Acceleration of heat shock-induced collagen breakdown in human dermal fibroblasts with knockdown of NF-E2-related factor 2

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INTRODUCTION

Heat shock increases skin temperature during sun exposure and some evidence indicates that it may be involved in skin aging. The antioxidant response mediated by the transcription factor NF-E2-related factor 2 (Nrf2) is a critically important cellular defense mechanism that serves to limit skin aging. We investigated the effects of heat shock on collagenase expression when the antioxidant defense system was downregulated by knockdown of Nrf2. GSH and collagenases were analyzed, and the expression of inducible Nrf2, HO-1, and NQO1 was measured. HS68 cells were transfected with small interfering RNA against Nrf2. Heat shock induced the downregulation of Nrf2 in both the cytosol and nucleus and reduced the expression of HO-1, GSH, and NQO1. In addition, heat-exposed Nrf2-knockdown cells showed significantly increased levels of collagenase protein and decreased levels of procollagen. Our data suggest that Nrf2 plays an important role in protection against heat shock-induced collagen breakdown in skin. [BMB Reports 2015; 48(8): 467-472]

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chinery involved in skin aging induced by heat-shock with knockdown of Nrf2.

RESULTS

Heat shock reduced the expression or translocation of Nrf2 proteins and expression of HO-1, NQO1, and GSH proteins in HS68 cells

Cell viability of HS68 cells was not affected by treatment at temperatures of 37-44°C, according to a MTT assay 48 h later. However, heat shock at 46°C caused increased cytotoxicity (Supplementary Fig. 1). Additionally, mitogen-activated protein kinase signaling and ROS generation were increased significantly by heat shock at 44°C (Supplementary Fig. 2 and 3). Thus, all the experiments described below were performed at a heat shock temperature of 44°C, similar to the procedures used in previous studies (5). To evaluate whether heat shock impaired Nrf2 regulation in HS68 cells, we measured Nrf2 protein levels. Exposure of HS68 cells to 44°C showed that expression and translocation of Nrf2 protein were increased sig-

![Fig. 1. Effects of heat shock on Nrf2 downregulation in HS68 cells. The levels of nuclear and cytosolic Nrf2 (A) were measured by Western blotting. Additionally, representative photomicrographs are shown (B). ^p < 0.01 and *** p < 0.001; one-way analysis of variance followed by Tukey’s post hoc test, performed using the GraphPad Prism software.](image1)

![Fig. 2. Effects of heat shock on HO-1, NQO1, and GSH downregulation in HS68 cells. The levels of HO-1 (A), NQO1 (B), and GSH (C) were measured by Western blotting. Representative photomicrographs are shown (D). Scale bar = 50 μm. *** and ^p < 0.001; one way analysis of variance followed by Tukey’s post hoc test, performed using the GraphPad Prism software.](image2)
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Effects of heat shock on HO-1, NQO1, and GSH downregulation and accelerated collagen breakdown in Nrf2-siRNA transfected HS68 cells. The levels of HO-1 (A), NQO1 (B), GSH (C), and MMP-1 and COL1A1 (D) were measured by Western blotting or a kit. *, #, and @ P < 0.05, ## and && P < 0.01 and &&& and ^^^P < 0.001; one-way analysis of variance followed by Tukey’s post hoc test, performed using the GraphPad Prism software.

Nrf2 knockdown reduced expression of HO-1, NQO1, and GSH and accelerated collagen breakdown induced by heat shock in Nrf2-siRNA transfected HS68 cells

To demonstrate the importance of Nrf2 upregulation, we developed a Nrf2-knockdown model in HS68 cells using siRNA transfection. HO-1, NQO1, and GSH levels were decreased significantly in Nrf2 siRNA-transfected cells by heat shock (14.43 ± 2.17, 10.28 ± 1.28, and 37.09 ± 8.25%, versus control siRNA-transfected cells (Fig. 3A, B, and C). Additionally, MMP-1 levels were increased significantly in Nrf2 siRNA-transfected cells by heat shock (135.23 ± 8.96%), versus control siRNA-transfected cells. Moreover, COL1A1 levels were decreased significantly in Nrf2 siRNA-transfected cells by heat shock (254.30 ± 16.84%), versus control siRNA-transfected cells (Fig. 3D).

Effects of Nrf2 regulators on heat shock-induced expression of GSH proteins and deceleration of collagen breakdown in Nrf2-siRNA transfected HS68 cells

Effects of Nrf2 regulators on heat shock-induced expression of HO-1, NQO1, and GSH proteins and deceleration of collagen breakdown in Nrf2-siRNA transfected HS68 cells

To further confirm the involvement of Nrf2, the effects of Nrf2 regulators, CSE, NAC, and quercetin, on heat-shock-induced GSH and MMP-1 levels were measured in Nrf2 knockdown HS68 cells. GSH levels were decreased significantly in Nrf2 siRNA-transfected cells by heat shock (36.05 ± 6.84%), versus control siRNA-transfected cells. In contrast, Nrf2 siRNA-transfected cells treated with 500 μg/mL CSE, 2 mM NAC, or 50 μM quercetin before exposure to heat shock were protected, in terms of GSH levels (267.87 ± 26.43%, 241.41 ± 14.08%, and 290.64 ± 19.10%, respectively), as compared to Nrf2 siRNA-transfected cells (Fig. 4B). Additionally, MMP-1 levels were increased significantly in Nrf2 siRNA-transfected cells by heat shock (200.34 ± 14.54%), as compared to control siRNA-transfected cells. In contrast, Nrf2 siRNA-transfected cells treated with 500 μg/mL CSE, 2 mM NAC, or 50 μM quercetin before exposure to heat shock showed decreased MMP-1 levels (57.06 ± 6.38%, 45.51 ± 12.21%, and 41.94 ± 7.13%, respectively).

significant after heat shock for 30 min (274.68 ± 13.57% vs. the 37°C value), and then declined at 24 h (46.67 ± 8.20% vs. the 37°C values) (Fig. 1). Next, to assess whether heat shock impaired Nrf2 target proteins in HS68 cells, we measured HO-1, NQO1, and GSH expression levels. HS68 cells exposed to a temperature of 44°C showed significantly reduced GSH, HO-1, and NQO1 levels at 24-48 h (71.86 ± 15.17 to 66.86 ± 6.51%, 55.66 ± 3.38 to 43.66 ± 5.23%, and 56.66 ± 6.17 to 40.66 ± 8.74% of the corresponding 37°C values, respectively) (Fig. 2).

Factors, the Nrf2 regulators CSE, NAC, and quercetin were used. Heat shock exposure significantly depleted GSH (by 27.60 ± 8.88%) and elevated MMP-1 (by 231.71 ± 21.72%), while 100 or 500 μg/ml CSE, 2 mM NAC, or 50 μM quercetin increased levels of GSH (by 39.02 ± 5.27 to 72.64 ± 9.97%, 50.67 ± 4.74%, or 58.54 ± 2.98% of the corresponding 37°C values, respectively) and decreased levels of MMP-1 (by 124.21 ± 69.28 to 49.40 ± 6.14%, 58.88 ± 25.56%, or 35.30 ± 11.57% of the corresponding 37°C values, respectively) (Fig. 4 A and C).
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**Fig. 4.** Effects of Nrf2 regulators on heat shock-induced upregulation of GSH and downregulation of MMP-1 levels in HS68 cells. The levels of GSH (A) and MMP-1 (C) were measured using commercial kits. Then, effects of Nrf2 regulators on heat-shock-induced upregulation of GSH and deceleration of collagen breakdown in Nrf2-siRNA transfected HS68 cells. The levels of GSH (B) and MMP-1 (D) were measured. *, **, and *** P < 0.05, 0.01, and 0.001, respectively, compared with Nrf2 siRNA-transfected cells (Fig. 4D).

**DISCUSSION**

Nrf2 can function as a direct oxidative stress sensor, and phase 2 detoxifying enzymes are major executors of cellular defense against oxidative stress. Many studies have shown that the Nrf2 signals plays an important role in protection on UV-induced aging by maintaining antioxidants levels (18). Further, Nrf2/heat-related studies reported that Nrf2 activation is the regulatory pathway of cytoprotective gene and proteins expression against heat shock in dental pulp and liver (19, 20). In fact, Sahin et al. reported that Nrf2 activators such as curcumin, epigallocatechin-3-gallate, and resveratrol modulated the Nrf2/HO-1 pathway in quail hepatocytes to counteract the damage caused by heat shock (21). However, no reported study has yet examined the effect of Nrf2 on skin aging induced by heat shock. Therefore, we focused on Nrf2 as a putative major component of the protective involved in skin aging induced by heat shock.

Oxidative stress, including UV, IR, and heat shock, causes depletion of antioxidants such as GSH, NQO1, and HO-1 in skin (22). Moreover, Nrf2 has been shown to be involved in the inducting phase II enzymes or antioxidants (22). In this studies, heat shock induced a certain concentration of ROS and it activates Nrf2 expression and translocation in initial stage, but excessive ROS generation inactivated Nrf2 (Fig. 1). Moreover, time dependently ROS generation and its downstream proteins of ERK, JNK, and P38 MAPKs signaling, demonstrating that Nrf2 activation may be associated with the regulation of heat shock in initial stage (Supplementary Fig. 2 and 3). This result is consistent with the previous report, Shin MH et al.; heat shock induces cellular levels of ROS generation and its downstream proteins of MAPKs signaling in keratinocytes (5). Also, Nrf2 expression in nucleus were increased rapidly after H2O2 for 30 min, and then declined at 24 h. In this study, furthermore, Nrf2 siRNA-transfected cells showed a significantly greater decrease in HO-1, NQO1, and GSH levels than siRNA-control cells after heat shock (Fig. 3A, B, and C). Additionally, to determine whether mechanisms of heat shock in collagen breakdown were regulated by Nrf2, we measured collagenase levels following Nrf2 knockdown. The family of COL gene products is composed of various chain types, such as collagens types I, II, III, IV, and V. Specially, COL1A1 is important for the skin development and for maintain physiological functions (23-25). MMP-1, a fibroblast-type or interstitial collagenase, is secreted by fibroblasts and macrophages (24). It degrades collagen and is thought to play a role in skin aging (24). Sahin et al. reported that heat shock induced increasing MMP-1 by production of ROS in skin cells (Fig. 3D and supplementary Fig. 2) (5). Further, as mentioned previously, Nrf2 is the sensory mediator for protection on ROS by induction of antioxidants, even at low levels of oxidative damage. Due to its central role in ROS detoxification, Nrf2 is an attractive target for pharmacological protection of the skin aging (26). However, no reported study has yet examined the effect of Nrf2 on collagen-relationships induced by heat shock. In our study, MMP-1 levels were significantly increased in Nrf2 siRNA-transfected cells by heat shock, compared with
siRNA-control cells (Fig. 3D). Also, COL1A1 was decreased significantly in Nrf2 siRNA-transfected cells by heat shock (Fig. 3D). No difference in MMP-1 and COL1A1 levels was observed between Nrf2 siRNA-transfected cells and siRNA-control cells. As the constitutively lower Nrf2 levels dependent antioxidant enzymes in Nrf2-knockdown cells were shown here to accelerate skin aging, heat shock-induced Nrf2 inactivation was not directly related to the results of the present study.

Furthermore, we measured the effects of Nrf2 regulators against heat-shock-induced GSH and MMP-1 levels in normal and Nrf2 knockdown HS68 cells. In our previous study, we demonstrated that Nrf2 activators (coriander, walnut, and green tea extract, sauchinone, and NAC) inhibited oxidative-stress-induced apoptosis and skin aging (14-16). In the present study, CSE, NAC, and quercetin significantly protected skin cells against heat shock-induced damage without inducing toxicity (data not shown). Additionally, heat shock caused significant depletion of GSH and elevation of MMP-1, whereas CSE, NAC, and quercetin induced GSH and reduced MMP-1 levels. Also, in Nrf2 siRNA-transfected cells treated with CSE, NAC, or quercetin showed significantly increased GSH expression levels and decreased MMP-1 expression levels (Fig. 4). These results indicate that Nrf2 plays an important role in preventing skin aging via upregulating antioxidants through Nrf2 activators.

Our findings show that Nrf2 plays a crucial role in protection against heat shock-induced skin aging. Based on this concept of thermal aging through the Nrf2 pathway, development of Nrf2-activator may provide effective therapeutic strategies for thermal skin aging.

MATERIALS AND METHODS

Cell culture and treatments
The cell culture system was established according to previously published methods (13). The HS68 cell line, human skin fibroblast cell, was obtained from the American Type Culture Collection (ATCC; Rockville, USA). Cells were maintained in DMEM supplemented with 10% heat inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in condition of 95% air and 5% CO₂ at 37°C. Then, for heat shock treatment, cells were cultured to 80% confluence and then maintained in culture medium without FBS for 24 h; the culture dishes were sealed with Parafilm (Pechiney Plastic; CA). After 30 min at room temperature, 300 μl of the mix was added to 300 μl of serum-free DMEM in each dish and incubated for 24 h (Supplementary Fig. 4).

Measuring Nrf2, HO-1, NQO1, and COL1A1 levels by Western blotting
Western blotting was performed according to previously published methods (16). For the detection of Nrf2, HO-1, NQO1, and COL1A1 proteins, the cells were lysed. The lysates were separated by 15% SDS-PAGE, and were then transferred to a membrane. The membranes were incubated with 5% skim milk in TBST for 1 h. Then they were incubated with primary antibody (1:1,000 dilutions) overnight at 4°C, followed by incubated with HRP-conjugated secondary antibody for 1 h. Immunoreactive bands were detected using an ECL detection kit and a LAS-4000 mini system (Fujifilm Corporation, Tokyo, Japan) was used for visualization.

Measuring Nrf2, HO-1, and NQO1 levels by immunofluorescence
Immunofluorescence was performed according to previously published methods (16). Cells were fixed through incubation with 4% PFA at room temperature for 30 min. Fixed cells were rinsed in PBS and then incubated overnight at 4°C with primary antiantibody (dilution 1:500). They were then incubated for 2 h with an Alexa Fluor conjugated secondary antibody (dilution 1:250). Cells were finally washed in PBS and mounted using Vectashield Mounting Medium containing DAPI. Confocal immunofluorescent images were captured using an LSM 700 confocal microscope (Carl Zeiss, Thornwood, USA) and the fluorescence intensity was measured using Axio Vision 4.4 (Carl Zeiss, Oberkochen, Germany). Data are presented as percentages of control values. For the assessment of intra-experimental variability, three independent experiments were carried out in triplicate.

Measuring MMP-1 and GSH levels
MMP-1 and total GSH levels were measured using a Human MMP-1 ELISA Kit (RayBiotech; USA) and Total Glutathione Quantification Kit (Dojingo; Japan), respectively, according to the instruction manuals.

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