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RESEARCH ARTICLE

High Glucose Enhances Skin Sensitizer-induced NRF2 Activation *In Vitro*

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Abstract:

Background:

Hyperglycemia has a potentially critical role in the promotion of sensitization, however, the clear mechanism of this phenomenon is unknown. The activation of NRF2 is a key event triggered by skin sensitizers. Therefore, we investigated the effects of high glucose on the activation of NRF2 by the skin sensitizers *in vitro*.

Methods:

The involvement of glucose levels in NRF2 activation by cinnamaldehyde, a skin sensitizer, was assessed in human MCF-7 breast cancer cells under normal glucose conditions (1.0 g/L D-glucose) and high glucose conditions (4.5 g/L D-glucose).

Results:

High glucose induced the NRF2 transactivation, *HMOX1* mRNA expression, and SOD-like activity. Nuclear NRF2 level was increased under high glucose conditions compared to normal glucose conditions. High glucose also enhanced the cinnamaldehyde-induced *HMOX1* mRNA expression and SOD-like activity.

Conclusion:

Oxidative stress caused by hyperglycemia induced additionally the activation of NRF2 signaling by skin sensitizers.

Keywords: Glucose, Skin sensitizer, Cinnamaldehyde, Oxidative stress, KEAP1-NRF2 signaling, ROS.

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1. INTRODUCTION

Hyperglycemia, blood glucose levels above 2.0 g/L, is one of the major pathophysiological factors causing late complications in diabetes [1, 2]. Reactive Oxygen Species (ROS) are increased by hyperglycemia [3]. Oxidative damage in individual cells may reach a sufficient threshold to cause DNA strand breaks and induce cell death [4]. Therefore, hyperglycemia-induced oxidative stress is one of the responsible factors for the pathology of diabetic complications.

The frequency of allergic diseases, such as allergic rhinitis, asthma, and food allergy, has been increased dramatically over the past decade [5]. Allergies are highly dependent on both environmental factors (allergens, tobacco smoke, and indoor and outdoor air pollution) and internal factors (hormones, diet, and circadian clock). Additionally, there is a high prevalence of allergic sensitization in Type 1 diabetic patients [6]. A recent study has shown that hyperglycemia promotes sensitization in a mouse model for asthma [7, 8]. Hyperglycemia is assumed to be a contributor to the increased prevalence of skin sensitization, however, the clear mechanism of this phenomenon is unknown.

Skin sensitizations are growing among the general population as a result of increased exposure to environmental and industrial compounds present in toiletry and household products; the key biological events underlying skin sensitization are well-known [9 - 11]. Skin sensitizer means a chemical that leads to an allergic response following skin contact. Nuclear Factor Erythroid 2-related Factor 2 (NRF2) activation is a key event triggered by skin sensitizers [12, 13]. The Kelch-Like ECH-Associated Protein 1 (KEAP1)-NRF2 pathway is one of the most important cell defense systems and survival pathways against oxidative stress [14 - 16] and is involved in dampening the induction of skin sensitization [15]. NRF2 is anchored in the cytoplasm by KEAP1 under normal

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conditions. Oxidative stress induces the translocation of NRF2 into the nucleus, and then NRF2 induces gene expression *via* the binding to Antioxidant Response Elements (AREs) in the regulatory region of target genes, including Heme Oxygenase 1 (HMOX1) and Superoxide Dismutase (SOD) [14]. These enzymes are a group of enzymes that catalyze the dismutation of ROS. Many reporter gene assays using a human breast cancer cell line (MCF-7 cells), a human keratinocyte cell line (HaCaT cells), and fibroblasts were developed for the evaluation of NRF2 activation [17 - 24].

In this study, we investigated the involvement of glucose levels in the activation of KEAP1-NRF2 signaling by the skin sensitizers, such as eugenol, Cinnamaldehyde (CA), and 2,4-Dinitrochlorobenzene (DNCB) using MCF-7 cells and mouse BALB/3T3 fibroblasts transfected with an ARE-driven reporter plasmid. We also determined the effects of D-glucose concentration in the medium on NRF2 signaling in MCF-7 cells.

2. MATERIALS AND METHODS

2.1. Reagents

Eugenol and isopropanol were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). CA and DNCB were obtained from Nacalai Tesque Inc. (Kyoto, Japan) and were cultured in Dulbecco's modified Eagle's medium-high glucose (4.5 g/L glucose) (DMEM-HG) supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 lg/mL streptomycin).

2.2. Cell Culture

MCF-7 cells and BALB/3T3 clone A31 cells (RIKEN Cell Bank, Ibaraki, Japan) and were maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 4.5 g/L D-glucose, 10% fetal bovine serum (FBS), 1.5 g/L $NaHCO_3$, 100 units/mL penicillin, and 100 μg/mL streptomycin (DMEM HG) at 37°C in 5% CO_2 and 95% air atmosphere at 100% humidity.

2.3. Cytotoxicity Test

The cytotoxic effects of skin sensitizers on MCF-7 cells and BALB/3T3 clone A31 cells were investigated by measuring the activity of Lactate Dehydrogenase (LDH) in the culture supernatant. In 96-well plates, MCF-7 cells were cultured in phenol red-free DMEM supplemented with 1.0 g/L D-glucose and 10% FBS (DMEM NG) with the various concentrations of skin sensitizers for 24 hours, and four replicate wells were prepared for each treatment. LDH activity assay was measured using LDH Cytotoxicity Detection Kit (Dojindo, Kumamoto, Japan) as described previously [25, 26].

2.4. Luciferase Assay

Luciferase reporter plasmid carrying ARE was constructed according to the method of Wang *et al*. [17]. In brief, the eight copies of the ARE (5′-GTGACAAAGCA-3′) were inserted into pGL 4.20 plasmid (Promega, Madison, WI, USA) together with an adenovirus E1b TATA sequence or simian virus 40 enhancer and early promoter, termed pAREx8-TATA and pAREx8-SV40, respectively. MCF-7 cells and BALB/3T3 clone A31 cells were cultured in Opti-MEM (Thermo Fisher Scientific) on 48-well plates, and transiently transfected with pAREx8-TATA or pAREx8-SV40 using HilyMax (Dojindo) for 24hours, and incubated in fresh DMEM NG or DMEM HG medium, containing a test substance for an additional 24 hours. Luciferase activity was determined as described previously [27, 28]. Transfection efficiency was normalized with *Renilla* luciferase expression vector (pGL 4.73 [hRluc/SV40], Promega), and data were expressed as Relative Light Units (RLU, firefly luciferase activity divided by *Renilla* luciferase activity).

2.5. Immunofluorescence Assay

MCF-7 cells were cultured in DMEM NG or DMEM HG medium for 24 hours, and fixed in 4% paraformaldehyde in PBS for 10 minutes, and permeabilized with 0.2% Triton X-100 in PBS for 5 minutes as described previously [26, 29]. Fixed cells were blocked and incubated with rabbit polyclonal NRF2 antibody (Proteintech Group Inc. Rosemont, IL, USA) at room temperature for 1 hour, followed by Alexa Fluor 594-conjugated anti-rabbit IgG (Thermo Fisher Scientific). After 1 hour, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Dojindo). Fluorescent images were captured using a fluorescence microscope (BX53, Olympus, Tokyo, Japan). The ratio of nuclear to cytoplasmic NRF2 fluorescence was measured.

2.6. Quantitative Real-time PCR

MCF-7 cells were cultured in DMEM NG or DMEM HG medium containing a test substance for 24 hours. Total RNA was extracted from MCF-7 cells and reverse transcribed with PrimeScript reverse transcription Reagent Kit (Takara Bio Inc., Shiga, Japan). The resultant cDNA was subjected to quantitative real-time PCR using the specific primers (Table **1**). The PCR profiles consisted of denaturation at 95°C for 30 seconds, primer-annealing at 55°C for 20 seconds, and primer extension at 72°C for 20 seconds. The final primer extension was performed at 72°C for 10 minutes. The PCR was performed with SYBR Premix Ex Taq II (Takara Bio Inc.) on Thermal Cycler Dice, TP-900 (Takara Bio Inc.) in triplicate. Ct values were transformed into relative quantification data by $2^{-\Delta\Delta}$Ct method. Data were normalized to the mRNA levels of *GAPDH,* which encodes glyceraldehyde-3-phosphate dehydrogenase.

2.7. Superoxide Dismutase (SOD) Assay

MCF-7 cells were cultured in DMEM NG or DMEM HG medium containing a test substance for 24 hours. Cells were suspended in phosphate-buffered saline (4.3 mM Na_2HPO_4, 1.4 mM KH_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), and sonicated for 3 minutes. Sonicated samples were then centrifugated at 10,000 x *g*, at 4°C, for 15 minutes. SOD activity assay was measured using SOD Assay Kit-WST (Dojindo).

Table 1. PCR primer sequences.

Gene	-	Sequence (5′-3′)
HMOX1	Forward	CTCAAACCTCCAAAAGCC
	Reverse	TCAAAAACCACCCCAACCC
GAPDH	Forward	CAACGGATTTGGTCGTATTGG
	Reverse	GCAACAATATCCACTTTACCAGAGTTAA

2.8. Statistical Analyses

The half-maximal cytotoxic concentration (CC_{50}) values were calculated by fitting to a standard 4-parameter logistic curve using the R (version 3.6.1). Data were statistically compared by Dunnett's test or Student *t*-test using the R software (version 3.6.1). Data were expressed as mean ± S.D., and the differences were considered statistically significant at a *p*-value of < 0.05 (n = 3 or n = 6).

3. RESULTS

3.1. Cytotoxicity Test of Skin Sensitizers in Normal or High Glucose Conditions

Cell viability was analyzed after 24 hours of exposure to different concentrations of three skin sensitizers, eugenol, CA, and DNCB to determine which concentrations of skin sensitizers have minimal toxicity against MCF-7 cells and BALB/3T3 clone A31 cells, isopropanol was used as a negative control. In both normal and high glucose conditions, cell viabilities were decreased in a dose-dependent manner by exposing eugenol, CA, and DNCB in MCF-7 cells (Fig. **1**) and BALB/3T3 clone A31 cells (Fig. **2**). Isopropanol did not affect the cell viabilities of both cell lines under all conditions (Figs. **1** and **2**). In normal glucose conditions, the CC_{50} values of eugenol, CA, and DNCB were 287.8 μM, 178.4 nM, and 10.5 μM in MCF-7 cells, and 453.1 μM, 90.1 nM, and 0.9 μM in BALB/3T3 clone A31 cells, respectively (Table **2**). In high glucose conditions, the CC_{50} values of eugenol, CA, and DNCB were 349.1 μM, 281.7 nM, and 7.3 μM in MCF-7 cells, and were 422.0 μM, 92.1 nM, and 4.6 μM in BALB/3T3 clone A31 cells, respectively (Table **2**).

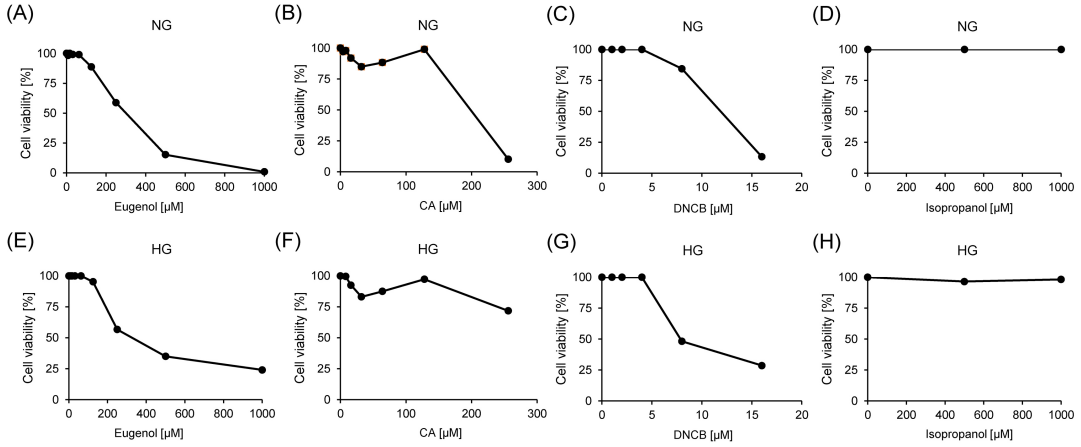

Fig. (1). The cytotoxicity of skin sensitizers in MCF-7 cells and BALB/3T3 clone A31 cells under normal or high glucose conditions. MCF-7 cells were incubated in various concentrations of eugenol (**A**, **C**), CA (**B**, **F**), DNCB (**C**, **G**), and isopropanol (**D**, **H**) under normal or high glucose conditions for 24 hours. Cytotoxicity was evaluated by LDH cytotoxicity assay. All experiments were performed in triplicate.

Table 2. The half of cytotoxicity concentration (CC_{50}).

Chemicals	MCF-7		BALB/3T3 clone A31	
	NG	HG	NG	HG
Eugenol	287.8 μM	349.1 μM	453.1 μM	422.0 μM
CA	178.4 μM	281.7 μM	90.1 μM	92.1 μM
DNCB	10.5 μM	7.3 μM	0.9 μM	4.6 μM
Isopropanol	>1000 μM	>1000 μM	>1000 μM	>1000 μM

NG: normal glucose conditions, HG: high glucose conditions, CA: cinnamaldhyde, DNCB: 2,4-dinitrochlorobenzene

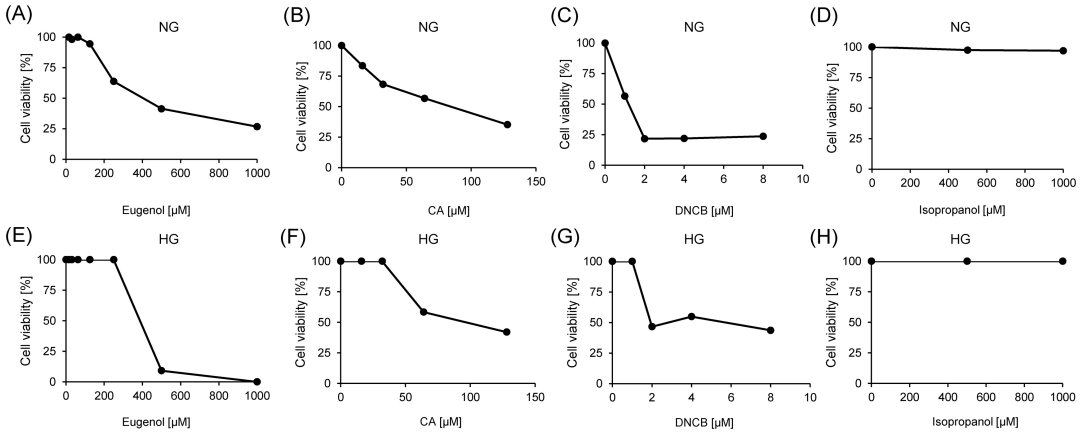

Fig. (2). The cytotoxicity of skin sensitizers in BALB/3T3 clone A31 cells under normal or high glucose conditions. BALB/3T3 clone A31 cells were incubated in various concentrations of eugenol (**A**, **E**), CA (**B**, **F**), DNCB (**C**, **G**), and isopropanol (**D**, **H**) under normal or high glucose conditions for 24 hours. Cytotoxicity was evaluated by LDH cytotoxicity assay. All experiments were performed in triplicate.

3.2. Effects of Skin Sensitizers on NRF2 Transcriptional Activity in Normal or High Glucose Conditions

MCF-7 cells were transfected with pAREx8-TATA or pAREx8-SV40 to determine the optimal reporter plasmid for evaluating NRF2 transactivation. The induction of luciferase activity by CA was 21.2-fold in pAREx8-TATA and 2.67-fold in pAREx8-SV40 (Supplementary Fig. **S1**), suggesting that the response sensitivity of pAREx8-TATA was higher than that of pAREx8-SV40. We investigated whether skin sensitizers activated the NRF2 signaling using MCF-7 cells or BALB/3T3 clone A31 cells transfected with pAREx8-TATA. Three skin sensitizers increased the luciferase activity dose-dependently in both cells under normal-glucose conditions (Figs. **3** and **4**). On the other hand, the luciferase activity was increased by CA, but not eugenol and DNCB, in both cell lines under high-glucose conditions (Figs. **3** and **4**). Isopropanol had no effects on reporter activation in both MCF-7 cells and BALB/3T3 clone A31 cells under all conditions (Figs. **3** and **4**).

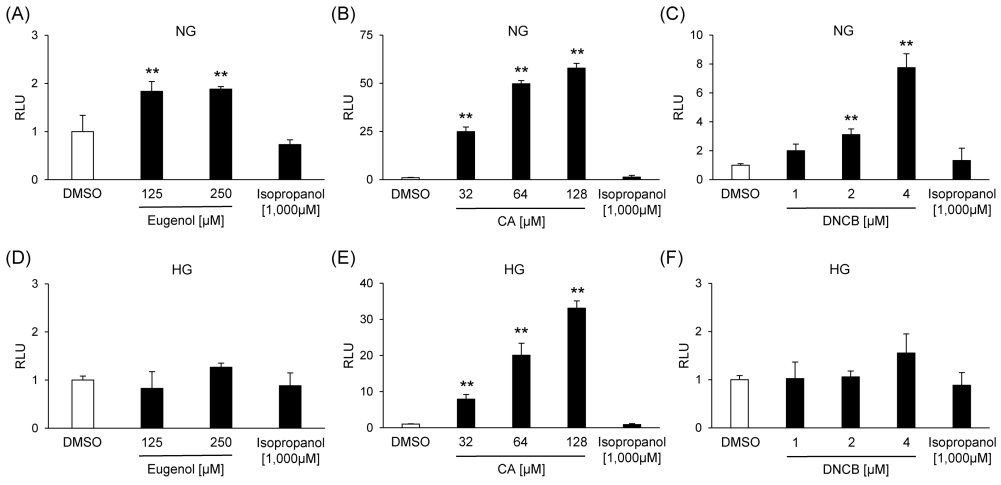

Fig. (3). Effects of skin sensitizers on NRF2 transcriptional activity in MCF-7 cells under normal or high glucose conditions. MCF-7 cells were transfected with pAREx8-TATA and further incubated in various concentrations of eugenol (**A, E**), CA (**B**, **F**), DNCB (**C**, **G**), and isopropanol (**D**, **H**) under normal or high glucose conditions for 24 hours. The luciferase activity was determined, and the data were expressed as Relative Light Units (RLU: firefly luciferase activity divided by *Renilla* luciferase activity). All experiments were performed in triplicate, and values are indicated as mean ± S.D. Statistically significant differences are indicated by an asterisk (**$p < 0.01$, *$p < 0.05$ by Dunnett's test).

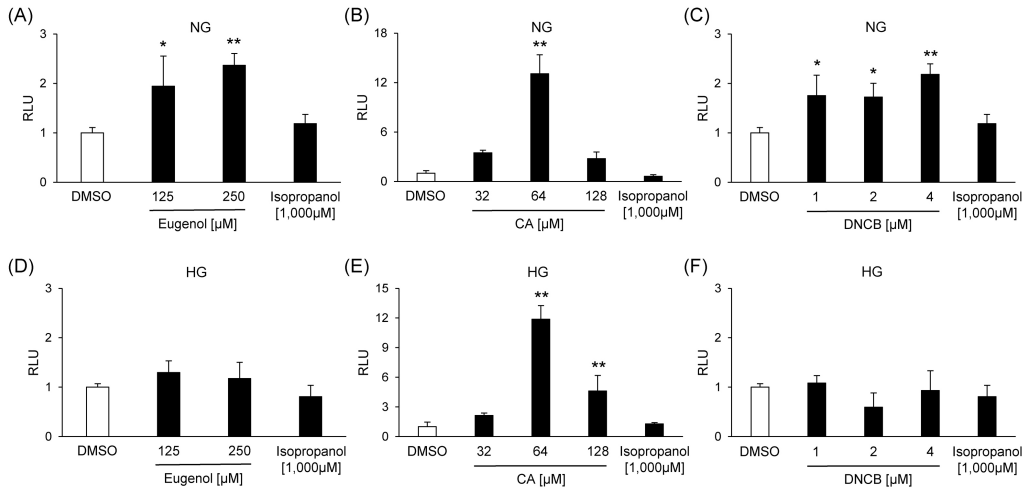

Fig. (4). Effects of skin sensitizers on NRF2 transcriptional activity in BALB/3T3 clone A31 cells under normal or high glucose conditions. BALB/3T3 clone A31 cells were transfected with pAREx8-TATA, and further incubated in various concentrations of eugenol (**A, E**), CA (**B**, **F**), DNCB (**C**, **G**), and isopropanol (**D**, **H**) under normal or high glucose conditions for 24 hours. The luciferase activity was determined, and data were expressed as relative light units (RLU: firefly luciferase activity divided by *Renilla* luciferase activity). All experiments were performed in triplicate, and the values were indicated as mean ± S.D. Statistically significant differences are indicated by an asterisk (**$p < 0.01$, *$p < 0.05$ by Dunnett's test).

3.3. Effects of Glucose Concentration on the Cellular Localization of NRF2

NRF2 localization was determined by immunofluorescence assay to investigate the effects of glucose concentration on the activation of NRF2 in MCF-7 cells and BALB/3T3 clone A31 cells. Most of the NRF2 proteins were observed in the cytoplasm under both normal and high glucose conditions, but nuclear NRF2 was significantly increased under high glucose conditions compared to normal glucose conditions in MCF-7 cells (Fig. **5A**). On the other hand, in BALB/3T3 clone A31 cells, nuclear NRF2 was also significantly increased under high glucose conditions compared to normal glucose conditions, but NRF2 proteins were almost localized at the nucleus (Fig. **5B**), thereby, it was decided to carry out the subsequent experiments using MCF-7 cells.

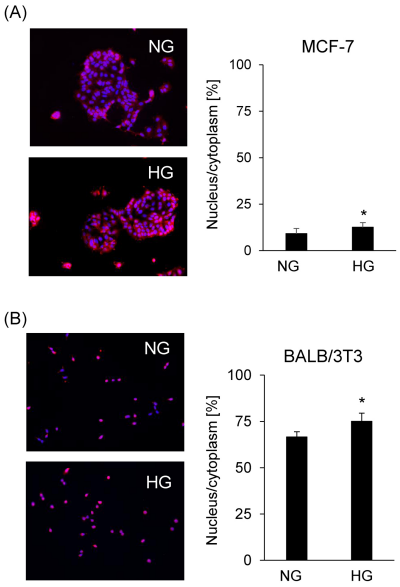

Fig. (5). Effects of glucose on the cellular localization of NRF2 in MCF-7 cells and BALB/3T3 clone A31 cells. MCF-7 cells (**A**) or BALB/3T3 clone A31 cells (**B**) were incubated in the medium containing various concentrations (1.0 or 4.5 g/L) of D-glucose for 24 hours. Fixed cells were visualized with anti-NRF2 antibody and fluorescent-labeled secondary antibody. The nuclei were counterstained with DAPI, and nuclear to cytoplasmic NRF2 ratios were calculated. Experiments were performed in sixtuplicate or triplicate, and values are indicated as mean ± S.D. Statistically significant differences are indicated by an asterisk (*$p < 0.05$ by Student's *t*-test).

3.4. Effects of Glucose Concentration on CA-Induced NRF2 Transcriptional Activity

The effect of glucose concentration on NRF2 transcriptional activity was assessed in MCF-7 cells transfected with pAREx8-TATA. High glucose conditions induced the NRF2-ARE reporter activity compared to normal glucose conditions (Fig. **6A**). CA-induced NRF2 activation was significantly increased in high glucose conditions compared to normal glucose conditions (Fig. **6B**).

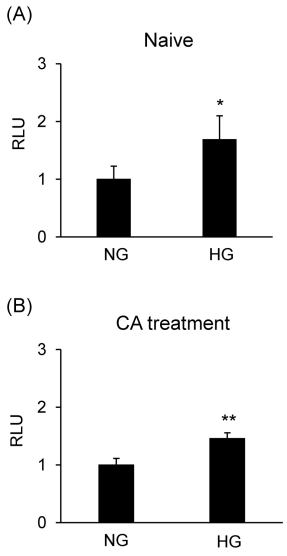

Fig. (6). Effects of glucose on CA-induced NRF2 activation in MCF-7 cells. (**A**): MCF-7 cells were transfected with pAREx8-TATA, and further incubated in the medium containing various concentrations (1.0 or 4.5 g/L) of D-glucose for 24 hours (Naive). (**B**): MCF-7 cells were transfected with pAREx8-TATA, and further incubated in the medium containing various concentrations (1.0 or 4.5 g/L) of D-glucose in the presence of 64 μM CA for 24 hours (CA treatment). The luciferase activity was determined, and data were expressed as relative light units (RLU: firefly luciferase activity divided by *Renilla* luciferase activity). All experiments were performed in triplicate, and values are indicated as mean ± S.D. Statistically significant differences are indicated by an asterisk (**$p < 0.01$, *$p < 0.05$ by Student's *t*-test).

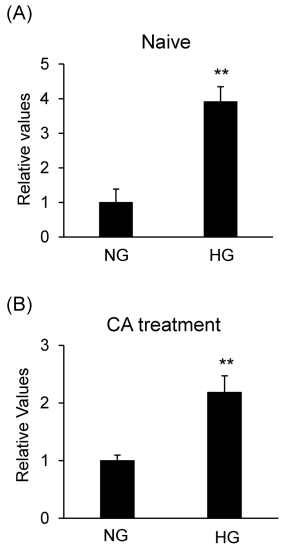

Fig. (7). Effects of glucose on CA increased the mRNA level of *HMOX1* in MCF-7 cells. (**A**): MCF-7 cells were incubated in the medium containing various concentrations (1.0 or 4.5 g/L) of D-glucose for 24 hours (Naive). (**B**): MCF-7 cells were incubated in the medium containing various concentrations (1.0 or 4.5 g/L) of D-glucose in the presence of 64 μM CA for 24 hours (CA treatment). The level of *HMOX1* mRNA was analyzed by quantitative real-time PCR. *GAPDH* mRNA expression was used as an internal control. All experiments were performed in triplicate, and values are indicated as mean ± S.D. Statistically significant differences are indicated by an asterisk (**$p<0.01$ by Student's *t*-test).

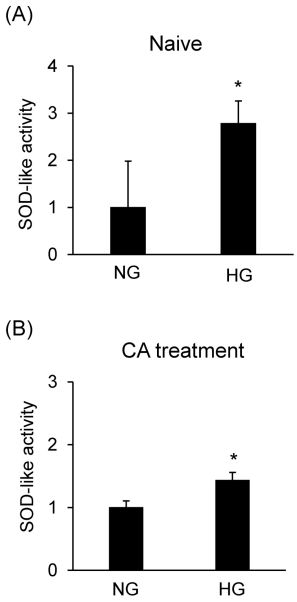

Fig. (8). Effects of glucose on CA-induced SOD-like activity in MCF-7 cells. (**A**): MCF-7 cells were incubated in the medium containing various concentrations (1.0 or 4.5 g/L) of D-glucose for 24 hours (Naive). (**B**): MCF-7 cells were incubated in the medium containing various concentrations (1.0 or 4.5 g/L) of D-glucose in the presence of 64 μM CA for 24 hours (CA treatment). The SOD-like activity was determined. All experiments were performed in triplicate, and values are indicated as mean ± S.D. Statistically significant differences are indicated by an asterisk (*p<0.05 by Student's t-test).

3.5. Effects of Glucose Concentration on CA-Induced Anti-Oxidative Gene

The mRNA level of *HMOX1* was evaluated in MCF-7 cells cultured in the medium containing various concentrations of glucose to investigate the effect of glucose concentration on the NRF2 target gene. High glucose conditions induced the level of *HMOX1* mRNA compared to normal glucose conditions (Fig. **7A**). CA-induced *HMOX1* mRNA expression was significantly increased in high glucose conditions compared to normal glucose conditions (Fig. **7B**).

3.6. Effects of Glucose Concentration on CA-Induced SOD-Like Activity

SOD-like activity was evaluated in MCF-7 cells cultured in the medium containing various concentrations of glucose to determine the effects of glucose on oxidative stress induced by CA. High glucose conditions induced the SOD-like activity compared to normal glucose conditions (Fig. **8A**). CA-induced SOD-like activity was significantly increased in high glucose conditions compared to normal glucose conditions (Fig. **8B**).

4. DISCUSSION

A recent study has reported that hyperglycemia has a potentially critical role in the promotion of allergy [7, 8], however, the clear mechanism of this phenomenon is unknown. KEAP1-NRF2 pathway is involved in the cellular processes in skin sensitization [12]. In this study, we demonstrated for the first time that high glucose enhanced NRF2 transcriptional activity by a skin sensitizer *in vitro*. This study indicates that high glucose enhanced the skin sensitizer-induced activation of NRF2. Although, the relative contribution of this mechanism to skin sensitization *in vivo* remains to be elucidated, diabetic patients may be more likely to develop an allergy to skin sensitizers.

DNCB, CA, and eugenol are classified as extreme, strong, and moderate sensitizers to reflect differing skin sensitization potency based on the results of local lymph node assay (LLNA) [30]. ARE-driven reporter assays have practical applications for detecting skin sensitizing [31 - 35]. We demonstrated that the ranking of the activities was DNCB > CA > eugenol in MCF-7 cells or BALB/3T3 clone A31 cells transfected with an ARE reporter plasmid under normal glucose conditions. Therefore, these indicate that our two assays can estimate the potency class (weak, moderate, strong, or extreme) of skin sensitizers similar to previous ARE-driven reporter assays.

HMOX1 mRNA and SOD-like activity in high glucose conditions were higher than in normal glucose conditions in both the absence and presence of CA. Furthermore, glucose induced the nuclear translocation and transactivation of NRF2 in MCF-7 cells 24 hours after the treatment. The production of H_2O_2 is induced by high glucose concentration within 24 hours *in vitro* [36]. High glucose increases the percentage of 8-hydroxy-2'-deoxyguanosine-positive cells compared to the control at 8 hours after the treatment [37]. Under oxidative stress, the conformation of KEAP1 is modified, leading to NRF2 release, which translocates to the nucleus [38]. Hyperglycemia induces NRF2 activation indirectly *via* ROS production [39, 40]. Therefore, this indicates that glucose causes oxidative stress and assists the CA-induced NRF2 activation *in vitro*.

NRF2 facilitates the repair of radiation-induced DNA damage in a ROS- independent manner [41]. Extreme high glucose (concentrations above 10 g/L) induces cytotoxic,

genotoxic, and apoptotic effects on MCF-7 cells [42]. It was hypothesized that NRF2 was activated in response to cell damage by glucose, however, the cytotoxicity of skin sensitizers in MCF-7 cells and BALB/3T3 clone A31 cells was not dependent on the glucose concentration in the medium in this study. Blood glucose concentrations are usually in the range between 2.0 and 4.5 g/L in diabetic patients [43, 44]. Therefore, these findings suggest that hyperglycemia-promoted oxidative stress probably leads to the activation of NRF2 without cytotoxicity in diabetes.

This study showed that the response sensitivity of MCF-7 cells was higher than that of BALB/3T3 clone A31 cells. Several skin sensitizers were not able to be evaluated in BALB/3T3 clone A31 cells. NRF2 is basally activated in human and mouse fibroblasts [45, 46]; by contrast, the basal activation of NRF2 is not observed in MCF-7 cells [47]. We also demonstrated that the nuclear ratio of NRF2 in BALB/3T3 clone A31 was higher than in MCF-7 cells. Thus, this indicates that the response to stimulation by skin sensitizers in MCF-7 cells is higher than in BALB/3T3 clone A31 cells.

Our reporter assays showed that eugenol and DNCB induced the reporter activity in both MCF-7 cells and BALB/3T3 clone A31 cells under normal glucose conditions, but not high glucose conditions. Furthermore, the luciferase activity induced by CA showed higher fold changes compared to vehicle control under normal glucose conditions than high glucose conditions. We also demonstrated that nuclear NRF2 levels in normal glucose conditions were lower than in high glucose conditions. In short, NRF2 activity under basal conditions was maintained at low levels under normal conditions. For ARE-driven reporter gene assays, our results propose that the lower concentrations of glucose in the medium lead to enhanced detection sensitivity for skin sensitizers.

CONCLUSION

We demonstrated for the first time that glucose enhanced skin sensitizers-induced NRF2 transcriptional activity and SOD-like activity *in vitro*. This indicate that oxidative stress caused by hyperglycemia additionally induced the activation of NRF2 signaling by skin sensitization. Further studies are needed to investigate the effects of blood glucose levels on skin sensitization *in vivo*.

LIST OF ABBREVIATIONS

ARE	=	Antioxidant Response Element
CC_{50}	=	Half-Maximal Cytotoxic Concentration
CA	=	Cinnamaldehyde
CS	=	Dextran-Coated Charcoal-Stripped
DMEM	=	Dulbecco's Modified Eagle Medium
DNCB	=	2,4-Dinitrochlorobenzene
GAPDH	=	Glyceraldehyde-3-Phosphate Dehydrogenase
HG	=	High Glucose
KEAP1	=	Kelch-like ECH-associated Protein 1
LDH	=	Lactate Dehydrogenase
LLNA	=	Local Lymph Node Assay
HMOX1	=	Heme Oxygenase 1
NG	=	Normal Glucose
NRF2	=	Nuclear Factor Erythroid 2-Related Factor 2
ROS	=	Reactive Oxygen Species
SOD	=	Superoxide Dismutase

AUTHOR'S CONTRIBUTION

The article has been written by Takeo Takeda and Masahiro Ogawa. The data has been analyzed by Takeo Takeda, Junya Kitamoto, Takahiro Kyoya, and Megumi Terada. The study has been designed by Takeda Takeo and Masahiro Ogawa. The data has been collected by Takeda Takeo.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIAL

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

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