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Kidney International, Vol. 63 (2003), pp. 464–473

# Advanced glycation end products inhibit de novo protein synthesis and induce TGF-β overexpression in proximal tubular cells

**SHO-ICHI YAMAGISHI, YOSUKE INAGAKI, TAMAMI OKAMOTO, SHINJIRO AMANO, KOHACHIRO KOGA, and MASAYOSHI TAKEUCHI**

*Division of Endocrinology and Metabolism, Department of Medicine, Kurume University School of Medicine, Kurume, and the Department of Biochemistry, Faculty of Pharmaceutical Science, Hokuriku University, Kanazawa, Japan*

**Advanced glycation end products inhibit de novo protein synthesis and induce TGF-β overexpression in proximal tubular cells.**

*Background.* We have shown previously that OPB-9195, a novel inhibitor of advanced glycation end products (AGE), significantly prevented renal tubular injury and tubulointerstitial fibrosis in spontaneous diabetic rats. However, the molecular mechanisms underlying this have not been fully elucidated.

*Methods.* Three immunochemically distinct AGE were prepared by incubating bovine serum albumin (BSA) with glucose, glyceraldehyde, or methylglyoxal. Then, the effects of AGE on human proximal tubular epithelial cells were examined. The intracellular formation of reactive oxygen species (ROS) was detected using the fluorescent probe CM-$H_2$DCFDA. DNA synthesis was evaluated by thymidine uptake, and de novo protein synthesis was determined by [$^3$H]leucine incorporation. Prostaglandin $E_2$ ($PGE_2$) and transforming growth factor-β (TGF-β) released into media were quantitatively analyzed in an enzyme-linked immunosorbent assay. TGF-β gene expression was analyzed by quantitative reverse transcription-polymerase chain reaction (RT-PCR).

*Results.* When these AGE-BSA were administered to tubular cells, each of them increased generation of intracellular ROS. All of the AGE-BSA, but not non-glycated BSA, were found to induce statistically significant decreases in de novo protein synthesis and $PGE_2$ secretion by tubular cells. Furthermore, AGE-BSA up-regulated the levels of mRNAs for TGF-β in tubular cells. The structural epitope designated glucose-derived AGE was found to have the greatest cytopathic effects on tubular cells. These AGE-induced inhibition of protein synthesis and $PGE_2$ secretion as well as the up-regulation of TGF-β mRNA were found to be completely prevented by *N*-acetylcysteine. Furthermore, $H_2O_2$ was shown to inhibit protein synthesis and $PGE_2$ secretion by proximal tubular cells in a dose-dependent manner.

*Conclusion.* The results suggest that AGE inhibits de novo protein synthesis and stimulates TGF-β mRNA expression in proximal tubular epithelial cells through overgeneration of intracellular ROS. Thus, AGE are involved in the pathogenesis of tubular injury in diabetic nephropathy.

**Key words:** glycation, tubular injury, diabetic nephropathy, reactive oxygen species, end-stage renal disease, prostaglandin $E_2$.

Received for publication December 3, 2001
and in revised form July 23, 2002
Accepted for publication September 10, 2002

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Diabetic nephropathy is a leading cause of end-stage renal disease, and accounts for disabilities and the high mortality rates in patients [1, 2]. Diabetic nephropathy is characterized by functional and structural changes in the glomerulus, such as glomerular hyperfiltration, thickening of glomerular basement membranes and an expansion of extracellular matrix in mesangial areas [3]. However, it has recently been recognized that changes within tubulointerstitium are more important than glomerulopathy in terms of renal prognosis in diabetic nephropathy [4–6]. These changes include proximal tubular cell atrophy and tubulointerstitial fibrosis [7, 8]. Indeed, such tubular changes have been reported to be the dominant lesion in about one third of patients with type 2 diabetes [9].

Reducing sugars, including glucose, fructose and trioses can react non-enzymatically with the amino groups of proteins to form reversible Schiff bases, and then Amadori products [10–12]. This early glycation reaction leads through complex reactions such as rearrangement, dehydration and condensation to a broad range of heterogeneous fluorescent derivatives termed advanced glycation end products (AGE) [13, 14]. The formation and accumulation of AGE in various tissues are known to irreversibly progress during normal aging and at an extremely accelerated rate in diabetes mellitus. Recent understandings of this process have confirmed that AGE are implicated in the development and progression of diabetic micro- and macrovascular complications [15–23].

We have shown previously that OPB-9195, a novel inhibitor of AGE, significantly prevented renal tubular injury and tubulointerstitial fibrosis in Otsuka Long-Evans-Tokushima Fatty rats, a model animal of type 2 diabetes [24, 25]. However, the molecular mechanisms underlying this action have not been fully elucidated. Very recently we found that AGE can arise not only from glucose, but also from short chain reducing sugars and dicarbonyl compounds in serum of diabetic patients

[26]. Therefore, for the present study, we prepared three immunochemically distinct AGE in vitro by incubating bovine serum albumin (BSA) with glucose (glu-AGE-BSA), glyceraldehyde (glycer-AGE-BSA), or methylglyoxal (methyl-AGE-BSA). Then we investigated the effects of AGE-BSA on de novo protein synthesis, prostaglandin $E_2$ ($PGE_2$) secretion and transforming growth factor-β1 (TGF-β1) gene expressions in human proximal tubular epithelial cells. We demonstrated that all of these different types of AGE cause tubular atrophy and elicit ischemic and fibrogenic damage to proximal tubular cells through intracellular overgeneration of reactive oxygen species (ROS).

## METHODS

### Materials

Bovine serum albumin (fraction V), methylglyoxal, and *N*-acetylcysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). D-glyceraldehyde and trichloroacetic acid were from Nakalai Tesque (Kyoto, Japan). D-glucose and $H_2O_2$ were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). $[^3H]$Thymidine, $[^3H]$leucine, $[\gamma\text{-}^{32}P]$ATP, Hybond-$N^+$ nylon membrane and $PGE_2$ enzyme immunoassay system were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Reverse transcriptase and T4 polynucleotide kinase were from Takara (Kyoto, Japan).

### Preparation of AGE-BSA and carboxymethyllysine (CML)-BSA

Advanced glycation end products-BSA were prepared as described previously [26]. Briefly, BSA was incubated under sterile conditions with D-glucose, D-glyceraldehyde or methylglyoxal in 0.2 mol/L phosphate buffer at 37°C for seven days, except for glucose (8-week incubation). CML-BSA was prepared by incubating BSA with glyoxylic acid and sodium cyanoborohydride as described previously [26]. Then unincorporated sugars were removed by dialysis against phosphate-buffered saline (PBS). Control non-glycated BSA was incubated in the same conditions except for the absence of reducing sugars. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable.

### Cells

Proximal tubular epithelial cells from human kidney were maintained in REBM medium supplemented with 5% fetal bovine serum (FBS), 0.5 μg/mL hydrocortisone, 10 ng/mL human epidermal growth factor (EGF), 0.5 μg/mL epinephrine, 6.5 ng/mL triiodo-L-thyronine, 10 μg/mL transferrin, 5 μg/mL insulin, and GA-1000 according to the supplier's instructions (Clonetics Corp., San Diego, CA, USA). Cells at three to five passages were used for the experiments. AGE and $H_2O_2$ treatments were carried out in a serum-free assay medium containing 10 μg/mL transferrin and GA-1000.

### Intracellular ROS

The intracellular formation of ROS was detected by using the fluorescent probe CM-$H_2$DCFDA (Molecular Probes Inc., Eugene, OR, USA) as described previously [27]. Briefly, cells ($1 \times 10^5$/96-well plates) were loaded with 10 μmol/L CM-$H_2$DCFDA, incubated for 45 minutes at 37°C, and analyzed in an EZS-FL fluorescent plate reader (Asahi Techno Glass, Tokyo, Japan) using the EZScan-FL for Windows program.

### Preparation of polyclonal antibodies against glu-AGE-BSA

Antibodies against glu-AGE-BSA were prepared as described previously [26].

### Preparation of AGE-containing serum from control and diabetic patients

Advanced glycation end products containing serum (molecular weight >10 kD) were prepared from control and diabetic patients as described previously [26].

### Measurements of $[^3H]$leucine and $[^3H]$thymidine incorporation

Cells were plated at $1 \times 10^5$ cells/well in 12-well plates. The next day, cells were rested in a serum-free assay medium for 24 hours, and then treated with or without AGE proteins in the presence or absence of various concentrations of $H_2O_2$ for 72 hours. For the last 16 hours of the culture, cells were pulsed with 2 μCi $[^3H]$leucine. After cells were washed with ice-cold PBS, they were fixed with ice-cold 10% (wt/vol) trichloroacetic acid for 15 minutes, and the resultant acid-insoluble materials were processed for liquid scintillation counting. $[^3H]$Thymidine incorporation was determined as described previously [28].

### Measurement of $PGE_2$

Cells were plated at $1 \times 10^5$ cells/well in 12-well plates. The next day, cells were rested in a serum-free assay medium for 24 hours, and then treated with or without AGE-BSA in the presence or absence of $H_2O_2$ for 48 hours. $PGE_2$ released into media was measured with an enzyme immunoassay system according to the manufacturer's instructions.

### Primers and probes

Sequences of the upstream and down stream primers and the internal probe for quantitative RT-PCR were 5′-CAGAAATACAGCAACAATTCCTGG-3′, 5′-TTG CAGTGTGTTATCCCTGCTGTC-3′ and 5′-CGATA CCTCAGCAACCGGCT-3′ (nucleotides 1358–1381, 1520–1543, and 1382–1401) for detecting human TGF-β mRNA [29]. The primers and probe used for β-actin mRNA detection were the same as described previously [30].

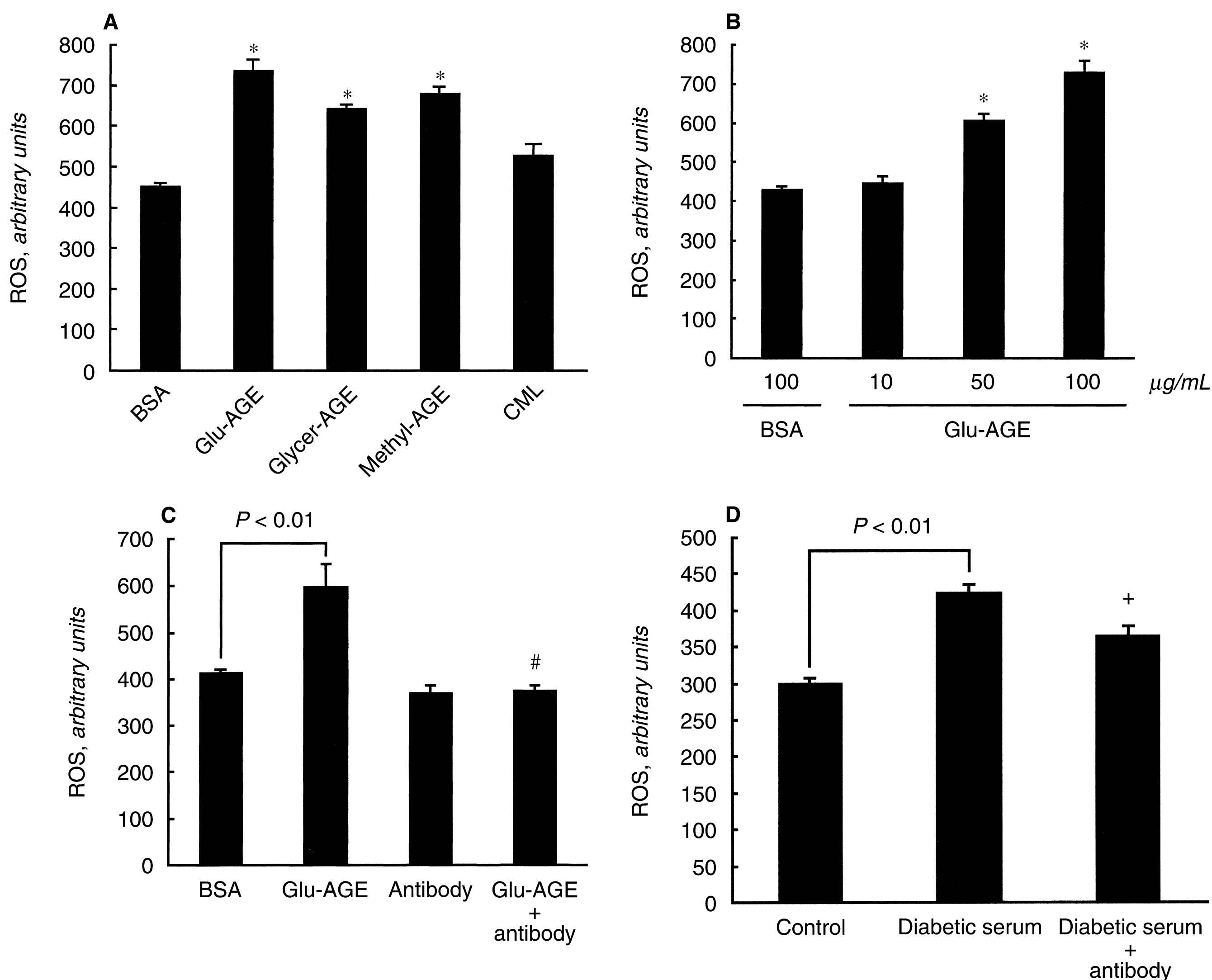


**Fig. 1. Effects of advanced glycation end products-bovine serum albumin (AGE-BSA) or diabetic serum on intracellular reactive oxygen species (ROS) generation in tubular cells.** (*A*) Cells were treated with 100 μg/mL of various types of AGE-BSA or non-glycated BSA, and then ROS were quantitatively analyzed. (*B*) Dose-effects of glu-AGE on ROS generation. (*C*) Cells were treated with 100 μg/mL of glu-AGE-BSA or non-glycated BSA in the presence or absence of 10 μg/mL of polyclonal antibody against glu-AGE, and then ROS were analyzed. (*D*) Cells were treated with control or diabetic serum in the presence or absence of 10 μg/mL of polyclonal antibody against glu-AGE, and then ROS were analyzed. *$P < 0.01$ compared to the value of the control with non-glycated BSA (Student *t* test). #$P < 0.05$ compared to the value with glu-AGE-BSA alone (Student *t* test). +$P < 0.01$ compared to the value with diabetic serum alone (Student *t* test).

### Quantitative RT-PCR

Poly(A)$^+$RNAs were isolated [31] from cells treated with or without AGE-proteins for the various time periods, and analyzed by reverse transcription-polymerase chain reaction (RT-PCR) as described previously [32]. Ten-microliter aliquots of each RT-PCR reaction mixture were electrophoresed on a 1.2% agarose gel, transferred to a Hybond-N$^+$ nylon membrane, and the membrane was hybridized with the respective $^{32}$P-end labeled probes [32]. The amounts of poly(A)$^+$RNA templates (30 ng) and cycle numbers (35 cycles) for amplification were chosen in quantitative ranges, where reactions proceeded linearly, which had been determined by plotting signal intensities as functions of the template amounts and cycle numbers [32]. Signal intensities of hybridized bands were measured by microcomputer-assisted NIH Image (Version 1.56).

### Measurement of active TGF-β proteins

Cells were plated at $1 \times 10^5$ cells/well in 12-well plates. The next day, cells were rested in a serum-free assay medium for 24 hours, and then treated with or without AGE-BSA for 24 hours. TGF-β released into media was measured with an enzyme immunoassay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

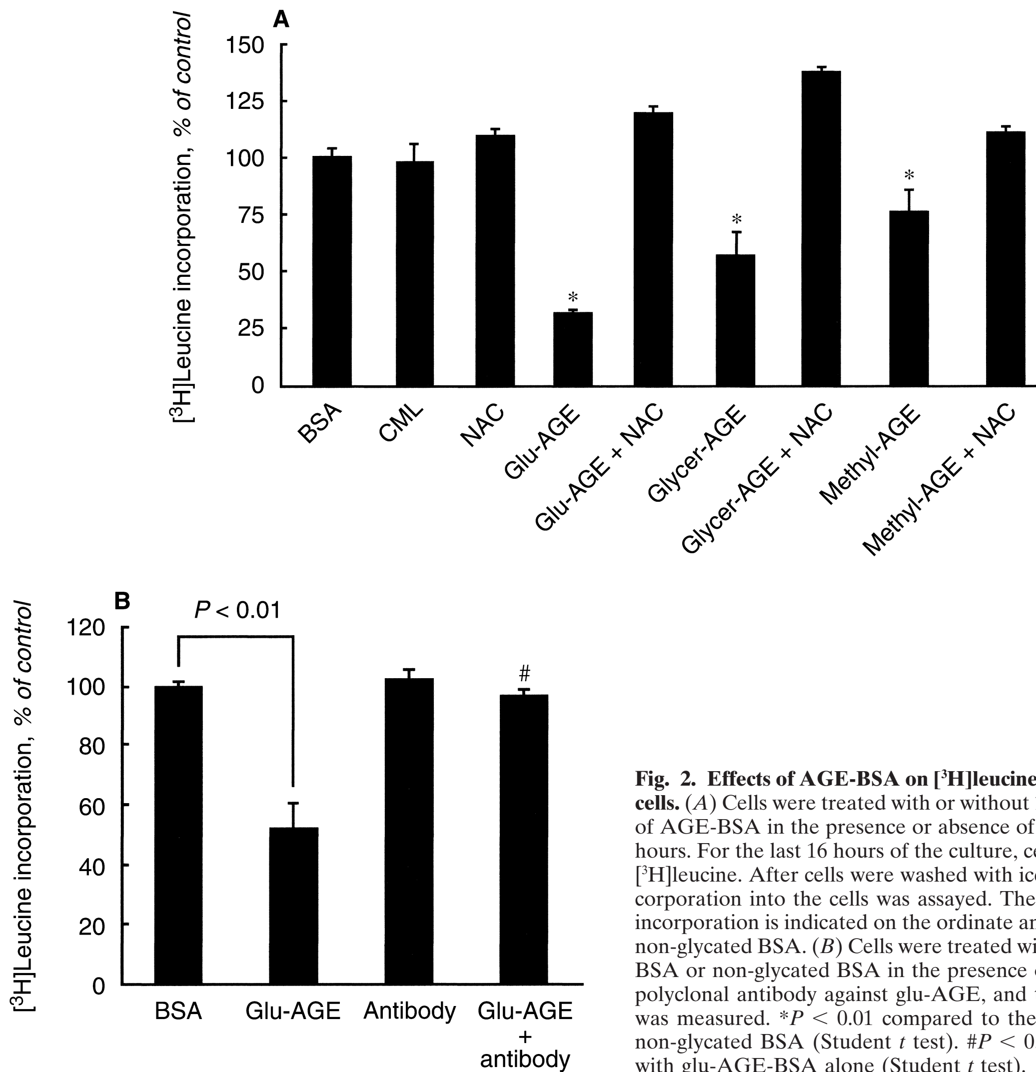


**Fig. 2. Effects of AGE-BSA on [$^3$H]leucine incorporation into tubular cells.** (*A*) Cells were treated with or without 100 μg/mL of various types of AGE-BSA in the presence or absence of 10 mmol/L of NAC for 72 hours. For the last 16 hours of the culture, cells were pulsed with 2 μCi [$^3$H]leucine. After cells were washed with ice-cold PBS, [$^3$H]leucine incorporation into the cells was assayed. The percentage of [$^3$H]leucine incorporation is indicated on the ordinate and related to the value with non-glycated BSA. (*B*) Cells were treated with 100 μg/mL of glu-AGE-BSA or non-glycated BSA in the presence or absence of 10 μg/mL of polyclonal antibody against glu-AGE, and then leucine incorporation was measured. *$P < 0.01$ compared to the value of the control with non-glycated BSA (Student *t* test). #$P < 0.01$ compared to the value with glu-AGE-BSA alone (Student *t* test).

### Statistical analysis

All values were presented as means ± SEM. Statistical significance was evaluated using the Student *t* test for paired comparison; $P < 0.05$ was considered significant.

## RESULTS

### Effects of AGE-BSA on intracellular ROS production in proximal tubular epithelial cells

As shown in Figure 1A, various synthetic AGE conjugated to BSA as a carrier protein, but not unmodified BSA, significantly increased the intracellular ROS generation in human proximal tubular epithelial cells. Further, glu-AGE were found to stimulate ROS production in tubular cells in a dose-dependent manner (Fig. 1B). Among the different types of AGE-proteins, 100 μg/mL of glu-AGE-BSA caused the most significant effects on tubular cells, increasing ROS production about 1.7-fold.

To confirm the specificity of glu-AGE effects on tubular cells, we next investigated whether antibodies against glu-AGE-BSA could actually neutralize the glu-AGE-induced increase in ROS generation. As shown in Figure 1C, the antibodies were found to completely block the glu-AGE-induced ROS generation in tubular cells.

### Effects of diabetic serum on intracellular ROS production in proximal tubular epithelial cells

Next the question of whether AGE-containing serum of diabetic patients could increase ROS generation in tubular cells was investigated. As shown in Figure 1D, compared with control serum, serum from diabetic patients significantly increased ROS generation in tubular cells. Antibodies against glu-AGE-BSA significantly prevented the diabetic serum-induced increase in ROS generation in tubular cells, suggesting that the immunological epitopes of in vitro-prepared glu-AGE-BSA might be involved in oxidative stress in proximal tubular cells in vivo.

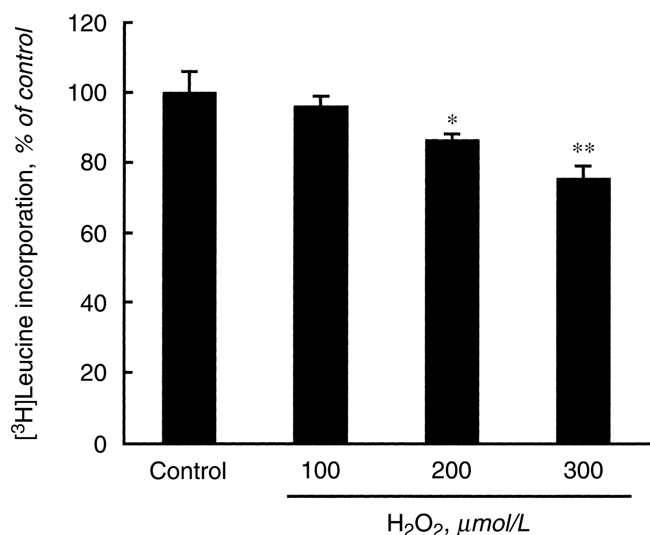


**Fig. 3. Effects of $H_2O_2$ on [$^3$H]leucine incorporation into tubular cells.** Cells were treated with or without various concentrations of $H_2O_2$ for 72 hours. For the last 16 hours of the culture, cells were pulsed with 2 μCi [$^3$H]leucine. After cells were washed with ice-cold PBS, [$^3$H]leucine incorporation into the cells was assayed. The percentage of [$^3$H]leucine incorporation is indicated on the ordinate and related to the value without additives. $*P < 0.05$; $**P < 0.01$ compared to the value of the control without additives (Student *t* test).

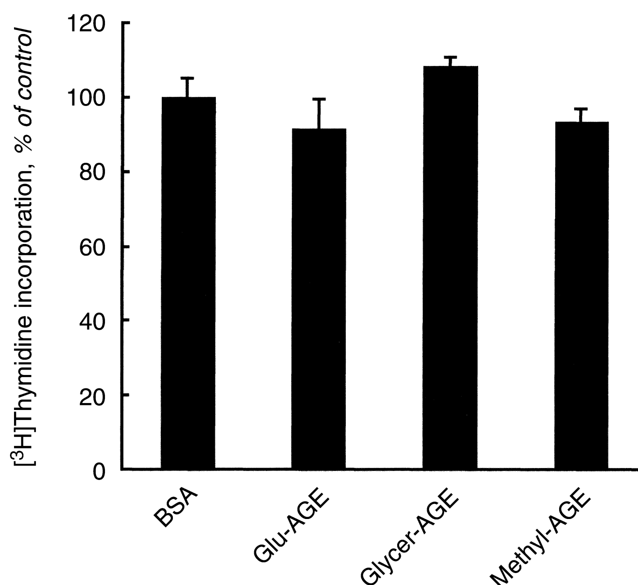


**Fig. 4. Effects of AGE-BSA on [$^3$H]thymidine incorporation into tubular cells.** Cells were treated with or without 100 μg/mL of various types of AGE-BSA for 48 hours. For the last 4 hours of the culture, cells were pulsed with 2 μCi [$^3$H]thymidine. After cells were washed with ice-cold PBS, [$^3$H]thymidine incorporation into the cells was assayed. The percentage of [$^3$H]thymidine incorporation is indicated on the ordinate and related to the value with non-glycated BSA.

### Effects of AGE-BSA and $H_2O_2$ on [$^3$H]leucine and [$^3$H]thymidine incorporation

Incorporation of [$^3$H]leucine into acid-precipitable proteins is used as a sensitive index of de novo protein synthesis in cultured cells. Thus, the effects of AGE on [$^3$H]leucine incorporation into tubular cells were investigated. As shown in Figure 2A, various types of AGE-BSA significantly inhibited de novo protein synthesis in tubular cells. Of note, tubular cells displayed a 70% decrease in leucine incorporation when exposed to glu-AGE-BSA for 72 hours. Co-treatment with 10 mmol/L NAC was found to completely prevent the effects of AGE (Fig. 2A). Furthermore, antibodies against glu-AGE-BSA completely blocked the glu-AGE-induced decrease in leucine incorporation in tubular cells (Fig. 2B).

To confirm that intracellular ROS generation was involved in protein synthesis, the effects of $H_2O_2$ on [$^3$H]leucine incorporation into tubular cells were studied. As shown in Figure 3, $H_2O_2$ inhibited [$^3$H]leucine incorporation in a dose-dependent manner. None of these AGE-proteins reduced [$^3$H]thymidine incorporation into tubular cells, demonstrating that AGE did not affect DNA synthesis in this type of cell (Fig. 4). We also confirmed that the viable cell numbers of tubular cells were not affected by these AGE treatments for two days (data not shown).

### Effects of AGE-BSA and $H_2O_2$ on $PGE_2$ secretion by proximal tubular cells

Advanced glycation end products-BSA significantly decreased $PGE_2$ secretion by proximal tubular cells; $PGE_2$ secretion was reduced to 40% of that of the control when exposed to glu-AGE-BSA for 48 hours (Fig. 5). Co-treatment with 10 mmol/L NAC completely prevented the effects of AGE, and further $H_2O_2$ significantly inhibited $PGE_2$ secretion by tubular cells in a dose-dependent manner (Fig. 6). These results suggest that intracellular ROS generation could be implicated in suppression of $PGE_2$ secretion by proximal tubular cells.

### Proximal tubular epithelial cells express mRNA for TGF-β in response to AGE-BSA

Poly(A)$^+$ RNAs were isolated from tubular cells treated with various types of AGE-proteins for various time periods in the presence or absence of NAC or antibodies against glu-AGE-BSA, and analyzed by a quantitative RT-PCR technique to determine the effects of AGE on the expression of TGF-β genes. As shown in Figure 7, A and B, when the tubular cells were exposed to various types of AGE-BSA, the levels of TGF-β mRNAs were found to be significantly increased; glu-AGE-BSA up-regulated TGF-β mRNA levels about fivefold (Fig. 7B). Co-treatment with 10 mmol/L NAC completely prevented the effects of AGE on TGF-β mRNA up-regulation, suggesting that intracellular ROS generation is involved in the induction of TGF-β genes in proximal tubular cells (Fig. 7C). Further, as the case in ROS generation, serum of diabetic patients significantly up-regulated TGF-β mRNA levels in tubular cells (Fig. 7D). Antibodies against glu-AGE were found to inhibit the diabetic

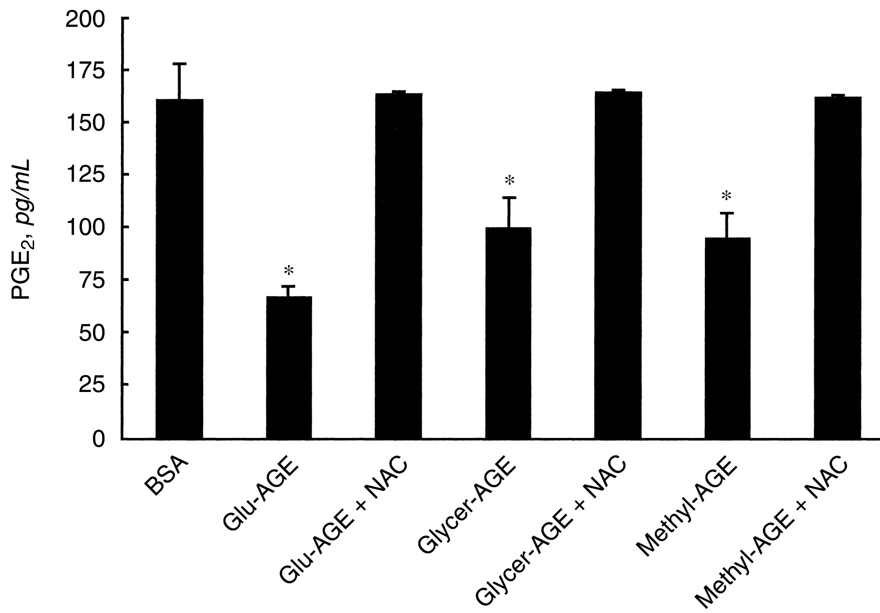


**Fig. 5. Effects of AGE-BSA on prostaglandin $E_2$ ($PGE_2$) secretion by tubular cells.** Cells were treated with or without 100 μg/mL of various types of AGE-BSA in the presence or absence of 10 mmol/L of NAC for 48 hours. $PGE_2$ released into media was measured with an enzyme immunoassay system. *$P < 0.01$ compared to the value of the control with non-glycated BSA (Student $t$ test).

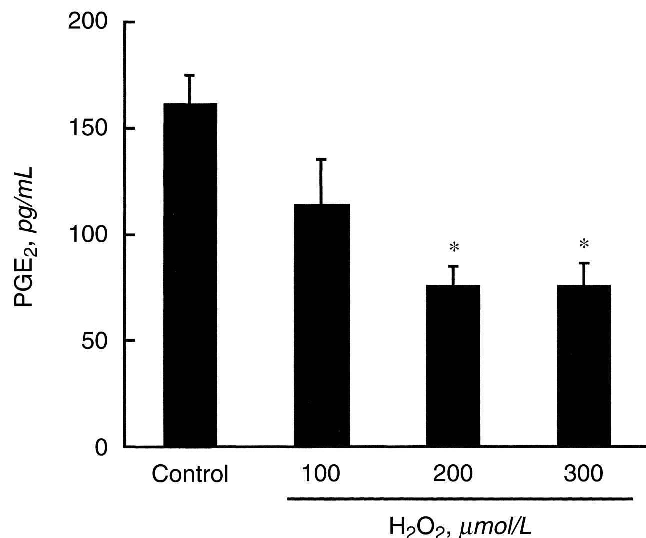


**Fig. 6. Effects of $H_2O_2$ on $PGE_2$ secretion by tubular cells.** Cells were treated with or without various concentrations of $H_2O_2$ for 48 hours. $PGE_2$ released into media was measured with an enzyme immunoassay system. *$P < 0.05$ compared to the value of the control without additives (Student $t$ test).

serum-induced TGF-β mRNA up-regulation, thus suggesting that the immunological epitopes of in vitro-prepared glu-AGE-BSA might participate in tubular injury in diabetes.

To confirm whether AGE actually increased the synthesis of active TGF-β proteins, an enzyme immunoassay was performed for active TGF-β proteins in a medium of the cells that had been treated with or without 100 μg/mL of various types of AGE-BSA for 24 hours. As shown in Figure 8, active TGF-β proteins were secreted more by cells exposed to AGE than the controls.

## DISCUSSION

Our previous study showed that OPB-9195, a novel inhibitor of AGE, significantly prevented renal tubular injury and tubulointerstitial fibrosis in spontaneous diabetic rats [24, 25]. The present study demonstrates, to our knowledge for the first time, that various types of AGE, non-enzymatically glycated protein derivatives formed at an accelerated rate under diabetes, inhibit de novo protein synthesis in human proximal tubular epithelial cells. Since the AGE-BSA did not affect DNA synthesis and viable cell numbers in these cells, AGE might be involved in the pathogenesis of tubular atrophy, one of the representative tubulointerstitial changes in diabetic nephropathy [7]. We recently showed that the structural epitope of in vitro-modified AGE-proteins actually existed in the serum of diabetic patients [33, 34]. Further, we have previously demonstrated that the serum concentrations of glu-AGE were about 30 to 50 μg/mL in diabetics, and its levels were increased two- to threefold in diabetic patients with uremia [33, 35]. These observations showed that the concentration of glu-AGE used in this experiment was comparable to that of the in vivo situation in diabetes. Since the serum concentrations of glycer-AGE and methyl-AGE were relatively low, being 1/5 to 1/3 of that of glu-AGE in diabetes [34], the immunological epitopes of glu-AGE rather than glycer-AGE or methyl-AGE may participate in tubular injury in diabetes. In support of this observation, the present study found that antibodies against glu-AGE-BSA that did not cross-react with glycer-AGE or methyl-AGE significantly inhibited the diabetic serum-induced ROS generation as well as TGF-β up-regulation in tubular cells. Antibodies against glu-AGE also did not to cross-

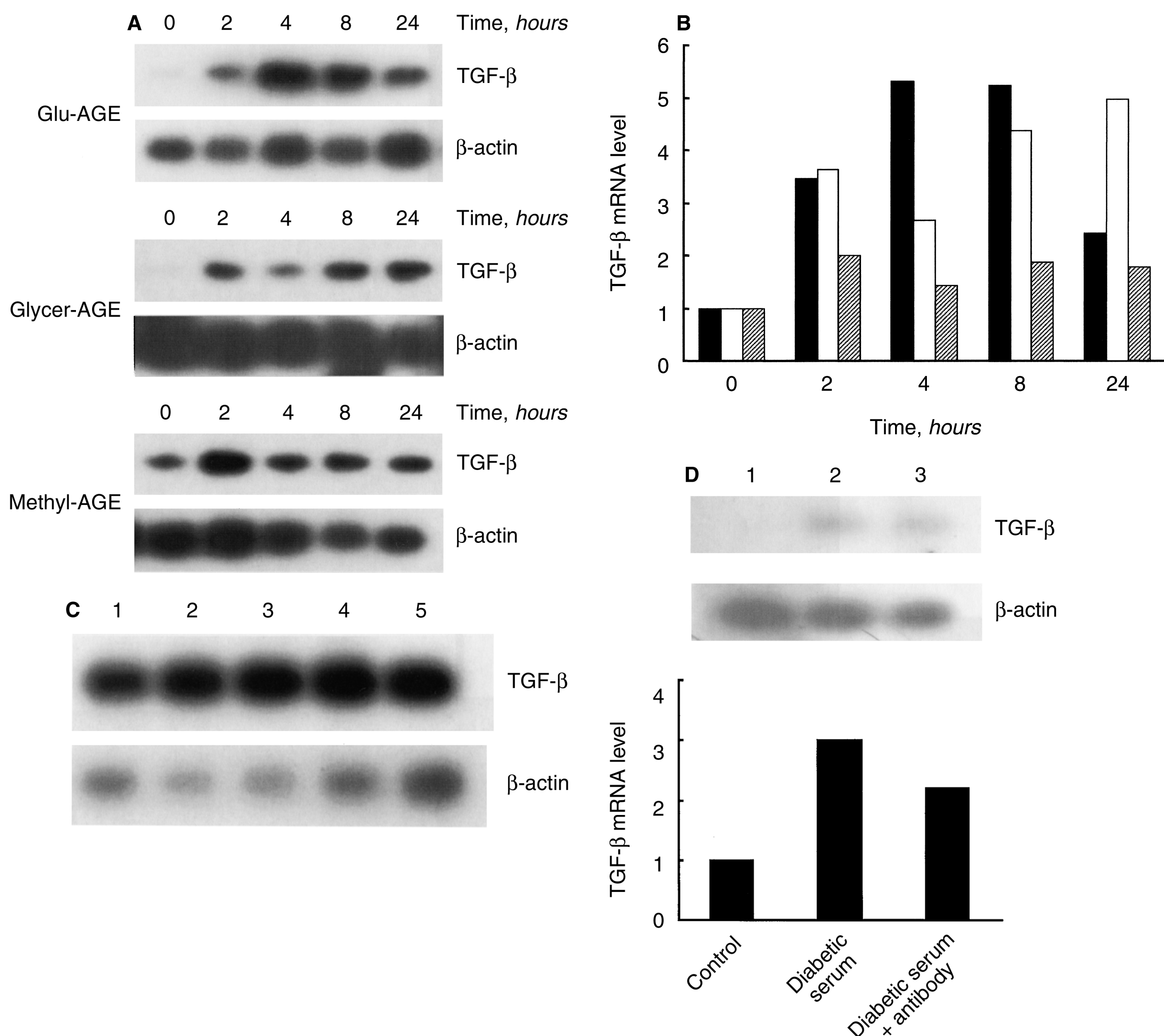


**Fig. 7. Expression of transforming growth factor-β (TGF-β) gene in tubular cells.** (*A*) Time course of TGF-β mRNA induction by various types of AGE-BSA. Cells were treated with or without 100 μg/mL of different types of AGE-BSA, and then 30 ng poly(A)$^+$RNAs were transcribed and amplified by PCR at the indicated times. Each lower panel shows expression of β-actin genes. PCR amplification for β-actin mRNA was performed for 20 cycles. (*B*) Quantitative representation of TGF-β gene induction by glu-AGE-BSA (■), glycer-AGE-BSA (□), or methyl-AGE-BSA (▨). Data were normalized by the intensity of β-actin mRNA-derived signals and related to the value of the control with non-glycated BSA. (*C*) Effects of NAC on AGE-induced TGF-β mRNA up-regulation. Cells were treated with or without 100 μg/mL of various types of AGE-BSA in the presence or absence of 10 mmol/L of NAC for four hours, and then 30 ng poly(A)$^+$RNAs were transcribed and amplified by PCR. Lane 1, control cells treated with non-glycated BSA; lane 2, cells treated with NAC alone; lane 3, cells treated with glu-AGE-BSA and NAC; lane 4, cells treated with glycer-AGE-BSA and NAC; lane 5, cells treated with methyl-AGE-BSA and NAC. (*D*) Effects of antibody against glu-AGE-BSA on TGF-β gene expression in tubular cells. Cells were treated with control or diabetic serum in the presence or absence of 10 μg/mL of polyclonal antibody against glu-AGE for four hours, and then poly(A)$^+$RNAs were transcribed and amplified by PCR. Lane 1, cells treated with control serum; lane 2, cells treated with diabetic serum alone; lane 3, cells treated with diabetic serum and antibody. Lower panel shows the quantitative representation of TGF-β gene induction. Data were normalized by the intensity of β-actin mRNA-derived signals and related to the value of the control. Similar results were obtained in two independent experiments.

react with structurally identified AGE-modified BSA, including pyrraline-BSA, pentosidine-BSA, argpyrimidine-BSA, 3-deoxyglucosone imidazolone-BSA, carboxymethyllysine-BSA (CML-BSA), glyoxal-lysine diamer, or methylglyoxal-lysine dimer [36]. These results suggest that structurally unidentified immunological epitopes in the in vitro-modified AGE preparations might exhibit deleterious effects on proximal tubular cells in diabetes.

That it was AGE-induced intracellular ROS that caused de novo protein synthesis inhibition was evidenced as

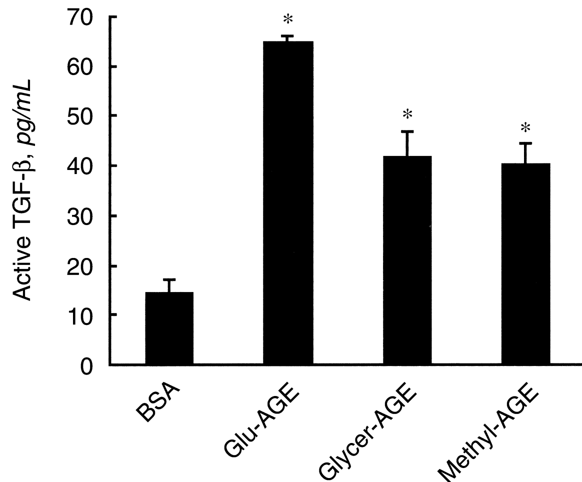


**Fig. 8. Effects of AGE-BSA on active TGF-β secretion by tubular cells.** Cells were treated with or without 100 μg/mL of various types of AGE-BSA for 24 hours. Active TGF-β released into media was measured with an enzyme immunoassay system. $^{*}P < 0.01$ compared to the value of the control with non-glycated BSA (Student *t* test).

follows. First, AGE-BSA, but not non-glycated BSA, increased intracellular ROS generation in proximal tubular cells (Fig. 1A). Second, NAC, an intracellular radical scavenger, completely prevented the inhibitory effects of AGE on protein synthesis (Fig. 2A). Third, $H_2O_2$ inhibited de novo protein synthesis in tubular cells in a dose-dependent manner (Fig. 3). Recently, Ohta et al reported that tubular atrophy and interstitial fibrosis are cardinal pathologic features of oxidative stress-mediated renal injury in patients with heme oxygenase-1 deficiency, supporting our speculation [37].

In our study, CML-BSA added to the culture medium failed to stimulate ROS generation, and it did not inhibit de novo protein synthesis in proximal tubular cells (Figs. 1A and 2A). Since CML adducts of proteins have been reported to be ligands for the receptor for AGE, termed RAGE [38], it is probable that AGE receptors other than RAGE might mediate the AGE effects. In our preliminary experiments, RAGE overexpression in tubular cells did not potentiate the AGE-induced ROS overgeneration or decrease in de novo protein synthesis, supporting our speculation (data not shown).

The present study also demonstrates that AGE inhibited $PGE_2$ secretion by proximal tubular cells through intracellular ROS generation. $PGE_2$ is a vasodilator and angiogenic prostanoid that can regulate blood supply to proximal tubules [39]. Since chronic tubulointerstitial injury including tubular atrophy and interstitial fibrosis is known to be associated with peritubular capillary loss [8, 40], the AGE-induced suppression of $PGE_2$ secretion may participate in peritubular capillary rarefaction and tubular ischemia, thus causing tubular atrophy in diabetic nephropathy. Oxidative stress impairs cyclooxygenase-2 activity, suppressing $PGE_2$ secretion by various types of cells and tissues [41–43], which may provide an explanation of the mechanism for AGE-induced inhibition of $PGE_2$ secretion by proximal tubular cells.

Evidence has implicated the TGF-β system as a major etiologic agent in the pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis in diabetic nephropathy [3, 44, 45]. TGF-β stimulates the synthesis of individual matrix components such as collagen, laminin, and simultaneously blocks matrix degradation by increasing concentrations of inhibitors of matrix metalloproteinase [46, 47]. In our study, AGE-induced intracellular ROS up-regulated the levels of mRNAs for TGF-β in proximal tubular cells. The AGE actually increased the secretion of active TGF-β proteins by tubular cells, suggesting the pathological involvements of AGE in the progression of diabetic nephropathy. The report that latent TGF-β can be activated in vitro upon oxidation with ROS supports our observation [48]. Recently, ROS were reported to act as signaling molecules in mesangial cells cultured under high glucose, leading to activation of redox-sensitive transcriptional factors, nuclear factor-κB (NF-κB) and activator factor-1 (AP-1) as well as up-regulation of TGF-β [49]. Therefore, AGE-induced ROS generation also might stimulate TGF-β genes in proximal tubular cells through the activation of NF-κB and AP-1. We have just recently found that all of the AGE preparations used in this experiment increased transcriptional activation of both NF-κB and AP-1 promoter in human microvascular endothelial cells (unpublished data).

Baynes et al recently demonstrated that AGE inhibitors, including aminoguanidine and OPB-9195, have potent antioxidative, chelating activity in vitro [50]. Therefore, we cannot entirely exclude the possibility that OPB-9195 directly improves renal tubular damage in OLETF rats. However, AGE sequestration and the resultant renal elimination by lysozyme recently were found to protect against renal damage in diabetic rats [51], confirming the causal role for AGE in renal abnormalities in diabetes. It is probable that renal improvement associated with OPB-9195 administration in OLETF rats is derived from inhibitions of AGE formation through its carbonyl trapping and chelation activity. The present observations suggest the active participation of AGE in tubular injury in diabetes, thus providing a basis for understanding the favorable molecular events in OLETF rats given OPB-9195.

## ACKNOWLEDGMENTS

This work was supported in part by grants of Venture Research and Development Centers from the Ministry of Education, Culture, Sports, Science and Technology, Japan; the Suzuken Memorial Foundation, Japan; and the Mochida Memorial Foundation for Medical and Pharmaceutical Research, Japan.

*Reprints requests to Sho-ichi Yamagishi, M.D., Ph.D., Division of Endocrinology and Metabolism, Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan. E-mail: shoichi@med.kurume-u.ac.jp*

## APPENDIX

Abbreviations used in this article are: AGE, advanced glycation end products; AP-1, activator factor-1; CML, carboxymethyllysine; glycer-AGE, glyceraldehyde-derived AGE; glu-AGE, glucose-derived AGE; methyl-AGE, methylglyoxal-derived AGE; NAC, *N*-acetylcysteine; NF-κB, nuclear factor-κB; $PGE_2$, prostaglandin $E_2$; ROS, reactive oxygen species.

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