Intracellular toxic advanced glycation end-products (TAGE) in myoblasts may cause sarcopenia: Research article of a non-clinical study

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Abstract

Background: Sarcopenia is a progressive disease that is characterized by decreases in skeletal muscle mass and function. Skeletal muscle consists of myotubes that differentiated from myoblasts. Although sarcopenia is associated with non-alcoholic steatohepatitis (NASH) and type 2 diabetes mellitus (T2DM), the underlying mechanisms remain unclear. We considered that glyceraldehyde (GA), a glucose/fructose metabolism intermediate, plays a crucial role in sarcopenia. We previously designated GA-derived advanced glycation end-products (AGEs) as toxic AGEs (TAGE) because of their cytotoxicity and involvement in lifestyle-related diseases (LSRD), such as NASH and T2DM. We hypothesized that TAGE induce cytotoxicity in myoblasts. Methods: C2C12 cells, which are murine myoblasts, were treated with 0, 0.5, 1, 1.5, and 2 mM GA for 24 h, and cell viability and intracellular TAGE were measured using WST-8 and slot blot assays. Cells were pretreated with 8 mM aminoguanidine (AG), an inhibitor of AGE production, for 2 h followed by 0, 1.5, and 2 mM GA for 24 h. Cell viability and intracellular TAGE were then measured. Serum TAGE levels in STAM mice, in which there were four stages (pre-simple steatosis, simple steatosis, steatohepatitis, and fibrosis), were measured using an enzyme-linked immunosorbent assay. Results were expressed as TAGE units (U) per milliliter of serum, with 1 U corresponding to 1.0 μg of GA-derived AGE-bovine serum albumin (BSA) (TAGE-BSA). The viabilities of cells treated with 20 μg/mL non-glycated BSA (NG-BSA) and TAGE-BSA for 24 h were assessed using the WST-8 assay. Results: In C2C12 cells treated with 1.5 and 2 mM GA, cell viability decreased to 47.7 and 5.0% and intracellular TAGE increased to 6.0 and 15.9 μg/mg protein, respectively. Decreases in cell viability and TAGE production were completely inhibited by 8 mM AG. Serum TAGE levels at the steatohepatitis and fibrosis stages were 10.51 ±1.16 and 10.44±0.95 U/mL, respectively, and increased from the pre-simple steatosis stage. The viabilities of C2C12 cells treated with 20 μg/mL NG-BSA and TAGE-BSA were 99.7 and 88.3%, respectively. Conclusion: Intracellular TAGE were generated in C2C12 cells and induced cell death more strongly than extracellular TAGE. Intracellular TAGE in myoblasts may cause sarcopenia in patients with LSRD.

Background

Skeletal muscle requires exercise, the synthesis of glycogen, and interactions with other organs, such

as the liver and adipose tissue [1]. Sarcopenia is a progressive disease that is characterized by decreases in skeletal muscle mass and function, resulting in the deterioration of activities of daily living and quality of life as well as increases in fall risk and mortality [2]. One of the mechanisms contributing to the loss of skeletal muscle is the death of or dysfunctions in myoblasts because skeletal muscle consists of myotubes that differentiated from myoblasts [2–6]. Although accumulating evidence has shown that patients with non-alcoholic steatohepatitis (NASH) and type 2 diabetes mellitus (T2DM) have an increased risk of sarcopenia [2, 7–10], the relationships between myoblast damage and NASH and T2DM remain unclear. We hypothesized that glyceraldehyde (GA), a glucose/fructose metabolism intermediate, plays a crucial role in sarcopenia. We previously designated GA-derived advanced glycation end-products (AGEs) as toxic AGEs (TAGE) because of their cytotoxicity, and involvement in lifestyle-related diseases (LSRD), such as NASH, T2DM, cardiovascular diseases, dementia, and cancer [11–15]. In a clinical study, we revealed that intracellular TAGE were generated in the livers of NASH patients, and also that serum TAGE levels were elevated in patients with LSRD, such as NASH and T2DM, as well as in healthy individuals at risk of LSRD [11–15]. Therefore, we speculated that TAGE contribute to the development/progression of LSRD. We hypothesized that intracellular TAGE may be generated in myoblasts and induce cytotoxicity based on previous findings showing their production in neuroblastoma cells [16], hepatic cells [17–20], pancreatic cells [21], and cardiac cells [22] as well as their induction of cell death and dysfunction. We focused on extracellular TAGE, such as TAGE in blood, which may be secreted or released from organs that generate intracellular TAGE, because they may induce cytotoxicity, such as inflammation and oxidative stress [11–13, 15, 23].

Methods

Reagents, cell lines, and serum of STAM mice

Dulbecco’s modified Eagle’s medium (D-MEM) and penicillin-streptomycin solution were obtained from Sigma-Aldrich (MO, USA). Fetal bovine serum (FBS) was purchased from Bovogen-Biologicals (VIC, Australia). GA was purchased from Nacalai Tesque Inc. (Kyoto, Japan). The WST-8 assay kit and 3-[(3-cholamido-propyl)-dimethyl-ammonio]-1-propane sulfonate) (CHAPS) were obtained from Dojindo

Laboratories (Kumamoto, Japan). Ethylene diamine-N,N,N’,N’-tetraacetic acid (EDTA)-free protease inhibitor cocktail was obtained from Roche Applied Science (Penzberg, Germany). C2C12 cells were obtained from KAC Co., Ltd. (Kyoto, Japan). The serum of STAM mice was purchased from SMC Laboratories, Inc. (Tokyo, Japan). The protein assay kit for the Bradford method was obtained from Takara Bio, Inc. (Otsu, Japan). A horseradish peroxidase (HRP)-linked molecular marker was obtained from Bionexus (CA, USA). A HRP-linked goat anti-rabbit IgG antibody was purchased from DAKO (Glostrup, Denmark). All other reagents and kits not indicated were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). GA-derived AGE-bovine serum albumin (BSA) (TAGE-BSA), non-glycated BSA (NG-BSA), and an anti-TAGE antibody were prepared as described previously [24].

Cell culture and cell seeds. C2C12 cells were incubated in D-MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin under standard cell culture conditions (humidified atmosphere, 5% CO_2, 37 °C). Cells were seeded (1.9×10^4 cells/cm^2) on 96-well microplates and culture dishes (Becton-Dickinson, NJ, USA).

GA and AG treatment of C2C12 cells. GA was dissolved in phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} ((PBS)(-)), and then filtrated before being added to C2C12 cells. All experiments were performed 24 h after the treatment with 0, 0.5, 1, 1.5, and 2 mM GA. The cell culture method before the treatment with AG, an inhibitor of AGE production, was the same as that described above. Cells were pretreated with 0 or 8 mM AG for 2 h followed by 0, 1.5, and 2 mM GA for 24 h.

Cell viability of C2C12 cells treated with GA and AG. Cell viability was assessed using the WST-8 assay. Ten microliters of WST-8 reagent was added to 96-well microplates in which C2C12 cells were cultured in medium (100 μL), and this was followed by an incubation at 37 °C for 2 h in a CO_2 incubator. Absorbance was measured at 450 and 655 nm using a microplate reader (Bio-Rad, CA, USA). Background absorbance was measured in medium without cells and subtracted from experimental values.

Slot blot (SB) analysis. This analysis was performed as described previously with some modifications [20–22]. Cells were washed with (PBS)(-), and lysed in buffer [a solution of 2 M thiourea, 7 M urea, 4%

CHAPS, and 30 mM Tris, and a solution of EDTA-free protease inhibitor cocktail (9:1)]. Cell extracts were then incubated on ice for 20 min, centrifuged at 10,000 × g at 4 °C for 15 min, and the supernatant was collected as cell extracts. Protein concentrations were measured using the protein assay kit for the Bradford method with BSA as a standard. Regarding the detection of TAGE, equal amounts of cell extracts, the HRP-linked molecular marker, and TAGE-BSA were loaded onto polyvinylidene difluoride (PVDF) membranes (0.45 µm; Millipore, MA, USA) fixed in the SB apparatus (Bio-Rad). PVDF membranes were cut to prepare two membranes and then blocked at room temperature (r.t.) for 1 h using 5% skimmed milk in PBS(-) containing 0.05% Tween 20 (SM-PBS-T). After this step, we used 0.5% of SM-PBS-T for washing or as the solvent of antibodies. After washing twice, membranes were incubated with (1) the anti-TAGE-antibody (1:1,000) or (2) neutralized anti-TAGE-antibody (a mixture of the anti-TAGE-antibody (1:1,000) and 250 µg/mL of TAGE-BSA) at 4 °C overnight. Membranes were then washed four times. Proteins on the membrane were incubated with the HRP-linked goat anti-rabbit IgG antibody (1:2,000) at r.t. for 1 h. After washing three times with PBS-T, membranes were moved into PBS(-). Immunoreactive proteins were detected with the ImmunoStar LD kit and band densities on the membranes were measured using the Fusion FX fluorescence imager (M&S Instruments Inc., Osaka, Japan). The densities of HRP-linked molecular marker bands were used to correct for differences in densities between membranes. The amount of TAGE in cell extracts was calculated based on a calibration curve for TAGE-BSA.

Analysis of serum TAGE levels in STAM mice. Briefly, each well of the 96-well microplate was coated with 1.0 µg/mL TAGE-BSA and incubated overnight in a cold room. Wells were washed three times with 0.3 mL of PBS containing 0.05% Tween 20 (PBS-T). Wells were then blocked by an incubation for 1 h with 0.2 mL of a solution of PBS containing 1% BSA. After washing with PBS-T, test samples (50 µL) were added to each well as a competitor for 50 µL of the anti-TAGE antibody (1:1,000), followed by an incubation at r.t. for 2 h with gentle shaking on a horizontal rotary shaker. Wells were then washed with PBS-T and developed with alkaline phosphatase-linked anti-rabbit IgG utilizing p-nitrophenyl phosphate as the colorimetric substrate. Results were expressed as TAGE units (U) per milliliter of serum, with 1 U corresponding to 1.0 µg of a TAGE-BSA standard as described previously

[24]. Sensitivity and intra- and interassay coefficients of variation were 0.01 U/mL and 6.2 and 8.8%, respectively [25].

NG-BSA and TAGE-BSA treatment of C2C12 cells and measure of cell viability. In total, 20 μg/mL of NG-BSA and TAGE-BSA were added to C2C12 cells, which were then incubated for 24 h. Cell viability was measured using the WST-8 assay. The ratio of cell viability was calculated based on the viability of cells treated with TAGE-BSA versus those treated with NG-BSA.

Statistical analysis. Stat Flex (ver. 6) software (Artech Co., Ltd., Osaka, Japan) was used for statistical analyses. Data were expressed as means ± S.D. When statistical analyses were performed on data, significant differences in the means of each group were assessed by a one-way analysis of variance (ANOVA). We then used Tukey's test for an analysis of variance. P-values < 0.05 were considered to be significant.

Results

Viability of C2C12 cells treated with GA. The viability of C2C12 cells treated with 0.5 and 1 mM GA did not decrease, whereas dose-dependent decreases to 47.7 and 5.0% were observed in those treated with 1.5 and 2 mM GA, respectively (Fig. 1a).

Quantity of intracellular TAGE in C2C12 cells treated with GA. Intracellular TAGE were not generated in C2C12 cells treated with 0, 0.5, and 1 mM GA (Fig. 1b). Intracellular TAGE dose-dependently increased to 6.0 and 15.9 μg/mg protein in C2C12 cells treated with 1.5 and 2 mM GA, respectively (Fig. 1b).

Effects of the AG pretreatment on the viability of C2C12 cells treated with GA. The viability of C2C12 cells treated with 1.5 and 2 mM GA without AG dose-dependently decreased to 35.0 and 3.0%, respectively (Fig. 1c). In C2C12 cells pretreated with 8 mM AG, cell viabilities were 71.7, 71.3, and 74.3% in those subsequently treated with 0, 1.5, and 2 mM GA, respectively. No significant differences were observed between each treatment (Fig. 1c). The AG pretreatment completely inhibited decreases in the viability of C2C12 cells treated with 1.5 and 2 mM GA.

Effects of the AG pretreatment on the quantity of intracellular TAGE in C2C12 cells treated with GA. GA concentrations of 1.5 and 2 mM without AG dose-dependently increased intracellular TAGE to 7.9

and 13.4 μg/mg protein, respectively (Fig. 1d). Intracellular TAGE levels in C2C12 cells pretreated with 0 mM AG followed by 0 mM GA and in those pretreated with 8 mM AG followed by 0, 1.5, and 2 mM GA were not significantly different. The AG pretreatment completely inhibited the generation of intracellular TAGE in C2C12 cells treated with 1.5 and 2 mM GA.

Serum TAGE levels in STAM mice. Serum TAGE levels in the pre-simple steatosis and simple steatosis stage groups were 7.27 ± 0.18 and 8.69 ± 1.01 U/mL, respectively (Fig. 2a). Serum TAGE levels in the steatohepatitis and fibrosis stage groups increased to 10.51 ± 1.16 and 10.44 ± 0.95 U/mL, which were higher than that in the pre-simple steatosis stage group.

Viability of C2C12 cells treated with NG-BSA and TAGE-BSA. The viabilities of C2C12 cells treated with 20 μg/mL NG-BSA and TAGE-BSA were 99.7 and 88.3%, respectively, and the ratio of cell viability was 88.6% (Fig. 2b).

Discussion

Sarcopenia is a progressive disease that is characterized by decreases in skeletal muscle mass and function, and skeletal muscle consists of myotubes that differentiated from myoblasts [2–6]. The death of or dysfunctions in myoblasts may lead to the loss of skeletal muscle [2–6].

We hypothesized that TAGE induce the loss of skeletal muscle because sarcopenia is associated with NASH and T2DM [2, 7–10], and we previously reported that TAGE contributed to the development/progression of LSRD, such as NASH and T2DM [11–15].

GA, which is a precursor of TAGE, is generated by the liver via three pathways [11, 15, 23]. (1) Glucose is metabolized to GA via glycolysis. (2) Fructose is metabolized to GA via the pathway involving fructokinase and aldolase B (fructolysis). (3) Glucose is metabolized to fructose via the sorbitol pathway, which regulates aldose reductase and sorbitol dehydrogenase, and this fructose is metabolized to GA via fructolysis. Since skeletal muscle uses glycolysis and has fructokinase [26], aldolase B [26], aldose reductase [27], and sorbitol dehydrogenase [28], we considered the three pathways of GA metabolism to occur in this tissue, similar to the liver.

We hypothesized that intracellular TAGE may be generated in and damage myoblasts cells because they were previously shown to be produced in neuroblastoma cells [16], hepatic cells [17–20],

pancreatic cells [21], and cardiac cells [22], and induced cell death.

We treated C2C12 cells with GA to rapidly generate intracellular TAGE. In the present study, C2C12 cells were treated with GA at a physiological concentration to generate TAGE within 24 h. Taniguchi et al. previously demonstrated that islets of the pancreas exposed to 20 mM glucose accumulated 0.025 pmol/islet GA, whereas exposure to 10 mM GA caused the accumulation of 0.12 pmol/islet GA [29]. Based on these findings, Takahashi et al. used 2 mM GA in their experiments, which is a similar concentration to those using 20 mM glucose [30]. On the other hand, in NASH and T2DM model mice, their plasma levels of glucose increased by more than 25 mM [31–33]. The viability of C2C12 cells treated with 1.5 and 2 mM GA for 24 h dose-dependently decreased (Fig. 1a). In contrast, intracellular TAGE were generated in a dose-dependent manner. (Fig. 1b). To demonstrate that the generation of TAGE decreased cell viability, C2C12 cells were pretreated with 8 mM AG, an inhibitor of the generation of AGEs, for 2 h followed by 1.5 and 2 mM GA for 24 h. AG inhibited decreases in cell viability as well as the generation of TAGE (Fig. 1c, d). To the best of our knowledge, this is the first study to show that intracellular TAGE were generated from GA at a physiological concentration in myoblasts, and strongly induced cell death. The death of myoblasts will lead to the loss of skeletal muscle. Living myoblasts that generate intracellular TAGE may also lead to the loss of skeletal muscle. In our previous study, when rat primary cardiomyocytes were treated with 4 mM GA for 6 h, cell viability decreased to 39.2% and intracellular TAGE were generated to 12.0 μg/mg protein [22]. Furthermore, living cardiomyocytes completely stopped beating. The viability of C2C12 cells treated with 1.5 mM GA was 47.7% and living cells generated intracellular TAGE levels of 6.0 μg/mg protein (Fig. 1a, b). The generation of skeletal muscle may be inhibited in myotubes with dysfunctional differentiation [2–6]. Collectively, these findings and the present results suggest that cell death or dysfunction of myoblasts that gain excess glucose or fructose and generate high levels of intracellular TAGE may inhibit the differentiation of myoblasts.

In STAM mice, which is a NASH model, serum TAGE levels were higher in the steatohepatitis and fibrosis stages than in the pre-simple steatosis stage (Fig. 2a). We previously reported that serum TAGE levels were higher in NASH patients than in healthy individuals [11–15]. To the best of our

knowledge, this is the first study to show that serum TAGE levels in NASH model mice were similar to those in NASH patients. Since TAGE in blood induces responses, such as inflammation and oxidative stress, in some organs [11–13, 15, 23], we investigated the cytotoxicity of TAGE-BSA, a model of extracellular TAGE, in myoblasts. We applied 20 μg/mL TAGE-BSA, which is approximately 2-fold that of serum TAGE levels in STAM mice that develop steatohepatitis and fibrosis (Fig. 2). To examine the effects of TAGE in C2C12 cells, we assessed the viability of C2C12 cells treated with both NG-BSA and TAGE-BSA (Fig. 2b). The effects of 20 μg/mL TAGE-BSA on cell viability were markedly weaker than those of intracellular TAGE generated from GA at a physiological concentration (Figs. 1a, c, and 2b).

Conclusion

The present study demonstrated that intracellular TAGE were generated in C2C12 cells and induced cell death more strongly than extracellular TAGE. These results suggest that intracellular TAGE induce the death of myoblasts and inhibit the generation of myotubes, which leads to the loss of skeletal muscle and, ultimately, sarcopenia in individuals with LSRD, such as NASH and T2DM.

Abbreviations

NASH:non-alcoholic steatohepatitis; T2DM:type 2 diabetes mellitus; GA:glyceraldehyde; AGEs:advanced glycation end-products; TAGE:toxic advanced glycation end-products; LSRD:lifestyle-related diseases; SB:slot blot; AG:aminoguanidine.

Declarations

Ethics approval and consent to particle

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

TT and MT designed the research, TT and AS-S performed the research, MT contributed the reagents that were indispensable for this investigation, TT and AS-S analyzed the data, and TT and MT wrote the manuscript.

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Not applicable.

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References

1. Ronden M, Shulman GI. The integrative biology of type 2 diabetes. Nature. 2019;576(7785):51-60.
2. Adachi N, Kanazawa I, Tanaka K, Takeno A, Notsu M, Tanaka S, et al. Insulin-like growth factor-I protects against the detrimental effects of advanced glycation end products and high glucose in myoblastic C2C12 cell. Calcif Tissue Int. 2019;105(1):89-96.
3. Sato H, Funaki A, Kimura Y, Sumitomo M, Yoshida H, Fukata H, et al. Ethanol extract of *Cyclolepis genistoides* Don (palo azul) induces formation of myotubes, which involves differentiation of C2C12 myoblast cells. Nutr Res. 2016;36(7):731-741.
4. Burks TN, Cohn RD. Role of TGF-β signaling in inherited and acquired myopathies. Skelet Muscle. 2011;1(1):19.
5. Byun SK, An TH, Son MJ, Lee DS, Kang HS, Lee EW, et al. HDAC11 inhibits myoblast differentiation through repression of MyoD-dependent transcription. Mol Cells. 2017;40(9):667-676.
6. Tanaka K, Kanazawa I, Yamaguchi T, Yano S, Kaji H, Sugimoto T. Active vitamin D possesses

beneficial effects on the interaction between muscle and bone. Biochem Biophys Res Commun. 2014;450(1):482-487.

7. Bhanji RA, Narayanan P, Moynagh MR, Takahashi N, Angirekula M, Kennedy CC, et al. Differing impact of sarcopenia and frailty in nonalcoholic steatohepatitis and alcoholic liver disease. Liver Transpl. 2019;25(1):14-24.
8. Petta S, Ciminnisi S, Di Marco V, Cabibi D, Cammà C, Licata A, et al. Sarcopenia is associated with severe liver fibrosis in patients with non-alcoholic fatty liver disease. Aliment Pharmacol Ther. 2017;45(4):510-518.
9. Mesinovic J, Zengin A, De Courten B, Ebeling PR, Scott D. Sarcopenia and type 2 diabetes mellitus: a bidirectional relationship. Diabetes Metab Syndr Obes. 2019;12:1057-1072.
10. Sarodnik C, Bours SPG, Schaper NC, van den Bergh JP, van Geel TACM. The risks of sarcopenia, falls and fractures in patients with type 2 diabetes mellitus. Maturitas. 2018;109:70-77.
11. Takeuchi M. Serum levels of Toxic AGEs (TAGE) may be a promising novel biomarker for the onset/progression of lifestyle-related diseases. Diagnostics. 2016;6:23.
12. Takeuchi M, Takino J, Sakasai-Sakai A, Takata T, Tsutsumi M. Toxic AGE (TAGE) theory for the pathophysiology of the onset/progression of NAFLD and ALD. Nutrients. 2017;9:634.
13. Sakasai-Sakai A, Takata T, Takino J, Takeuchi M. The relevance of Toxic AGEs (TAGE) cytotoxicity to NASH pathogenesis: A mini-review. Nutrients. 2019;11:462.
14. Hyogo H, Yamagishi S, Iwamoto K, Arihiro K, Takeuchi M, Sato T, et al. Elevated levels of serum advanced glycation end products in patients with non-alcoholic steatohepatitis. J Gastroenterol Hepatol. 2007;22(7):1112-1119.
15. Takino J, Nagamine K, Hori T, Sakasa-Sakai A, Takeuchi M. Contribution of the toxic advanced glycation end-products-receptor axis in nonalcoholic steatohepatitis-related hepatocellular carcinoma. World J Hepatol. 2015;7(23):2459-2469.
16. Koriyama Y, Furukawa A, Muramatsu M, Takino J, Takeuchi M. Glyceraldehyde caused

Alzheimer's disease-like alterations in diagnostic marker levels in SH-SY5Y human neuroblastoma cells. Sci Rep. 2015;5:13313.

17. Takino J, Kobayashi Y, Takeuchi M. The formation of intracellular glyceraldehyde-derived advanced glycation end-products and cytotoxicity. J Gastroenterol. 2010;45(6):646-655.

18. Takino J, Nagamine K, Takeuchi M, Hori T. *In vitro* identification of nonalcoholic fatty liver disease-related protein hnRNPM. World J Gastroenterol. 2015;21(6):1784-1793.

19. Sakasai-Sakai A, Takata T, Takino J, Takeuchi M. Impact of intracellular glycation end-products on human hepatocyte cell death. Sci Rep. 2017;7(1):14282.

20. Takata T, Sakasai-Sakai A, Takino J, Takeuchi M. Evidence for toxic advanced glycation end-products generated in the normal rat liver. Nutrients. 2019;11:1612.

21. Takata T, Ueda T, Sakasai-Sakai A, Takeuchi M. Generation of glyceraldehyde-derived advanced glycation end-products in pancreatic cancer cells and the potential of tumor promotion. World J Gastroenterol. 2017;23(27):4910-4919.

22. Takata T, Sakasai-Sakai A, Ueda T, Takeuchi M. Intracellular toxic advanced glycation end-products in cardiomyocytes may cause cardiovascular disease. Sci Rep. 2019;9(1):2121.

23. Takeuchi M, Takino J, Yamagishi S. Involvement of the toxic AGEs (TAGE)-RAGE system in the pathogenesis of diabetic vascular complications: A novel therapeutic strategy. Curr Drug Targets. 2010;11(11):1468-1482.

24. Takeuchi M, Makita Z, Bucala R, Suzuki T, Koike T, Kameda Y. Immunological evidence that non-carboxymethyllysine advanced glycation end-products are produced from short chain sugars and dicarbonyl compounds in vivo. Mol Med. 2000;6(2):114-125.

25. Jinno M, Takeuchi M, Watanabe A, Teruya K, Hirohama J, Eguchi N, et al. Advanced glycation end-products accumulation compromises embryonic development and achievement of pregnancy by assisted reproductive technology. Hum Reprod. 2011;26(3):604-610.

26. Bais R, James HM, Rofe AM, Conyers RAJ. The purification and properties of human liver

ketohexokinase. Biochem J. 1985;230(1):53-60.

27. Vander Jagt DL, Robinson B, Taylor KK, Hunsaker LA. Aldose reductase from human skeletal and heart muscle. J Biol Chem. 1990;265(34):20982-20987.

28. Lee FK, Lee AYW, Lin CXF, Chung SS, Chung SK. Cloning, sequencing, and determination of the sites of expression of mouse sorbitol dehydrogenase cDNA. Eur J Biochem. 1995;230(3):1059-1065.

29. Taniguchi S, Okinaka M, Tanigawa K, Miwa I. Difference in mechanism between glycelaldehyde- and glucose-induced insulin secretion from isolated rat pancreatic islets. J Biochem. 2000;127(2):289-295.

30. Takahashi H, Tran POT, LeRoy E, Harmon JS, Tanaka Y, Robertson RP. D-glyceraldehyde cause production of intracellular peroxide in pancreatic islets, oxidative stress, and defective beta cell function via non-mitochondrial pathways. J Biol Chem. 2004;279(36):37316-37323.

31. Jojima T, Tomotsune T, Iijima T, Akimoto K, Suzuki K, Aso Y. Empagliflozin (an SGLT2 inhibitor), alone or in combination with linagliptin (a DPP-4 inhibitor), prevents steatohepatitis in a novel mouse model of non-alcoholic steatohepatitis and diabetes. Diabetol Metab Syndr. 2016;8:45.

32. Khoshi A, Goodarzi G, Mohammadi R, Arezumand R, Moghbeli M, Najariyan M. Reducing effect of insulin resistance on alpha-synuclein gene expression in skeletal muscle. Diabetol Metab Syndr. 2019;11:99.

33. Glastras SJ, Chen H, Teh R, McGrath RT, Chen J, Pollock CA, et al. Mouse model of diabetes, obesity and related kidney disease. PLoS One. 2016;11(8):e0162131.

Figures

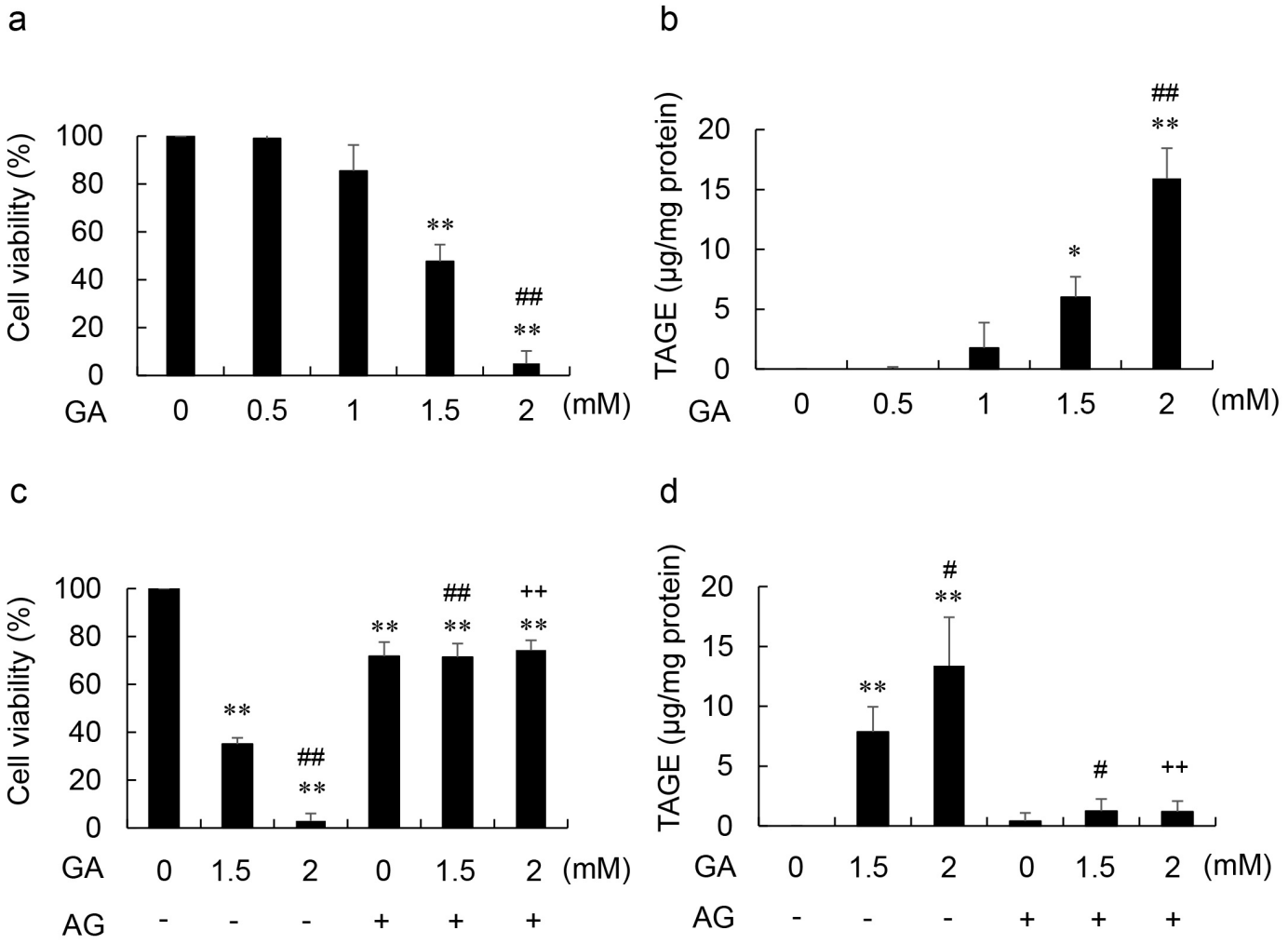

Figure 1

Cell viability and quantity of intracellular TAGE in C2C12 cells treated with GA and AG. (a, b) Cells were treated with 0, 0.5, 1, 1.5, and 2 mM GA for 24 h. (c, d) Cells were pretreated with 0 or 8 mM AG for 2 h, followed by 0, 1.5, and 2 mM GA for 24 h. (a, c) Cell viability was assessed by the WST-8 assay, which was performed in three independent experiments. One experiment was performed using 7 wells to calculate the average. Data are shown as means ± S.D. (N=3). P-values were based on Tukey's test. (b, d) Intracellular TAGE were analyzed with a slot blot (SB) analysis. Cell lysates (2.0 μg of protein/lane) were blotted onto a polyvinylidene difluoride membrane. The amount of TAGE was calculated based on a calibration curve for TAGE-BSA. A SB analysis was performed in three independent experiments. Data

are shown as means ± S.D. (N=3). P-values were based on Tukey's test. (a, b) *p<0.05 vs. 0 mM GA. **p<0.01 vs. 0 mM GA. ##p<0.01 vs. 1.5 mM GA. (c, d) **p<0.01 vs. 0 mM GA without AG. #p<0.05 vs. 1.5 mM GA without AG. ##p<0.01 vs. 1.5 mM GA without AG. ++p<0.01 vs 2 mM GA without AG.

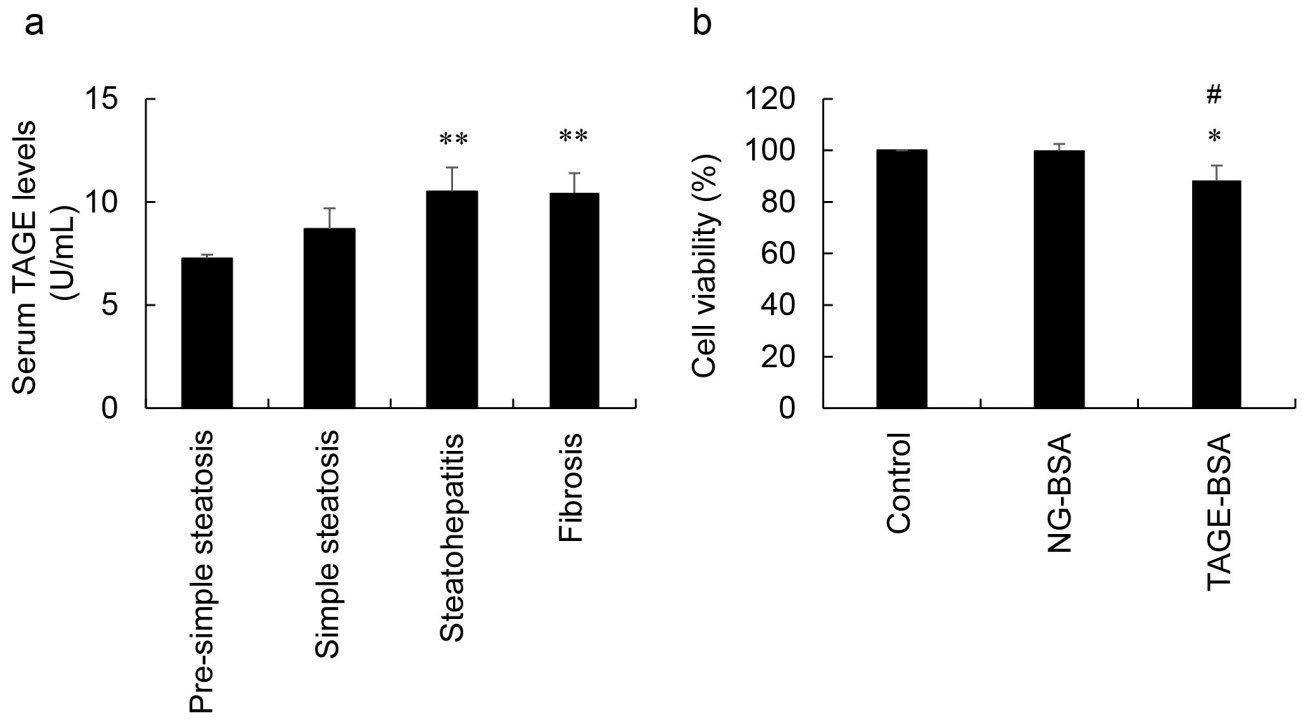

Figure 2

Serum TAGE levels in STAM mice and cytotoxicity of NG-BSA and TAGE-BSA against C2C12 cells. (a) Serum TAGE levels of the four stage groups of STAM mice. There were 4 mice in each group. Data are shown as means ± S.D. (N=4). P-values were based on Tukey's test. **p<0.01 vs. the pre-simple steatosis stage. (b) Cells were treated with 0 and 20 μg/mL NG-BSA and TAGE-BSA for 24 h. Cell viability was assessed by the WST-8 assay. This assay was performed in three independent experiments. One experiment was performed using 7 wells to calculate the average. Data are shown as means ± S.D. (N=3). P-values were based on Tukey's test. *p<0.05 vs. the control. #p<0.05 vs. the NG-BSA treatment.