

The production of cross-reactive autoantibodies that bind to bovine serum albumin in mice administered reducing sugars by subcutaneous injection

JI-HUN PARK, TAE-SAENG CHOI

Department of Microbiology, College of Medicine, Dankook University, Cheonan, Korea
Department of Nanobiomedical Science and BK21 PLUS NBM Global Research Center for Regenerative Medicine, Dankook University, Cheonan, Korea

Abstract

Introduction: In a previous study, we identified the formation of cross-reactive autoantibodies that bound to bovine serum albumin (BSA) in a D-galactose-induced aging mouse model.

Aim of the study: In this study, we investigated the effect of other reducing sugars (namely, glucose and fructose) on the formation of autoantibodies. The effects of concentration and route of administration on the formation of autoantibodies were examined in detail.

Material and methods: Three concentrations (100, 500, and 1,000 mg/kg) of reducing sugars were tested. The effects of different routes of administration (subcutaneous, oral, and intraperitoneal) on the formation of autoantibodies were also analysed. The immunoreactivities of serum samples from mice treated with reducing sugars were analysed by an enzyme-linked immunosorbent assay (ELISA) using BSA or mouse serum albumin antigens (MSA).

Results: Repeated subcutaneous administration of all reducing sugars lead to autoantibody formation in a concentration-dependent manner. However, these autoantibodies did not cross-react with MSA, and simultaneous treatment of aminoguanidine with reducing sugars did not show any inhibitory effects on the formation of autoantibodies. No autoantibodies were detected after oral or intraperitoneal administration of reducing sugars. Immunohistochemistry data showed that the target antigen(s) of the autoantibodies were present only in the skin tissue of mice treated with reducing sugars.

Conclusions: Our results show that administration of reducing sugars by subcutaneous injection leads to the formation of autoantibodies that cross-react with BSA; the formation and target antigen(s) of the autoantibodies may originate from within the skin tissue treated with the reducing sugars.

Key words: autoantibody, reducing sugar, bovine serum albumin (BSA), advanced glycation end products (AGEs).

(Centr Eur J Immunol 2015; 40 (1): 25-29)

Introduction

Sugar is a vital and major energy source for the body. However, an excessive intake of sugar can lead to increased blood glucose levels that can damage various physiological systems, resulting in harmful effects on health [1, 2]. Over the last 20 years, sugar consumption has increased worldwide, paralleling the rise in obesity and chronic disease, including diabetes and cardiovascular disease [3, 4]. Advanced glycation end products (AGEs), which are formed by the Maillard reaction (the non-enzymatic reaction of amino groups in proteins with reducing sugars) are among the harmful substances created when high levels of sugar are continuously present

in the body [5]. The advanced glycation end products induce cross-linking of long-lived proteins and expression of pro-inflammatory cytokines via interaction with AGE receptors [6]. Many studies have shown that accumulation of AGEs in the body leads directly or indirectly to various diseases, including diabetes, Alzheimer's disease, atherosclerosis, renal failure, and degenerative diseases [5]. More recently, it has been reported that several AGE products are antigenic, leading to the formation of antibodies specific for some AGEs or intermediate glycation products [7-9].

The D-galactose (D-gal) induced animal model, which is established by the consecutive injection of D-gal for ~6 weeks, has frequently been used to investigate the influence of excessive AGEs on the body [10-13]. Previously,

Correspondence: Tae-Saeng Choi, Room 403, Department of Microbiology, College of Medicine, Dankook University, 119 Dandae-ro, Dongnam-gu, Cheonan 330-714, Korea, phone +82-41-550-3872, fax +82-41-550-3864, e-mail: tschoi@dankook.ac.kr

we identified the formation of cross-reactive autoantibodies binding to bovine serum albumin (BSA) in the D-gal induced mouse model [14]. We considered whether the formation of autoantibody was D-gal specific. In this study, the effects of two further reducing sugars (glucose and fructose) on the formation of autoantibodies were investigated. The effects of concentration and route of administration on the formation of autoantibodies were also examined.

Material and methods

Animals and reducing sugar treatments

This study used female ICR mice aged 6-8 weeks. The mice were given free access to water and a normal diet, and were housed on a 12-hour light-dark cycle at $24 \pm 1^\circ\text{C}$ and 50% humidity. The mice were randomly assigned into experimental groups, each containing five mice. After a one-week adaptation period, mice from each group were administered a daily dose of 100-, 500-, or 1,000 mg/kg of reducing sugars by subcutaneous (SC), oral, or intraperitoneal (IP) routes, or a vehicle (0.9% saline) as a control for ~6 weeks. The reducing sugars used were D-gal, D-glucose (D-glu), and D-fructose (D-fruc) (Sigma-Aldrich, St. Louis, MO, USA). One group of mice undergoing each reducing sugar treatment was continually administered with 0.1% aminoguanidine (AG), an AGE-blocking agent, via the drinking water. All animal studies were conducted in compliance with the Guidelines for the Care and Use of Research Animals established by Dankook University Animal studies Committee.

For the blood serum preparation, whole blood obtained from an incision in the tail vein with a sharp surgical blade was allowed to clot at room temperature. It was then centrifuged at $1,500 \times g$ for 15 minutes. The serum was aliquoted into microcentrifuge tubes and stored at -80°C until use.

Enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay (ELISA) was performed on 96-well polystyrene plates. The plates were coated with 100 μl of 20 $\mu\text{g}/\text{ml}$ BSA or mouse serum albumin (Sigma Aldrich) in a 0.05 mol/l carbonate-bicarbonate buffer, at pH 9.6. The plates were incubated for 2 hours at 37°C or overnight at 4°C . Unbound antigen was removed by washing the plates three times with PBS-T (20 mM PBS, pH 7.4 containing 0.05% Tween-20), and unoccupied sites were blocked with 2% fat-free skimmed milk in PBS-T for 1 hour at room temperature. After incubation, the plates were washed a further three times with PBS-T. To quantify the antibodies present, serum was diluted serially to $\times 400$ and $\times 1,600$. Diluted test serum was added to the antigen-coated wells and incubated for 2 hours at room temperature or overnight at 4°C . Bound IgG was measured using an ELISA colorimetric detection kit (BluePhos[®] Mi-

crowell Phosphatase Substrate System; KPL, Gaithersburg, Maryland, USA). The absorbance of each well was monitored at 595 nm on an automatic microplate reader, and the mean of duplicate readings for each sample was recorded.

Skin tissue preparation and staining

Mice were sacrificed by cervical dislocation ~6-7 weeks after the reducing sugar treatments. Skin tissues, including the injection sites, were dissected and fixed in 10% formalin overnight. The fixed tissues were embedded in paraffin blocks using automated processing and embedding equipment. Tissue sections of 5- μm thickness were cut and mounted onto glass slides. These were stained with Harris's haematoxylin and eosin (H&E) or subjected to immunohistochemical staining.

Haematoxylin and eosin staining was performed according to the standard protocol. Immunohistochemical staining was carried out with an automatic staining system (Leica ST5020; Leica Microsystems, Wetzlar, Germany) and the Bond Intense R Detection kit (Leica Microsystems), according to the manufacturer's instructions. In brief, skin tissue sections were dewaxed and rehydrated by successive incubation at 72°C in Bond Dewax Solution (Leica Microsystems), ethanol, and distilled water. Antigens were retrieved by heating sections for 20 minutes at 100°C in Bond Epitope Retrieval Solution 2 (Leica Microsystems). Endogenous peroxidase was blocked by the addition of 3% hydrogen peroxide prior to 15-minute incubation with a 1,000-fold dilution of serum samples from the control- or reducing sugar-treated mice. Antibody-antigen reactions were visualised using diaminobenzidine and counterstained with haematoxylin.

Statistical analyses

Statistical differences were subjected to one-way analysis of variance (ANOVA). Data are shown as means \pm the standard error of the mean (SEM), and significance was defined as $p < 0.05$.

Results

Antibody production against bovine serum albumin in reducing-sugar-treated mice

The immunoreactivity against BSA of serum samples collected from mice injected with reducing sugars (1,000 mg/kg) at 2, 4, and 6 weeks was tested. At 2 weeks, little immunoreactivity was detected in the serum of mice treated with any of the three reducing sugars. However, immunoreactivity was significantly higher at 4 and 6 weeks after treatment (Fig. 1A). It was interesting to note that 6-week simultaneous treatment of aminoguanidine with the reducing sugars exerted no inhibitory effect on immunoreactivity (Fig. 1A). To investigate the effects of concentration on immunoreactivity, 100, 500, and 1,000 mg/kg reducing

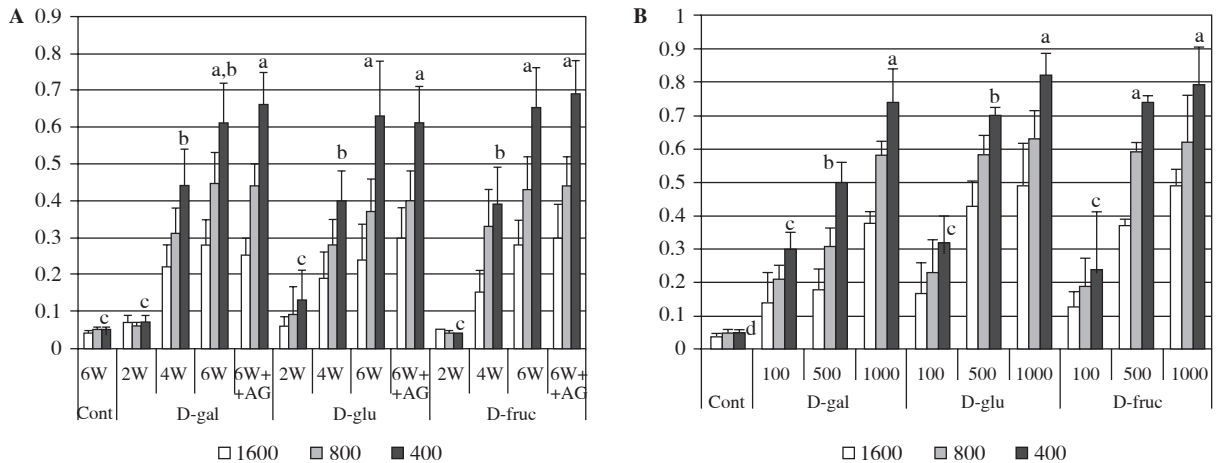


Fig. 1. Immunoreactivity to BSA of serum from mice treated with reducing sugars. **A)** Serum samples from each mouse group were obtained at 2, 4, and 6 weeks and diluted as indicated in the right-hand column before ELISA for immunoreactivity to BSA. Data represent the means \pm SEM of five mice per group. AG – aminoguanidine. **B)** Effects of reducing sugar concentration on immunoreactivity. Serum samples were obtained 6 weeks following treatment with 100, 500, or 1,000 mg/kg reducing sugar. Superscript figures indicate significantly different values ($p < 0.05$)

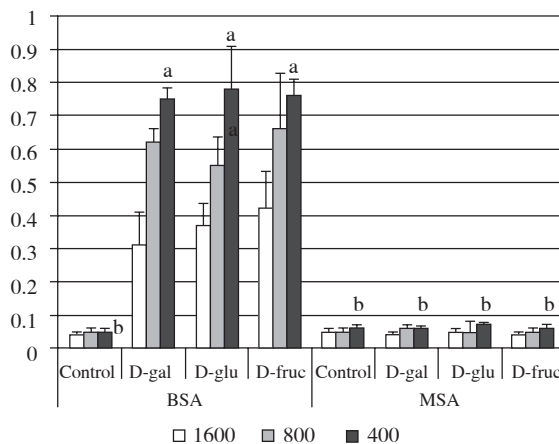


Fig. 2. Immunoreactivity to MSA of serum from mice treated with reducing sugars. Serum samples were obtained 6 weeks following treatment with 1,000 mg/kg reducing sugars. BSA or MSA were used as antigens in the ELISA. Data represent means \pm SEM of five mice per group. Superscript figures indicate significantly different values ($p < 0.05$)

sugars were used. There was no significant difference between the 500 and 1,000 mg/kg groups, but immunoreactivity was significantly lower following administration of 100 mg/kg (Fig. 1B).

Reducing-sugar-induced antibodies did not cross-react with mouse serum albumin antigens

In a previous study, we showed that autoantibodies induced by D-gal injection cross-reacted with BSA but not

with mouse serum albumin antigens (MSA). To determine whether the autoantibodies induced by injection of D-glu or D-fruc had the same characteristics as those induced by D-gal, immunoreactivity was tested using MSA as the antigen. The same serum samples as shown in Figure 1 were used (i.e. treatment with 1,000 mg/kg reducing sugars for 6 weeks). All of the autoantibodies (D-gal, D-glu and D-fruc) cross-reacted with BSA but not with MSA (Fig. 2).

Effects of administration route on autoantibody production

To investigate the effect of administration route on autoantibody production, autoantibody was administered orally and IP. The reducing sugar concentration was 1,000 mg/kg and the serum samples were obtained 6 weeks after treatment. No autoantibody formation was detected after oral or IP administration (Fig. 3).

Skin immunohistochemistry

The strong immunoreactivity observed when the subcutaneous injection of reducing sugar suggested that unknown proteins in the mouse skin functioned as antigens to produce autoantibodies. To investigate this, H&E staining and immunohistochemical analyses were performed using the autoantibody on mouse skin tissues. Haematoxylin and eosin staining showed localisation of granulation tissue beneath the muscle layers and infiltration of immune cells, especially in mouse skin treated with reducing sugars (Fig. 4A). The immunohistochemical analysis showed autoantibody reactivity by some cell types, including macrophage-like cells, and the stratum corneum in skin tissues treated with reducing sugars (Fig. 4B).

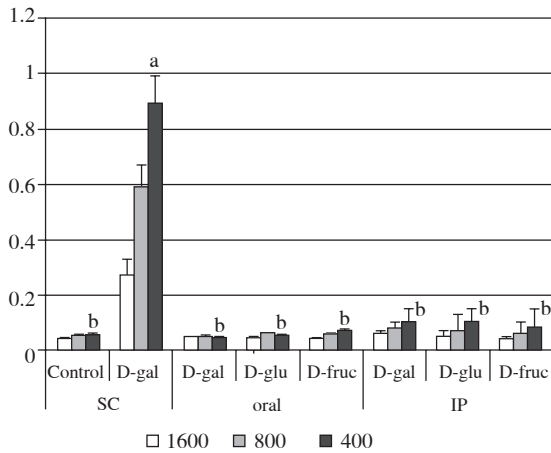


Fig. 3. Effects on immunoreactivity of the route of reducing sugar administration. Serum samples were obtained 6 weeks following SC, oral, or IP administration of 1000 mg/kg reducing sugars. Superscript figures indicate significantly different values ($p < 0.05$)

Discussion

Recently, we reported on the formation of cross-reactive autoantibodies binding to BSA but not to MSA, in a D-gal induced mouse model [14]. In this study, we investigated the effects of other reducing sugars (glucose and fructose) on the formation of autoantibodies. We also examined the effects of concentration and route of administration on autoantibody formation. Our results showed that glucose- and fructose-induced formation of autoantibodies, but only following subcutaneous injection.

An ELISA was performed to investigate immunoglobulin (Ig) G antibody formation. Immunoreactivity levels

were very low at 2 weeks, but significantly higher at 4 and 6 weeks after reducing sugar treatment (Fig. 1A). The immune system produces IgM initially, followed by IgG by isotype switching. Although IgM levels were not investigated in this study, the absence of immunoreactivity at 2 weeks may be explained by a delay in isotype switching from IgM to IgG.

Reducing sugars react with the amino groups of proteins, leading to the formation of advanced glycation end products (AGEs) *in vitro* and *in vivo* [15, 16]. Recent studies have reported that intermediate glycated products or AGEs possess immune potential and behave like haptens [8, 17]. Our initial hypothesis was that AGEs or certain intermediate glycated products produced due to excessive intake of reducing sugars acted as antigens *in vivo*, triggering autoantibody production. Aminoguanidine inhibits AGE formation *in vivo* in a variety of animal models [18, 19]. Thus, we expected that inhibition of AGE synthesis by aminoguanidine would prevent or reduce autoantibody formation. However, our results showed no inhibitory effects of treatment with aminoguanidine on autoantibody production (Fig. 1A), even when tested at the lower reducing sugar concentration of 500 mg/kg (data not shown). These results led to the supposition that the production of AGEs was not directly relevant to the production of autoantibodies. The possibility that an intermediate glycated product may act as an antigen cannot be ruled out because the formation of early glycation products is not inhibited by aminoguanidine [20].

In this study, autoantibody production occurred only following subcutaneous administration of reducing sugars. This result suggests that autoantibody production may require stimulation of subcutaneous tissues by reducing sugars, resulting in production of antigens. The immunohistochemistry results showed autoantibody cross-reactiv-

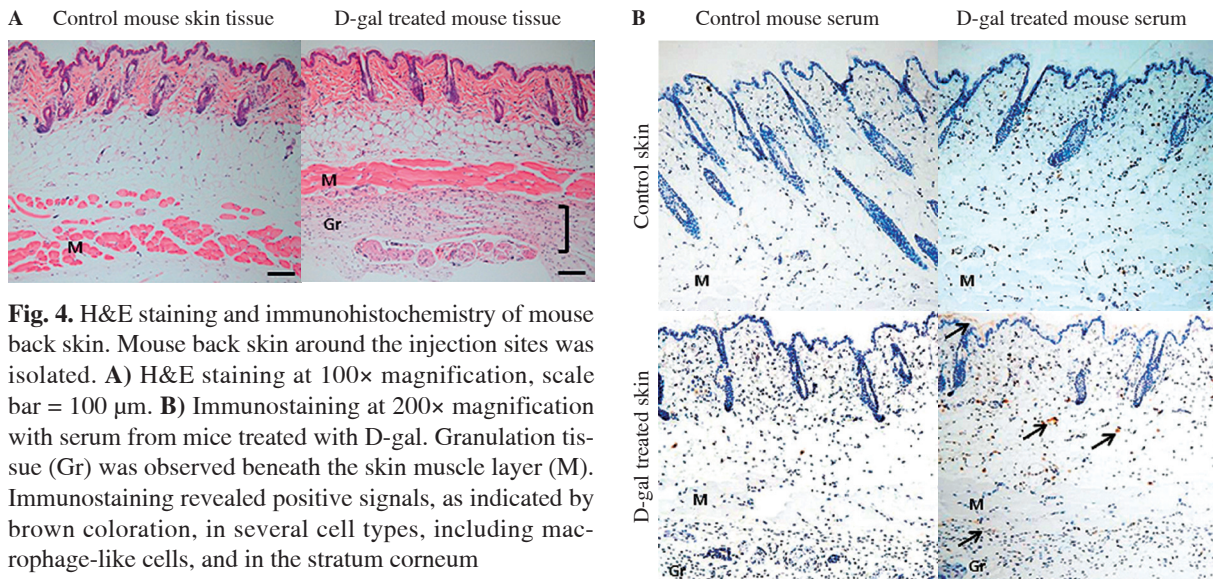


Fig. 4. H&E staining and immunohistochemistry of mouse back skin. Mouse back skin around the injection sites was isolated. **A)** H&E staining at 100 \times magnification, scale bar = 100 μ m. **B)** Immunostaining at 200 \times magnification with serum from mice treated with D-gal. Granulation tissue (Gr) was observed beneath the skin muscle layer (M). Immunostaining revealed positive signals, as indicated by brown coloration, in several cell types, including macrophage-like cells, and in the stratum corneum

ity with some cell types in subcutaneous tissues following treatment with reducing sugars (Fig. 4B). Interestingly, there was no cross-reactivity in the subcutaneous tissue of the control group. This could be explained by the creation of new antigens in the subcutaneous tissue through transcription and post-translational modification (including glycation), or by infiltration of new cells following stimulation with reducing sugars. Our results also showed the formation of granulation tissues and immune cell infiltration in mouse skin after repeated injection of reducing sugars, and autoantibody cross-reactivity with macrophage-like cells in the skin tissue (Fig. 4B).

The autoantibodies induced by D-glu and D-fruc showed identical characteristics to those induced by D-gal, i.e. non-reactivity with MSA but reactivity with BSA. This suggests that in mice, reducing sugars induce production of antibodies in response to unknown antigens, aside from MSA, and that these unknown antigens may have sequences or three-dimensional structures similar to those of BSA.

To our knowledge, this is the first report showing that autoantibody production is caused by excessive reducing sugars in vivo. This research may expand our understanding of autoantibody production arising from a variety of causes.

Further studies should aim to identify these antigens using synthetic peptides derived from BSA sequences that are not present in MSA.

The authors declare no conflict of interest.

We would like to thank Professor Won-Ae LEE for his technical assistance with tissue staining. The authors declare no conflict of interest.

References

1. Moreira PI (2013): High-sugar diets, type 2 diabetes and Alzheimer's disease. *Curr Opin Clin Nutr Metab Care* 16: 440-445.
2. Karalius VP, Shoham DA (2013): Dietary sugar and artificial sweetener intake and chronic kidney disease: a review. *Adv Chronic Kidney Dis* 20: 157-164.
3. Tappy L, Le KL (2010): Metabolic effects of fructose and the worldwide increase in obesity. *Physiol Rev* 90: 23-46.
4. Richelsen B (2013): Sugar-sweetened beverages and cardio-metabolic disease risks. *Curr Opin Clin Nutr Metab Care* 16: 478-484.
5. Semba RD, Nicklett EJ, Ferrucci L (2010): Does accumulation of advanced glycation end products contribute to the aging phenotype? *J Gerontol A Biol Sci Med Sci* 65: 963-975.
6. Chuah YK, Basir R, Talib H, et al. (2013): Receptor for advanced glycation end products and its involvement in inflammatory diseases. *Int J Inflamm* 2013: 403460.
7. Shibayama R, Araki N, Nagai R, et al. (1999): Autoantibody against N(epsilon)-(carboxymethyl)lysine: an advanced glycation end product of the Maillard reaction. *Diabetes* 48: 1842-1849.
8. Bozhinow A, Handzhiyski Y, Genov K, et al. (2012): Advanced glycation end products contribute to the immunogenicity of IFN- β pharmaceuticals. *J Allergy Clin Immunol* 129: 855-858.
9. Chikazawa M, Otaki N, Shibata T, et al. (2013): Multispecificity of immunoglobulin M antibodies raised against advanced glycation end products: involvement of electronegative potential of antigens. *J Biol Chem* 288: 13204-13214.
10. Song X, Bao M, Li D, et al. (1999): Advanced glycation in D-galactose induced mouse aging model. *Mech Ageing Dev* 108: 239-251.
11. Cui X, Zuo P, Zhang Q, et al. (2006): Chronic systemic D-galactose exposure induces memory loss, neurodegeneration, and oxidative damage in mice: protective effects of R-alpha-lipoic acid. *J Neurosci Res* 83: 1584-1590.
12. Wu L, Sun Y, Hu YJ, et al. (2012): Increased p66Shc in the inner ear of D-galactose-induced aging mice with accumulation of mitochondrial DNA 3873-bp deletion: p66Shc and mtDNA damage in the inner ear during aging. *PLoS One* 7: e50483.
13. Park JH, Choi TS (2012): Polycystic ovary syndrome (PCOS)-like phenotypes in the D-galactose-induced aging mouse model. *Biochem Biophys Res Commun* 427: 701-704.
14. Park JH, Choi TS (2014): Production of cross-reactive autoantibody binding to bovine serum albumin in the D-galactose-induced aging mouse model. *Am J Immunol* 10: 3-9.
15. Kaneto H, Fujii J, Myint T, et al. (1996): Reducing sugars trigger oxidative modification and apoptosis in pancreatic beta-cells by provoking oxidative stress through the glycation reaction. *Biochem J* 320: 855-863.
16. Luers L, Rysiewski K, Dumpitak C, et al. (2012): Kinetics of advanced glycation end products formation on bovine serum albumin with various reducing sugars and dicarbonyl compounds in equimolar ratios. *Rejuvenation Res* 15: 201-205.
17. Buongiorno AM, Morelli S, Sagratella E, et al. (2008): Immunogenicity of advanced glycation end products in diabetic patients and in nephropathic non-diabetic patients on hemodialysis or after renal transplantation. *J Endocrinol Invest* 31: 558-562.
18. Ulrich P, Cerami A (2001): Protein glycation, diabetes, and aging. *Recent Prog Horm Res* 56: 1-21.
19. Vlassara H, Fuh H, Makita Z, et al. (1992): Exogenous advanced glycosylation end products induce complex vascular dysfunction in normal animals: a model for diabetic and aging complications. *Proc Natl Acad Sci U S A* 89: 12043-12047.
20. Thornalley PJ (2003): Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts. *Arch Biochem Biophys* 419: 31-40.