〈Brief Note〉

Effect of high glucose concentration on aging and glycation in *Caenorhabditis elegans*

Ou Setoyama

Summary High glucose levels reportedly cause obesity and type 2 diabetes, and hyperglycemia promotes the production of advanced glycation end (AGE) products in the body. In this study, to clarify the effects of high glucose conditions on in vivo glycation and aging in *Caenorhabditis elegans* (*C.elegans*), the nematodes were cultured in a high-glucose condition medium containing a final concentration of 200 mmol/L glucose. We revealed that *C. elegans* cultured under high glucose conditions exhibited increased in vivo intracellular glucose concentrations and, consequently, increased levels of ROS, carbonylated proteins, and fluorescent AGEs.

Key words: *Caenorhabditis elegans*, Glycation, High glucose condition, Aging

1. Introduction

Dietary glucose metabolism is essential for vital activities. However, high glucose levels reportedly cause obesity and type 2 diabetes, and hyperglycemia promotes the production of advanced glycation end (AGE) products in the body[1]. Moreover, global sugar consumption has been increasing in recent years. Therefore, it is imperative to understand the biological consequences of excessive glucose intake[2].

Caenorhabditis elegans (*C. elegans*) is an ideal research model organism for aging and has been recently studied as a model for diabetes[3]. Previously reported effects of high glucose conditions in *C. elegans* include life span shortening via the induction of apoptosis[4], neurological disorders[5], and increased fat accumulation[6]. Furthermore, it has been reportedly linked to increased mitochondrial reactive oxygen species (ROS) formation[3]. However, the *in vivo* effects of high glucose conditions on glycation and aging in *C. elegans* remain largely unknown. In this study, we investigated the changes in ROS formation, as well as carbonylated protein and fluorescent AGE levels in *C. elegans* cultured under high glucose conditions in order to reveal the *in vivo* effects of such conditions on glycation and aging in *C. elegans*.

Kanagawa Institute of Industrial Science and Technology, 705-1 Shimoimaizumi, Ebina, Kanagawa 243-0435 Japan.
Tel: +81-46-236-1500
Fax: +81-46-236-1525
E-mail: setoyama@kistec.jp

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2. Materials and Methods

Nematode culture conditions

The wild type *C. elegans* (N2, Bristol) was maintained at 20°C on nematode growth medium (NGM) seeded with *Escherichia coli* (*E. coli*) feeding strain OP50[7].

Synchronized worm cultures

Age-synchronous animals were obtained by the bleaching of gravid adults. Gravid adults were rinsed off the NGM agar plates with S-buffer into collection tubes, the solution volume was adjusted to 4.5 mL with S-buffer (5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, H_2O to 1000 mL), then 0.5 mL bleaching solution (0.4 mL fresh bleach, 0.1 mL 10 N NaOH solution) was added into the tube and thoroughly mixed. The worm-containing solutions were then vortexed at 25°C for 3 min. The eggs were then washed in S-buffer, centrifuged at 3000 rpm for 1 min, and the supernatant was discarded. The eggs were washed three times with S-buffer, resuspended in 5 mL of fresh S-complete medium, allowed to hatch overnight, and their concentration in the solution was set to 2000 larvae per mL. The day of hatching was designated as day 1[8,9]. For feeding bacteria preparation, *E. coli* (OP50) cultures were grown overnight in 200 mL of Luria-Bertani (LB) broth, then centrifuged at 8000 rpm for 3 min, and the pellets were weighed in 50 mL pre-weighed sterile centrifuge tubes. The pellets were suspended in S-complete medium to maintain a concentration of 100 mg wet weight in 1 mL of medium. Heat-killed bacteria were prepared by exposing the pellets to 75°C for 1 h[10].

High-glucose condition worm cultures

On day 1, 100 mL culture flasks were seeded with L1 stage larvae at a concentration of 5000 worms per flask, and then heat-killed *E. coli* (OP50) bacteria were added to a final concentration of 10 mg/mL. The total culture volume was set to 20 mL. The culture conditions were maintained at 20°C at 100 rpm continuous shaking.

On day 2, glucose was added to the medium to a final concentration of 200 mmol/L in order to model high glucose conditions. Cultures with no added glucose were used as controls. 5-fluorodeoxyuridine (5-FUdR) was added to each medium to a final concentration of 40 μmol/L. All experimental set for each treatment condition was performed in triplicates.

C. elegans homogenization and protein extraction

On day 10, worms were collected from each culture flask, washed in cold S buffer, and centrifuged at 800 rpm for 1 min. The supernatant was discarded, the pellet was washed five times with cold S-buffer, and stored on ice until further use. Prior to protein extraction, the collected worms were resuspended in commercial RIPA Buffer (Nacalai Tesque, Japan), sonicated, homogenized, centrifuged at 15000 rpm for 15 min, and the supernatant was collected to obtain lysates. The protein concentration was determined using a commercial TaKaRa BCA Protein Assay Kit (Takara Bio, Japan), following the manufacturer’s protocol.

Intracellular glucose level measurement

The quantitative analysis of intracellular glucose levels was performed using a commercial Glucose Assay Kit (STA-680, Cell Biolabs, USA), following the manufacturer’s protocol.

Quantitative fluorescent AGE accumulation analysis

For the quantitative fluorescent AGE accumulation analysis, 150 μL of each worm extract was adjusted to a protein concentration of 100 μg/mL, and added to each well of a 96-well black plate. Each sample well underwent spectrofluorometric analysis at 340 nm excitation and 430 nm emission spectra using a multimode plate reader (Infinite® 200 PRO, Tecan Japan)[11,12].

Carbonyl protein level measurement

Each worm extract was adjusted to a protein concentration of 500 μg/mL. The quantitative analysis of the carbonyl protein level was performed using the commercial QuantiChrom Carbonyl Assay

Kit (DCAR-100, BioAssay Systems, USA), following the manufacturer's protocol.

Quantitative analysis of the intracellular reactive oxygen species (ROS) level

Each worm extract was adjusted to a protein concentration of 500 μg/mL. The intracellular ROS analysis was performed using CM-H_2DCFDA (Thermo Fisher, USA)[13], following the manufacturer's protocol.

Data analysis

The data are presented as mean ± standard error of the mean, as indicated. The statistical significance of differences between the control and treated groups were analyzed by Student's t-test.

3. Results

Glucose concentrations in *C. elegans*

Fig. 1 shows the glucose concentrations in *C. elegans* cultured under high glucose conditions (at final concentrations of 200 mmol/L). The results indicate that worm extracts from high glucose culture conditions exhibited more than twice higher intracellular glucose concentrations compared to the control.

Accumulation of fluorescent AGEs in *C. elegans*

The accumulation of fluorescent AGEs in *C. elegans* cultured under high glucose conditions (final concentration of 200 mmol/L) was measured in homogenized worm extracts (Fig. 2). The results of this experiment indicate approximately 1.7 times higher fluorescent AGE accumulation levels in worms cultured under high glucose concentration than in controls.

ROS formation and carbonyl protein amounts in *C. elegans*

The ROS formation and carbonylated protein amounts in *C. elegans* cultured under high glucose conditions (at a final concentration of 200 mmol/L) were measured in homogenized worm extracts (Fig.

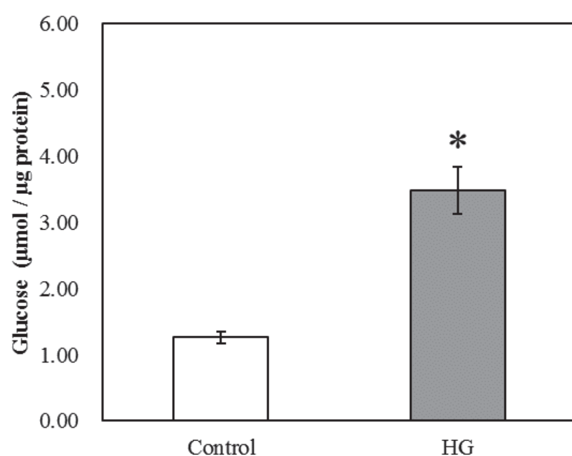

Fig. 1 Effect of High glucose condition on intracellular glucose levels in *C. elegans*. Glucose was added to the medium to achieve a HG (high glucose) condition at a final concentration of 200 mmol/L. Controls were treated without glucose. The glucose level is expressed in μmol/mg protein.
$*p < 0.05$ (n = 3, Student's t-test)

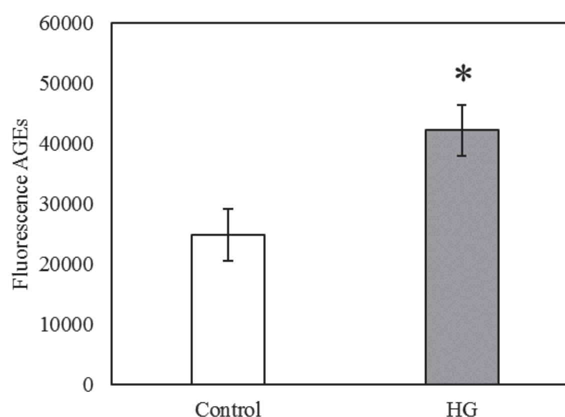

Fig. 2 Effect of high glucose condition on fluorescent AGE accumulation in *C. elegans*. Glucose was added to the medium to achieve a HG (high glucose) condition at a final concentration of 200 mmol/L. Controls were treated without glucose. The fluorescent AGE accumulation was analyzed using spectrofluorometry at 340 nm excitation and 430 nm emission spectra.
$*p < 0.05$ (n = 3, Student's t-test)

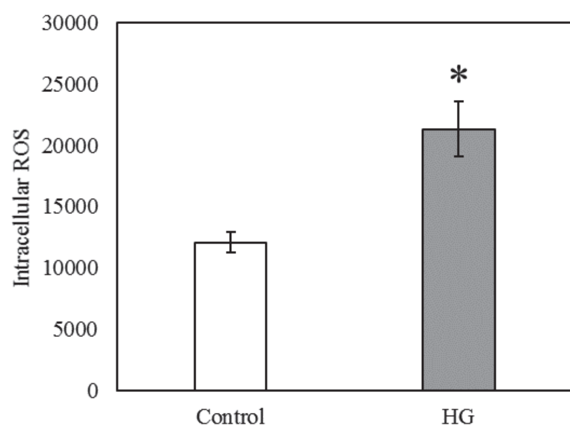

Fig. 3 Effect of high glucose condition on intracellular ROS levels in *C. elegans.* Glucose was added to the medium to achieve a HG (high glucose) condition at a final concentration of 200 mmol/L. Controls were treated without glucose. The intracellular ROS analysis was performed using CM-H_2DCFDA.
$*p < 0.05$ (n = 3, Student's t-test)

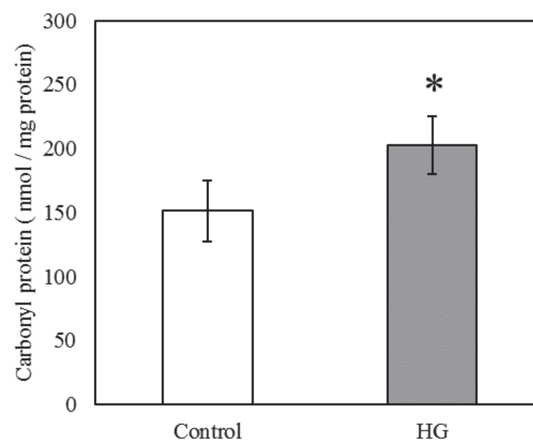

Fig. 4 Effect of high glucose condition on carbonyl protein levels in *C. elegans.* Glucose was added to the medium to achieve a HG (high glucose) condition at a final concentration of 200 mmol/L. Controls were treated without glucose. The carbonyl protein levels are expressed in nmol/mg protein.
$*p < 0.05$ (n = 3, Student's t-test)

3, Fig. 4). The results show approximately twice higher ROS-derived fluorescence intensities and 1.3 times higher carbonylated protein amounts in the extracts of *C. elegans* cultured under high glucose conditions compared to the control.

4. Discussion

In order to clarify the effects of high glucose conditions on *in vivo* glycation and aging in *C. elegans*, the nematodes were cultured in a high-glucose condition medium containing a final concentration of 200 mmol/L glucose. We observed significantly increased intracellular glucose concentrations in *C. elegans* cultured under high glucose conditions. Our result was similar to previous findings[3,14], suggesting higher levels of glucose uptake in *C. elegans* cultured in a medium of high glucose concentrations compared with the control.

A substantial increase in ROS formation was also observed in *C. elegans* cultured under high glucose conditions. This increase could likely be attributed to the increased glucose concentration in *C. elegans*. Mitochondrial dysfunction is reportedly responsible for the increased ROS formation in *C. elegans* under high glucose conditions[3]. A similar mechanism is likely underlying the increased ROS formation observed in this study.

An increased ROS formation reflects an increase in oxidative stress. Oxidative stress is well-known to target proteins in the body and is thus involved in aging and diseases[15,16]. Carbonyl proteins represent a class of oxidatively modified proteins. Carbonyl protein is a generic term for proteins, in which an aldehyde group is introduced into the amino acid side chain. Carbonyl proteins have been implicated in various human diseases and are used as oxidative stress biomarkers[17,18]. Regarding the effects of glucose concentration in the culture medium on the carbonyl protein levels in *C. elegans*, a previous study has shown that it remains unchanged at a final glucose concentration of 10 mmol/L but increases at a final glucose concentration of 100 mmol/L[19]. The present study also showed that the carbonyl protein levels in *C. elegans* increased under high glucose conditions compared to the control. This is presumably due to the high glucose condition-induced increased ROS formation,

which in turn promotes oxidative protein modifications in *C. elegans*.

In general, when the body is at a high glucose state, as found in patients with diabetes, protein glycation is promoted, and larger amounts of AGEs are produced in the body[1]. The AGE precursor methylglyoxal levels reportedly increase in *C. elegans* in the presence of 40 mmol/L glucose[3]. The present study also showed that the fluorescent AGE levels in *C. elegans* under high glucose conditions increased compared to those under the control conditions. This result suggests that *in vivo* protein glycation is enhanced in *C. elegans* cultured under high glucose conditions. A close association has been established between AGE formation and ROS formation. For example, methylglyoxal induces ROS formation and AGE formation occurs under oxidative conditions[20,21,22]. The increased *in vivo* ROS formation observed under high glucose conditions in the present study is likely to contribute to the increased AGE production.

In this study, we revealed that *C. elegans* cultured under high glucose conditions exhibited increased in vivo intracellular glucose concentrations and, consequently, increased levels of ROS, carbonylated proteins, and fluorescent AGEs. ROS and carbonylated protein are well-known to aging-related substances and indicators of aging. The increased ROS and carbonylated protein levels indicate accelerated aging, while the increased levels of fluorescent AGEs reflect enhanced glycation in *C. elegans* under high glucose conditions. This study clarified some of the relationships between *in vivo* glycation and aging of *C. elegans*.

Conflicts of interest

The author have no conflict of interest.

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