressing TTase expression according to our pre-experiment. siRNA duplexes were transfected into HLE-B3 cells with siRNA transfection reagents (Lipofectamine 2000, Invitrogen, USA; Opti-MEM, Gibco, USA) in line with manufacturer's instructions. After 6h of transfection, different treatments were given as mentioned above.

Quantitative Reverse Transcription Polymerase Chain Reaction The specific oligonucleotide primers which were synthesized at Takara (Dalian, China) are showed in Table 1. For qPCR, total RNA was isolated using Trizol (Invitrogen, USA) and converted to cDNA immediately using the PrimeScript™ RT Master mix (Perfect Real-Time; Takara, Dalian, China) following the manufacturer's instructions and stored at -20℃ until use. Each cDNA was amplified with the previously listed primers and SYBR Premix Ex Taq™ II (Tli RNaseH Plus; Takara) for 40 cycles, and results were analyzed using the Mx3000P System Software (ABI Stratagene, La Jolla, CA, USA).

Western Blot Analysis Cells were harvested and washed with phosphate-buffered saline (PBS). The lysates were achieved through RIPA buffer (Beyotime Biotechnology, China) with 1 mmol/L protease inhibitor (PMSF, Beyotime Biotechnology, China), followed with centrifugation (12 000 rpm, 4℃, 20min). Total protein concentration in the supernatant was determined with Bicinchoninic Acid assay kit (Beyotime Biotechnology, China). Samples with equal amounts of total protein were separated by 15% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA), which were then blocked for 1h at room temperature in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% fat-free milk. Membranes were then incubated with anti-TTase (1:250), anti-β-actin (1:2000) in PBST at 4℃ overnight. The membranes were then incubated with secondary anti-IgG antibody (1:10 000) at 4℃ for 1h in dark environment and washed with TBST three times. The immunoblot was analyzed and visualized with an infrared imaging system (Odyssey, LI-COR, USA).

Enzyme Assays TTase activity was determined with HEDS as substrate in the presence of GSH, following the method of Raghavachari and Lou[16]. Enzyme activities of catalase (CAT) and superoxide dismutase (SOD) were detected separately with CAT assay kit (Beyotime, China) and total SOD assay kit with NBT (Beyotime, China), according to the manufacturer's instructions.

Table 1 Sequences of forward and reverse primers used in qRT-PCR

RNA species	Primer pairs
TTase	Forward 5'-CCTGGGAAGGTGGTTGTGTT-3'
	Reverse 5'-TTAGTGTGGTTGGTGGCTGTG-3'
GAPDH	Forward 5'-GTGGGGCGCCCCAGGCACCA-3'
	Reverse 5'-CTCCTTAATGTCACGCACGATTT-3'

Reactive Oxygen Species Assay Total intracellular reactive oxygen species (ROS) was determined by staining cells with dichlorofluorescin diacetate (DCFH-DA, Beyotime Biotechnology, China). Briefly, cells were washed with PBS and incubated with 10 μmol/L DCFH-DA at 37℃ for 20min. Cells were then washed three times with PBS and trypsinized and resuspended in PBS. The fluorescence was analyzed by multi-mode microplate reader (Synergy HTX, BioTek, USA) with excitation at 488 nm and emission at 525 nm.

GSH Disulfide and Total GSH Assay As oxidative factors, GSH disulfide (oxidized GSH, GSSG) and total GSH were (T-GSH) detected using GSSG/T-GSH assay kit (Beyotime Biotechnology, China) according to the manufacturer's instructions. After detection, GSSG/T-GSH ratio was calculated.

Statistical Analysis All data were expressed as the mean±standard deviation (SD). Statistical analysis of the data was subject to one-way ANOVA followed by Dunnett's *T* test. All statistical calculations were performed using SPSS version 19 software. Significance level was set at $P<0.05$.

RESULTS

Effect of High Glucose and AGEs on TTase mRNA Expression of HLECs Figures 1 and 2 summarized the relative TTase mRNA expression in cells incubated with high glucose and AGEs-BSA, respectively. As shown in Figure 1, there was a sharp increase in TTase mRNA expression on the 1st day in cells treated with high glucose, about 6.24 times of control ($P<0.05$). It gradually increased and reached the peak on the 2nd day, about 9.28 times of control ($P<0.01$). On the 3rd day, it fell to 3.12 times of control ($P<0.05$) and returned to baseline on the 4th day. We set the mannitol group here to eliminate the influence of osmotic pressure, and there were no significant differences between the mannitol group and the normal control group. As shown in Figure 2, after AGEs-BSA treatment, the expression of TTase mRNA in HLECs increased significantly on the 2nd day, about 5.06 times of that in the normal control group ($P<0.01$), and began to decrease on the 3rd day of incubation, but it was still 3.53 times higher than that of the normal control group ($P<0.01$). BSA group was set as the nonglycated control of AGE, and there were no differences between the BSA group and the control group.

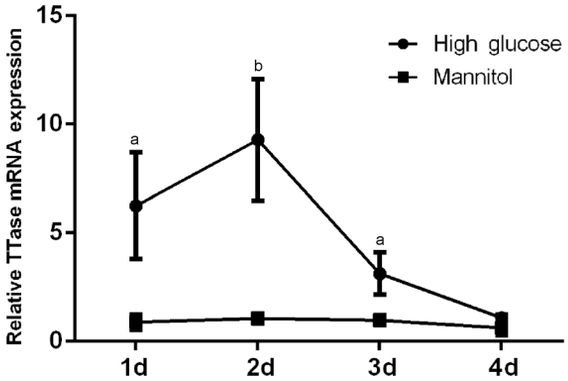

Figure 1 Effect of high glucose on TTase mRNA expression In high glucose group, TTase mRNA tended to be up-regulated on the 1st day, and reached the peak on the 2nd day compared with the normal control group. Mannitol group was set to eliminate the influence of osmotic pressure. $^{a}P<0.05$, $^{b}P<0.01$ *vs* control group; $n=3$.

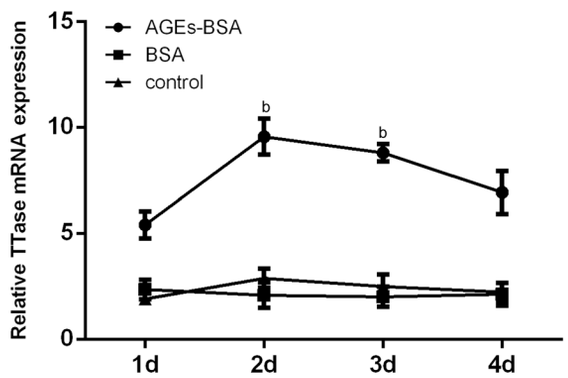

Figure 2 Effect of AGEs on TTase mRNA expression In AGEs-BSA group, the tendency of TTase mRNA expression had reached the peak on the 2nd day. There were no differences between the BSA group and the control group. $^{b}P<0.01$ *vs* control group; $n=3$.

Effect of High Glucose and AGEs on TTase Activities of HLECs The specific activity of TTase in both groups is shown in Figure 3. In high glucose group, TTase activities were 8.37±1.24, 10.19±0.66, 11.23±1.75, and 10.50±0.70 mU/mg protein from the 1st day to the 4th day, respectively. On the 2nd, 3rd and 4th day, the activity of TTase was significantly higher than that of the normal control groups (approximate 1.52, 1.68, and 1.57 times, respectively). In AGEs-BSA group, TTase activities were 8.03±0.86, 10.82±0.51, 11.05±0.83, and 8.63±0.77 mU/mg protein from the 1st day to the 4th day, respectively. On the 2nd and 3rd day, the activity of TTase was 1.53 and 1.69 times higher than that of the normal control group, and the differences were significant.

Effect of High Glucose and AGEs on TTase Expression of HLECs To further examine the expression level of TTase protein in both groups, Western blot analysis was performed by using specific antibody for human TTase. In high glucose group, the bands gradually increased in intensity from the 2nd to the 4th day (Figure 4A). Similarly, the bands in AGEs-BSA group intensified on the 3rd and 4th day (Figure 4B).

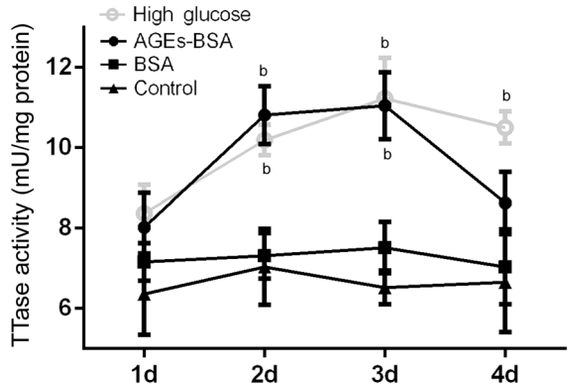

Figure 3 TTase activity in high glucose group and AGEs-BSA group Contrasted with the control group, TTase activity in both high glucose group and AGEs-BSA group increased significantly on the 2nd day, and reached the peak on the 3rd day. $^{b}P<0.01$ *vs* control group; $n=3$.

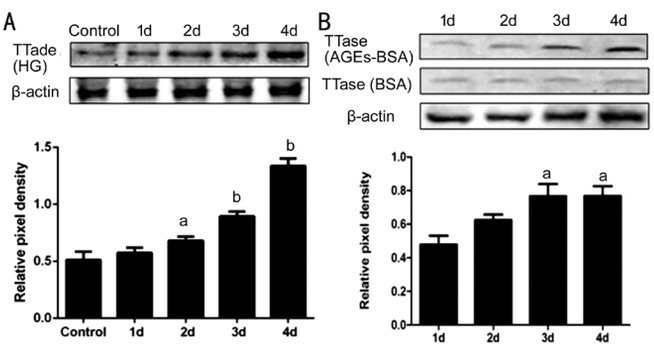

Figure 4 The tendency of TTase expression in HLECs A: Western blot analysis showed TTase expression started to rise from the 2nd day in high glucose group compared with the control group; B: Western blot analysis showed TTase expression started to elevate from the 3rd day in AGEs-BSA group compared with the control group. $^{a}P<0.05$, $^{b}P<0.01$ *vs* control group; $n=3$.

Effect of High Glucose and AGEs on the Activities of Oxidative Defense Enzymes and ROS Content in HLECs To preliminarily evaluate the role of TTase in oxidative stress induced by high glucose and AGEs, TTase siRNA was used to transfected into HLECs to knock down TTase expression. After incubation for 3d, cells were collected for the following analysis. We chose 3d as the time point because TTase protein expression and its activity were relatively higher than other days when incubated with high glucose or AGEs. The mean activity of CAT in high glucose group (8.33±1.14 U/mg protein) and AGEs-BSA group (9.27±0.73 U/mg protein) was significantly lower than that of normal control group (12.13±1.39 and 15.07±0.78 U/mg protein). The lowest activity of CAT was observed in high glucose+TTase siRNA group (4.46±0.74 U/mg protein) and AGEs-BSA+TTase siRNA group (4.01±0.94 U/mg protein) when compared with their negative control counterpart (7.71±0.80 and 9.01±1.52 U/mg protein), respectively (Figure 5A and 5B). The activity of SOD showed the similar tendency to CAT. As shown in Figure 5C

and 5D, the mean activity of SOD were 114.80±9.49, 88.17±7.92, 90.76±12.03, and 47.15±5.41 U/mg protein in high glucose related groups, and 119.77±8.42, 92.98±8.98, 79.78±6.05, and 54.15±9.06 U/mg protein in AGEs related groups. ROS accumulation was measured by the molecular indicator DCF and quantified in relative fluorescence units. As shown in Figure 5E and 5F, ROS produced in the high glucose group (186.30±11.59) and the AGEs-BSA group (190.30±9.84) was much higher compared with its control group (155.70±11.50 and 93.33±21.85). More importantly, the ROS accumulation in high glucose+TTase siRNA group (265.30±16.50) and AGEs-BSA+TTase siRNA group (280.32±18.35) was more conspicuous than that in their counterpart group with siRNA negative control (200.70±8.08 and 188.02±19.31).

Effect of High Glucose and AGEs-BSA on GSSG/T-GSH Ratio The GSSG/T-GSH ratio indicates the redox status of the cells. Based on our previous results, we hypothesized GSSG would accumulate in HLECs when incubated with high glucose or AGEs, which resulted in the rise of the GSSG/T-GSH ratio. The results confirmed our conjecture. As shown in Figure 6A and 6B, The ratio was (8.97±0.95)% and (11.35±0.62)% in high glucose group and AGEs-BSA group, respectively, which was increased significantly than that of counterpart control [(3.81±1.08)% and (3.20±0.47)%]. Moreover, by knocking down of TTase, the ratio raised much higher with significant differences compared with their siRNA negative control. It was (14.15±1.48)% in high glucose+TTase siRNA group and (16.45±0.64)% in AGEs-BSA+TTase siRNA group. The (7.96±1.75)% and (10.86±1.50)% were the ratio of their TTase siRNA negative control counterpart.

DISCUSSION

It is well established that the reduced state of lens crystallin plays an important role in maintaining its transparency. Under physiological condition, the lens is in the dynamic state of redox balance. There are two sets of strong antioxidant systems in the lens to maintain the stability of redox: one is the high level of antioxidants in the cell, including GSH, vitamin C, vitamin E, carotenoid, *etc*, and the other is endogenous antioxidant enzymes and repair enzymes[34]. As one of these repair enzymes, TTase plays an important role in repairing protein thiol, resisting oxidative stress and maintaining the reduced state of lens. Changes associated with oxidative damage and with restoration of cellular homeostasis often lead to activation or silencing of genes encoding regulatory transcription factors, antioxidant defense enzymes, and structural proteins[35]. Raghavachari *et al*[36] have demonstrated that both TTase mRNA level and enzyme activity were doubled when the HLECs were exposed to a low level of H_2O_2, and then followed by a gradual down-regulation of both when the oxidant in the culture media is totally detoxified.

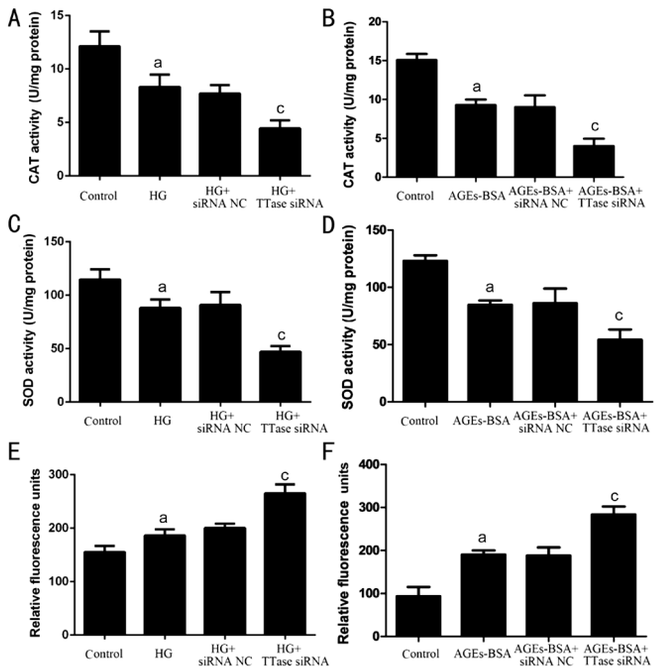

Figure 5 Effect of high glucose and AGEs-BSA on the activities of oxidative defense enzymes and ROS content in HLECs CAT (A and B) and SOD (C and D) activities were obviously lowered in both high glucose group and AGEs-BSA group. ROS content (E and F) elevated in both high glucose group and AGEs-BSA group. These biochemical alterations were more prominent in the groups with TTase siRNA. [a]P<0.05 *vs* control group, [c]P<0.05 *vs* high glucose+siRNA negative control group or AGEs-BSA+siRNA negative control group; n=3.

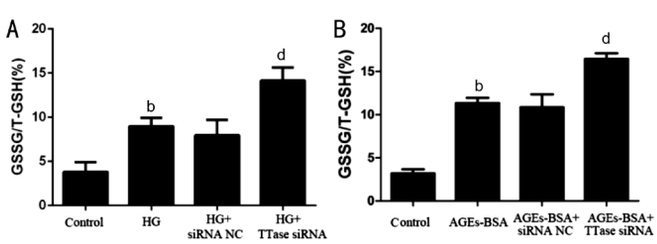

Figure 6 Effect of high glucose and AGEs-BSA on GSSG/T-GSH ratio GSSG/T-GSH increased in both high glucose and AGEs-BSA group, and the ratio was much higher in the groups with TTase siRNA. [b]P<0.01 *vs* control group, [d]P<0.01 *vs* high glucose+siRNA negative control or AGEs-BSA+siRNA negative control; n=3.

Our group has previously reported TTase in the lens of young mice showed a transient increase during the initial insult of ultraviolet exposure and gradually returned to baseline at day 8[23]. Similar to the findings reported before, our experiment showed the up-regulation of TTase in both high glucose and AGEs-BSA groups in the early stage. This suggests that the HLECs have a very sensitive mechanism that responds to the need for protection and repair of oxidizable sulfhydryl groups of proteins by a rapid up-regulation of TTase gene expression to dethiolate and restore the functions of the damaged enzymes and other proteins. We speculate that the up-regulation of

TTase expression in lens epithelial cells may be an adaptive response of the cells to combat oxidative stress in order to restore vital functions of lens proteins and enzymes. This oxidative stress existed persistently and celluar antioxidants exhausted, which resulted to the gradual decrease of TTase mRNA and its activity. TTase protein showed an increasing trend during 4d, but the decrease was not obvious. This may be due to the protein expression slightly lagged behind the mRNA alteration.

ROS, including superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2), are toxic, harmful by-products of living in an aerobic environment[37]. As the primary antioxidant enzymes in the lens, SOD specifically catalyses superoxide radicals to hydrogen peroxide which is in turn catalyzed into ground-state oxygen and water by CAT. Studies have shown both SOD and CAT can be inactivated by glucose and glycation in a time-dependent manner[38]. SOD and CAT in lenses from diabetic cataract patients showed lower activities compared with lenses from senile cataractous subjects, and increased production of high levels of ROS is linked to glucose oxidation and non-enzymatic glycation of proteins[39]. The lens from SOD1 knock out mice developed more cataract and showed raised biochemical markers of oxidative damage after exposure to high glucose *in vitro* or in streptozotocin-induced DM *in vivo*[40-41]. Overexpression of SOD in intact lenses could prevent cataract formation induced by oxidative stress[42]. In our study, the mean activities of SOD and CAT in HLECs significantly decreased and ROS content raised obviously in the groups treated with high glucose or AGEs-BSA, and these alterations were exaggerated in groups with down-regulation of TTase. This indicates high glucose and AGEs increases ROS production in HLECs, which is responsible for the inactivation of SOD and CAT. TTase can inhibit the generation of ROS in HLECs in an indirect way, thus protecting the cells from oxidative stress.

As the first line of defense against oxidative stress, GSH plays a vital role in the protection of lens[43]. There is a large amount of GSH in the lens, especially in the epithelium and outer cortex of human lens[44]. Studies have shown GSH significantly decrease in human age-related nuclear and cortical cataract[45]. GSH can be oxidized to GSSG when it contacts with oxidants, and GSSG can be reduced to GSH under the catalysis of GR. Evidence has shown TTase can partially revive GR activity in cataract or clear human aged lenses[18]. If this cycle is disturbed, GSSG accumulates and the dimer can oxidize a neighboring protein thiol nonenzymatically to form protein-S-S-glutathione (PSSG) which is believed to represent an early cataractous state[46]. TTase may be expected to play an important role in this protection by maintaining reduced states of thiols in proteins and by reducing their glutathionylated cysteine residues with the concurrent oxidation of GSH to GSSG[44]. Under normal physiological conditions, the GSSG/T-GSH ratio maintains at a low level since the intracellular milieu is predominately in a reduced state. When incubated with high glucose or AGEs-BSA, HLECs showed an obvious increase in the GSSG/T-GSH ratio, and more significant rise in the cells with down-regulation of TTase, indicating that GSH was indeed severely depleted by oxidative stress together with the loss of TTase. We also speculate that GR activity might be impaired by the oxidative stress and the absence of TTase in the cells might weaken the repair effect on GR, but these need further evidence.

Our previous studies have indicated that oxidative stress occurred in the mouse lens under hyperglycemia conditions both *in vitro* and *in vivo*. To emphasize the involvement of TTase in diabetes-induced cataract, we use TTase knock out mice but TTase appears to play a minor role in hyperglycemia induced cataract than in senile cataract[47]. This may partly because the diabetic duration is not long enough, and the lens are less sensitive to oxidative stress induced by hyperglycemia or AGEs than the cell lines. Although our present study suggests that oxidative stress occurs in HLECs when incubated with high glucose and AGEs, and TTase plays an important role in protecting HLECs from oxidative damage through its involvement in reducing ROS level initially, there are still many questions to explore. Whether TTase is involved in the repair of antioxidant enzymes under hyperglycemia condition? Are there any differences of total cellular protein-thiol mixed disulfides (PSSG) among these groups? All these subjects deserve more attention for future studies.

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