Heat Stress Activates Interleukin-8 and the Antioxidant System via Nrf2 Pathways in Human Dental Pulp Cells

Seok-Woo Chang, DDS, MSD, Sang-Im Lee, BS,‡ Won-Jung Bae,‡ Kyung-San Min, DDS, PhD,§ Eun-Sang Shin, DDS,‡ Gi-Su Oh, PhD,‖ Hyun-Ock Pae, PhD,‖ and Eun-Cheol Kim, DDS, PhD†*

Abstract

Introduction: This study tested whether heat stress (42°C for 30 minutes) induces reactive oxygen species (ROS), proinflammatory cytokines, Nrf2 activation, and Nrf2 target genes such as antioxidant enzymes in human dental pulp (HDP) cells. **Methods:** ROS was evaluated by using flow cytometry. Proteins and messenger RNA levels for cytokines and antioxidant genes were determined by using Western blotting and reverse transcription-polymerase chain reaction (RT-PCR) analysis, respectively. **Results:** Heat stress induced the production of ROS and the increased expression of the interleukin (IL)-8 and IL-8 receptor genes. Exposure of cells to heat stress resulted in the nuclear translocation of Nrf2 and increased expression of Nrf2 target genes including heme oxygenase-1. Pretreatment with an exogenous antioxidant inhibited the heat-induced expression of IL-8 and Nrf2 target genes and Nrf2 translocation. **Conclusion:** Collectively, these results show that heat-induced Nrf2 activation is the major regulatory pathway of cytoprotective gene expression against oxidative stress in HDP cells. *(J Endod 2009;35:1222–1228)*

Key Words

Antioxidants, defense system, heat stress, Nrf2, pulp cells

From the *Department of Conservative Dentistry, The Institute of Oral Health Science, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea; †Department of Operative Dentistry, Samsung Medical Center, College of Medicine, Sungkyunkwan University College of Dentistry, Seoul, South Korea; Departments of ‡Oral and Maxillofacial Pathology and §Conservative Dentistry, College of Dentistry, Wonkwang University, Iksan, South Korea; and ‖Department of Microbiology and Immunology, College of Medicine, Wonkwang University, Iksan, South Korea.

This study was supported by a grant from the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea. (A084458).

Address requests for reprints to Dr Eun-Cheol Kim, Department of Oral and Maxillofacial Pathology, College of Dentistry, Wonkwang University. 344-2 Shinyong, Iksan, Republic of Korea 570-749. E-mail address: eckwkop@wonkwang.ac.kr.

0099-2399/$0 - see front matter

doi:10.1016/j.joen.2009.06.005

Various dental treatments used in clinical practice result in an increased temperature of the tooth surface and thereby the tooth pulp (1–4). Temperature changes in pulp tissue can induce inflammatory reactions and hypersensitivity, leading to pulpal necrosis requiring root canal treatment (5). However, the extent of thermal injury that can be tolerated by dental pulp and the defense mechanisms against injury are unknown.

In animal models, heat stress has been shown to be beneficial in attenuating the inflammatory response. For example, heat shock response inhibits the expression of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-1, IL-12, IL-10, and IL-18 (6, 7). By contrast, heat shock induced IL-1 and TNF-α expression in the brain and blood of heat-stroked rats (8, 9) and increased IL-6 production in the gut mucosa *in vivo* (10) and in Caco-2 cells (11). Therefore, the results are conflicting as to whether heat stress exerts anti-inflammatory or inflammatory effects.

In pulp cells, heat stress increases leukotriene B4 (12), apoptosis (13), heat shock protein (HSP) 70 (14), alkaline phosphatase activity and HSP25 (15), HSP27 and heat shock transcription factor-1 (16), and HSP25 (17). Nevertheless, pulp is capable of surviving various injuries, including heat stress (14), which suggests that pulp cells have a recovery defense system.

The transcription factor NF-E2–related factor 2 (Nrf2)-mediated antioxidant response is a critically important cellular defense mechanism that serves to maintain intracellular redox homeostasis and to limit oxidative damage (18). Nrf2 can activate transcription of several antioxidant enzymes, including glucose-6-phosphate dehydrogenase (G6PD), superoxide dismutase (SOD), and heme oxygenase-1 (HO-1) as well as phase II enzymes such as glutathione peroxidases (GPX), γ-glutamylcysteine lygase (GCL), glutathione reductase (GR), and glutathione-S-transferase (GST) (19). Previously, we reported that HO-1 is a cytoprotective antioxidant enzyme essential for both the adaptation of human dental pulp (HDP) cells to stressful conditions and their recovery from injurious events (20–23).

When cells are exposed to electrophiles or reactive oxygen species (ROS), Nrf2 is released from Keap1 cytoplasmic capture, leading to its translocation to the nucleus where Nrf2 activates the transcription of target genes (24). Although ROS are toxic, they also function as signaling molecules. When the ROS level exceeds the defense capacity, damage may be induced (25). By contrast, low levels of ROS may stimulate defense networks and play an essential role in adaptation, leading to the prevention of further damage (26). In this context, intracellular antioxidants are important for regulating cell adaptation in response to oxidative stress. Although previous studies have reported that heat stress induced ROS in various cells (27, 28), the cellular response to heat stress has not been clearly elucidated in HDP cells.

Furthermore, the induction of endogenous antioxidant enzymes by activators of the Nrf2/antioxidant response element (ARE) pathway is an interesting approach to obtain sufficient levels of antioxidants to interfere with inflammation (24). The activation of cytoprotective Nrf2/ARE-regulated genes can suppress inflammatory responses, whereas decreased expression of these genes results in autoimmune disease and enhanced inflammatory responses to oxidant insults. Modulation of these cytoprotective genes has profound effects on the immune and inflammatory responses (29). Based on evidence of oxidative injury, we hypothesized that Nrf2-dependent ARE activation plays

TABLE 1. Primer sets and PCR conditions

Genes	Sequence (5′-3′)	Size (bp)	Tm (°C)	cycle
IL-1β	Forward: GGA TAT GGA GCA ACA AGT GG Reverse: ATG TAC CAG TTG GGG AAC TG	288	60	35
TNF-α	Forward: CTC TGG CCC AGG CAG TCA GA Reverse: GGC GTT TGG GAA GGT TGG AT	519	60	35
IL-6	Forward: TAG CCG CCC CAC ACA GAC AG Reverse: GGC TGG CAT TTG TGG TTG GG	408	60	35
IL-8	Forward: ATG ACT TCC AAG CTG GCC CGT GGC T Reverse: TCT CAG CCC TCT TCA AAA ACT TCT C	289	62	25
Il-8R	Forward: TGG GCA ACA ATA CAG CAA ACT Reverse: GCA CTT AGG CAG GAG GTC TTA	501	60	35
GST	Forward: CGC TTA CCT GCA CAC AG Reverse: AGT AGA GGT TAA GCA GGA ACG	558	55	30
GR	Forward:GATCCTGTCAGCCCTGGGTTCTAAGA Reverse:CGTCTACGATGATATGACCCTTGTCATC	337	54	41
GCL	Forward: ACC TGC AGA CCG GGA ACC TG Reverse: CGC AGT AGC CAC AGA GGC ACC	691	55	30
GAPDH	Forward: CGGAGTCAACGGATTTGGTCGTAT Reverse: AGCCTTCTCCATGGTGGTGAAGAC	306	62	25

a role in preventing the cell injury or inflammation resulting from heat stress. In this study, we sought to determine whether Nrf2-dependent antioxidants are key molecules in the heat stress response with oxidative stress and proinflammatory cytokines in HDP cells.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and the other tissue culture reagents were obtained from Gibco BRL (Grand Island, NY). Monoclonal antibodies for HO-1 (catalog no. 374987, human) and GPx (catalog no. ST1000, human) were purchased from Calbiochem (San Diego, CA). The monoclonal antibody for SOD (catalog no. H86141 M, human) was obtained from Biodesign International (Kennebunk, ME), and the polyclonal Nrf2 antibody was purchased from Abcam (catalog no. ab31163, human; Cambridge, UK). The monoclonal antibody for β-actin (catalog no. A1978, human), *N*-acetylcysteine (NAC), and other chemicals were purchased from Sigma (St Louis, MO).

Cell Culture

Normal HDP tissues were obtained from the intact teeth of a 15-year-old boy who needed orthodontic treatment under approved guidelines set out by the Institutional Review Board of Wonkwang University Hospital (Iksan, Republic of Korea) and conformed to the provisions set forth by the Declaration of Helsinki. The tooth was cracked open, and the pulp was removed and immersed in DMEM containing 10% FBS, 200 U/ml penicillin, 200 mg/mL streptomycin, and 1 mg/mL amphotericin B at 37°C in a humidified incubator containing 5% CO_2. Cell cultures between the fifth and seventh passages were used in this study. Where indicated, the cells were treated with 20 mmol/L NAC (Alexis, San Diego, CA), 20 μmol/L vitamin E, and 10 mmol/L glutathione for 2 hours before the heat treatment.

Thermal Stimulation

The method described by Amano et al (14) and Lee et al (15) was used to simulate heat stress. Briefly, the cultured cells were maintained at 37°C until subconfluent growth was attained. Then, heat stress was induced by incubating the cells at 42°C for 30 minutes, after which the cells were returned to the 37°C humidified incubator (time point 0). The samples were harvested at specific time points (0, 1, 3, 6, 9, 12, 16, and 24 hours postheating). In addition, samples were obtained 1 hour before heat stress as a control.

Measuring ROS

Intracellular ROS generation was measured using the fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes, Eugene, OR) as described previously (30). For experiments, cells were preincubated in phenol red-free DMEM medium (to avoid the interference of phenol red) without FBS for 16 hours. Cells were treated with heat stress for 30 minutes and then incubated with 10 μmol/L CM-H_2DCFDA for 20 minutes at 37°C in the dark. Hydrogen peroxide (H_2O_2, 0.2 mmol/L, 30 minutes) was used as a positive control and unstimulated cells as a negative control. In all experiments, the background fluorescence was determined. ROS formation was assessed using flow cytometry in a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). The mean CM-H_2DCFDA fluorescence was registered at 530 nm (bandwidth 30 nm) with excitation at 488 nm using a 15-mW argon laser. Ten thousand events were evaluated for every analysis. Each sample was tested in six wells, and each experiment was performed in triplicate.

G6PD Assay

The G6PD activity was determined by monitoring the generation of NADPH using the Vybrant Cytotoxicity Assay Kit (Molecular Probes) according to the manufacturer's instructions. In this assay, NADPH reduces resazurin to produce the fluorescent product resorufin. Substances containing the fluorescent dye were always processed while protected from light. Then, the tissue culture plates (96-well) were shaken gently; 50 μL of medium from each well was transferred to separate plates, and 50 μL/well of two-fold concentrated resazurin/reaction mixture was added to the collected medium. After light-protected incubation for 20 minutes at 37°C, measurements were made using a fluorescence microplate reader with excitation at 530 nm and emission detection at 590 nm. Triplicate experiments were performed, and the results were reported as the average $\pm$ standard deviation.

RNA Isolation and RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Gaithersburg, MD) according to the manufacturer's protocol. A volume containing 1 μg of total RNA from each sample was subjected to reverse transcription using an AccuPower RT PreMix kit (Bioneer, Daejeon, Korea) according to the manufacturer's

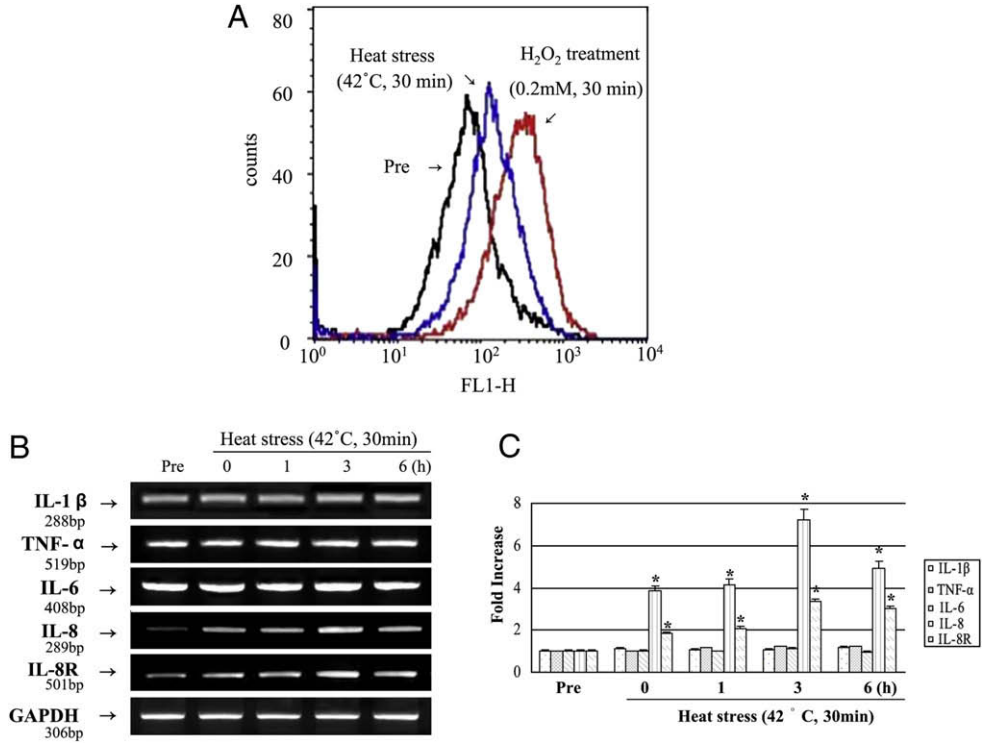

Figure 1. (*A*) The effect of heat stress on the production of reactive oxygen species and (*B* and *C*) the expression of proinflammatory cytokines in human dental pulp cells. The levels of proinflammatory cytokines, including IL-1β, TNF-α, IL-6, and IL-8, were assayed by semiquantitative RT-PCR. The generation of intracellular ROS was detected using flow cytometry after CM-H_2DCFDA staining for 2 hours. Cells were treated with hydrogen peroxide as a positive control. The x-axis shows the log FL-1 fluorescence intensity; the y-axis indicates the cell number. The amount of ROS production was quantified as the percentage of cells with increased fluorescence relative to the control. The results shown are representative of the results from triplicate experiments. (*C*) Levels of cytokine mRNA genes were measured by densitometry. The relative level of gene expression was normalized against the GAPDH mRNA signal, and the control was set as 1.0. Optical density values represent the mean ± standard deviation. *Statistically significant difference compared with the control group ($p < 0.05$).

instructions. Then, the RT-generated DNA (2-5 μL) was amplified using AccuPower PCR PreMix (Bioneer). The primer sequences for cytokines and antioxidants are detailed in Table 1. The PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. The semiquantitative RT-PCR method was validated in preliminary experiments. The PCR cycle number was optimized for each experimental condition and primer set. Representative samples were run for different numbers of cycles (22-40 cycles), and the optimal cycle number was selected from the region of linearity between the cycle number and PCR product intensity. To confirm the linear relationship between the template and PCR product intensity at the optimal cycle number, PCR was run for different concentrations of complementary DNA obtained from representative samples. All reactions included a negative control from which complementary DNA was omitted from the PCR. To show sample equality, amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The intensity of each band after normalization with GAPDH messenger RNA (mRNA) was quantified on the photographed gels with a densitometer (Quantity One; Bio-Rad, Richmond, CA).

Preparation of Nuclear and Cytosolic Protein Extracts

For nuclear proteins, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 400 μL of Buffer A (10 mmol/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [pH 7.9], 10 mmol/L KCl, 0.1 mmol/L ethylenediaminetetraacetic acid, 0.1 mmol/L ethylene glycol tetraacetic acid, 1 mmol/L dithiothreitol, and 0.5 mmol/L phenylmethylsulfonyl fluoride). After 15 minutes, Nonidet P-40 (Sigma, St Louis, MO) was added to a final concentration of 0.6%. The samples were centrifuged to collect the supernatant containing cytosolic proteins. The pelleted nuclei were resuspended in 50 μL of Buffer B (20 mmol/L 2-hydroxyethyl]-1-piperazineethanesulfonic acid [pH 7.9], 0.4 M NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L ethylene glycol tetraacetic acid, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride). After 30 minutes at 4°C, the lysates were centrifuged, and the supernatants containing the nuclear proteins were stored at –80°C.

Western Blot Analysis

The protein samples (cell extracts, 50 μg) were separated by SDS-discontinuous polyacrylamide gel electrophoresis and blotted onto a membrane. The membrane was blocked with 5 w/v% bovine serum albumin (BSA), 1 w/v% milk powder in 10 mmol/L Tris-HCl containing 150 mmol/L NaCl, and 0.5 w/v% Tween 20 for 1 hour and incubated overnight with diluted antibodies against Nrf2 (1:1000), HO-1 (1;1000), GPx (1;2000), SOD (1:1000), and β-actin (1;4000). After washing three times for 10 minutes each with washing solution, the membranes were incubated with goat antirabbit immunoglobulin (Ig)G-HRP (1:2,000, catalog no. 10004301; Cayman, Ann Arbor, MI) or goat antimouse IgG-HRP (1:2,000, catalog no. 10004302, Cayman) in blocking solution for 1 hour at room temperature. Bands were detected using a chemiluminescence system (Amersham, Barcelona,

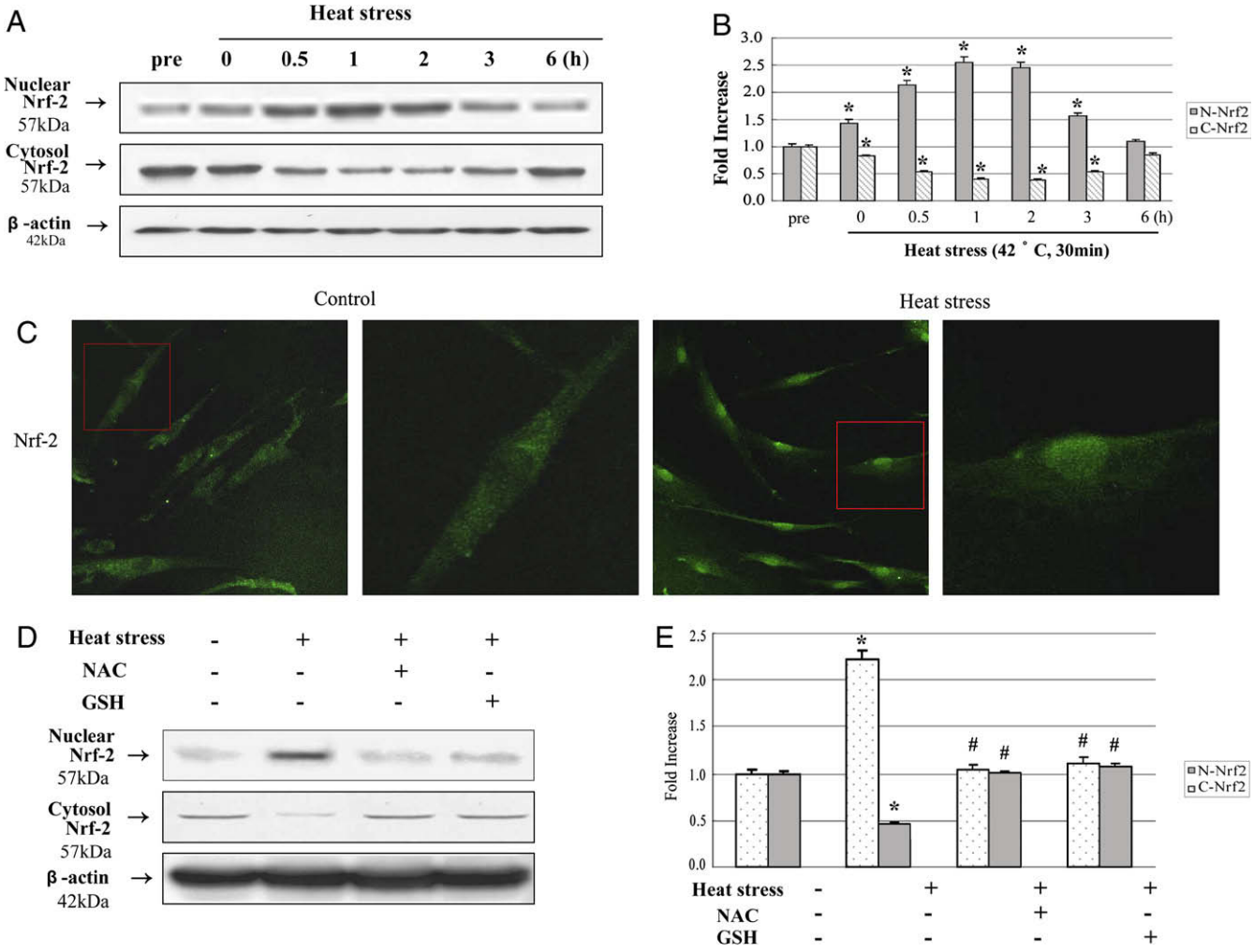

Figure 2. Accumulation and distribution of Nrf2 protein after treatment of HDP cells with heat and antioxidants. (*A* and *B*) Time course expression of Nrf2 protein in response to heat treatment. (*C*) A confocal image of fluorescein isothiocyanate–-conjugated secondary antibody staining indicates the location of Nrf2 (green). (*D* and *E*) Effects of NAC and glutathinone on heat-induced Nrf2 expression. Cells were pretreated with NAC (20 mmol/L) and glutathione (10 mmol/L) for 2 hours, after which Nrf2 expression was evaluated by Western blotting. Results are representative of three independent experiments. (*B* and *E*) Densitometric analysis of Nrf-2 proteins. *Statistically significant difference compared with the control group ($p < 0.05$). #Statistically significant difference compared with the heat-treated group ($p < 0.05$).

Spain) according to the manufacturer's instructions and exposed to X-ray film.

Immunocytochemistry

For immunostaining, the cells were fixed in 100% methanol for 30 minutes and washed three times with PBS. After blocking in 5% BSA in PBS for 1 hour at room temperature or overnight at 4°C, the cells were incubated for 1 hour with polyclonal rabbit anti-Nrf2 antibody (1:100) in PBS containing 0.5% BSA. The cells were incubated with fluorescein isothiocyanate–-conjugated goat antirabbit IgG antibody (1:100, catalog no. A11034, Molecular Probes) after serial washings with PBS. The coverslips were mounted on slides using mounting solution. Fluorescent images were obtained using laser scanning confocal microscopy (DM IRBE; Leica, Wetzlar, Germany).

Statistical Analysis

We performed these experiments using samples from at least three different cell preparations, and the data were confirmed using the same cell samples at least in triplicate. Differences among groups were analyzed by using one-way analysis of variance combined with the Bonferroni test.

Results

Effects of Heat Stress on the Production of ROS and Proinflammatory Cytokines

Because ROS are known to contribute to inflammatory reactions, changes in the ROS level were examined in heat-stimulated HDP cells. As shown in Figure 1*A*, the level or intensity of intracellular ROS in pulp cells with heat was higher than in control cells. Heat stress upregulated the levels of IL-8 and IL-8 receptor mRNA in HDP cells, whereas the expression of IL-1β, TNF-α, and IL-6 mRNAs remained unchanged (Fig. 1*B*).

Effects of Antioxidants on Heat Stress–Induced Nrf2 Expression

Nrf2 protein can function as a direct oxidative stress sensor, and phase II detoxifying enzymes are major executors of the cellular defense

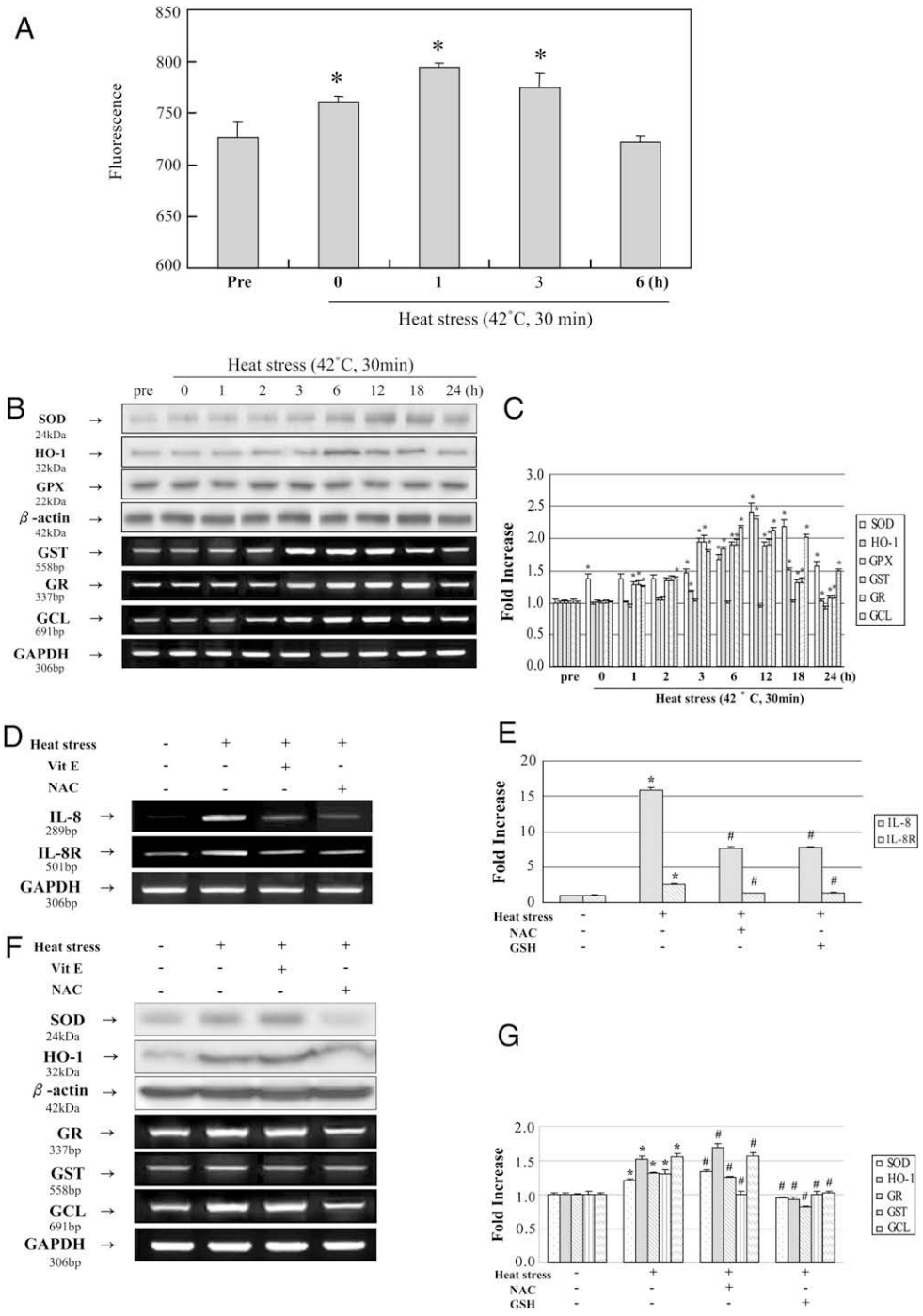

Figure 3. (*A-C*) The effects of heat stress on the expression of Nrf2 target genes or cytoprotective genes such as G6PD, SOD, HO-1, GPX, GCL, GR, and GST and the effects of exogenous antioxidants on (*D* and *E*) heat stress-induced proinflammatory cytokine and (*F* and *G*) Nrf2detoxifying ge2nes in HDP cells. (*A*) The effect of heat stress on the release of G6PD from human pulp cells was assessed using a G6PD assay kit. *Statistically significant difference compared with the control group ($p < 0.05$). (*C*, *E*, and *G*) Gene expression was evaluated by RT-PCR and Western blotting. Densitometric analysis of proteins and mRNA. Results are representative of three independent experiments. *Statistically significant difference compared with the control group ($p < 0.05$). #Statistically significant difference compared with the heat-treated group ($p < 0.05$).

system against oxidative stress. Because we found that heat stress could activate ROS and IL-8, we next investigated the effects of heat stress on the Nrf2 accumulation in the cytosol and nuclear fraction of HDP cells. As shown in Figure 2*A*, Nrf2 was detected in the cytosolic fraction of the control unstimulated cells, but treatment with heat stress resulted in a pronounced increase in nuclear Nrf2 along with a concomitant decrease in cytosolic Nrf2 protein levels on Western blotting, and the translocation peaked at 1 hour.

To determine whether the level of Nrf2 protein is affected by heat stress, confocal microscopy was used. Immunocytochemistry revealed that Nrf2 was located predominantly in the cytoplasm of the control cells. In cells treated with heat stress for 1 h, Nrf2 had a perinuclear

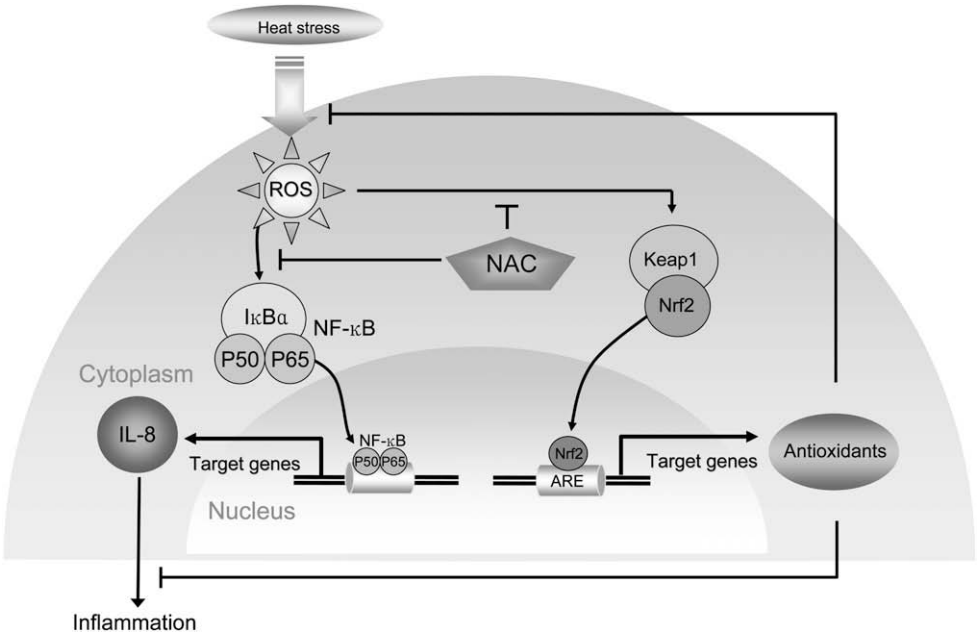

Figure 4. A schematic diagram showing the activation of antioxidants via Nrf2/ ARE pathway and IL-8 via NF-κB pathway triggered by exposure to heat stress in HDP cells. ROS are involved as second messengers in the NF-κB and ARE activation pathways. According to previous reports (41), NF-κB plays a major role in IL-8 secretion of the HDP cells.

and nuclear localization, indicating that Nrf2 had moved into the nucleus (Fig. 2*C*).

To examine the role of oxidation in mediating Nrf2 translocation, we pretreated cells with 20 mmol/L of the antioxidant NAC, 20 μmol/L of vitamin E, and 10 mmol/L of reduced glutathione for 2 hours before heat stress exposure. Figure 2*D* and *E* shows that the heat-mediated nuclear accumulation of Nrf-2 was blocked by NAC and glutathione.

Effects of Heat Stress on the Expression of Nrf-2 Target Genes or Cytoprotective Genes

To assess whether heat treatment can induce Nrf2 target genes, antioxidant genes, or phase II detoxifying genes, we examined the levels of G6PD, SOD, HO-1, GPX, GCL, GR, and GST. G6PD activity increased with heat from 0 to 3 hours; the response peaked at 1 hour followed by a gradual decrease (Fig. 3*A*). SOD, HO-1, GST, GCL, and GR upregulation was detected in cells after incubation under heat stress conditions for 3 or 6 hours, but the levels returned to baseline by 16 or 24 hours after incubation. The level of GPX was not affected by heat treatment (Fig. 3*B* and *C*).

Effects of Antioxidants on Heat Stress–Induced Proinflammatory Cytokines and Detoxifying Genes

To verify that antioxidants protect cells from heat stress–induced proinflammatory cytokines and stress genes, the exogenous antioxidants NAC and vitamin E were used. As shown in Figure 3*D* and *E*, NAC and vitamin E blocked the heat-induced increases in IL-8 and IL-8R. NAC also significantly attenuated the heat stress–induced increases in SOD, HO-1, GR, GST, and GCL, whereas vitamin E did not (Fig. 3*F* and *G*).

Discussion

To our knowledge, this is the first report on the Nrf-2-mediated defense system in response to oxidative stress induced by heat stress in HDP cells. The temperature within pulp tissues varies between 36.5°C and 45°C depending on the dental application to which they are subjected. For example, the temperatures within the dental pulp tissues that resulted from dental procedures ranged from 37°C to 40°C for hard tissue preparations (31), 40°C to 45°C for light curing of composite materials (32), 38°C to 41°C for laser irradiation (33), and reached 44°C while manufacturing temporary crowns (31). Therefore, we induced heat shock by subjecting cells to 42°C for 30 minutes, which is similar to the procedures used in previous studies (14, 15).

Heat shock increases the concentration of intracellular ROS in Chinese hamster cells and bovine endothelial cells, suggesting that ROS play a role in heat-induced cell injury (34). A previous study on cultured rat vascular smooth muscle cells showed that the intracellular increase in superoxide anion on exposure to the inhibitor of soluble guanylate cyclase, LY83583, led to early transient (10 minutes) and late sustained activation (120 minutes) of extracellular signal-regulated kinase 1/2 and stimulated vascular smooth muscle cell proliferation (35). Furthermore, Liao et al (36) showed that the early phase was activated by ROS directly and the late was mediated via secreted oxidative-stressed factors involving HSP90. In our study, the heat stress–induced generation of ROS began at 10 minutes, peaked at 1 or 2 hours, and returned to baseline at 3 hours (data not shown); this was accompanied by the induction of HSP32 (HO-1, Fig. 3*B*). However, the mechanism of the oxidative stress factors mediating ROS requires further study.

Heat shock markedly enhances TNF-α–induced IL-8 secretion in human A549 cells and human airway epithelial cells (37). The results of this study showing that heat stress upregulates proinflammatory cytokines such as IL-8 and IL-8R are consistent with these previous findings (37). Our results showed that heat stress increased intracellular ROS levels, which contributed to increased IL-8 in HDP cells.

Although Nrf2 has been established as a key player in the cellular adaptive response to oxidative stress (18, 19), the involvement of Nrf2 in the oxidative stress response induced by heat stress in HDP cells had not been assessed. In this study, confocal analysis and immunoblot data showed that Nrf2 was translocated into the nucleus after heat treatment in HDP cells (Fig. 2*A-C*). Our studies also showed that NAC and

glutathione inhibited Nrf2 translocation in heat-treated HDP cells. This suggests that ROS are involved in modulating heat-induced Nrf2 turnover. Collectively, these results are in strong agreement with the profile of Nrf2 activation induced by other pro-oxidants in various cell types (38), suggesting that heat exposure activates Nrf2.

To further evaluate the transcriptional effects of heat-induced Nrf2 activation, the expression of several Nrf2 target genes was assessed. Consistent with the results showing enhanced Nrf2 translocation in response to heat exposure, the expression levels of SOD, HO-1, GST, GR, and GCL were increased. These results are consistent with the results of another study showing that the self-defense system in adult dental pulp cells involves the expression of self-defense proteins (39).

Interestingly, in our study, the upregulated levels observed for these antioxidant enzymes returned almost to the basal condition by 24 hours after the heat stress. This suggests that under normal conditions, the generation and scavenging of ROS remain in balance, preventing ROS accumulation by antioxidant enzymes.

Vitamin E is a major lipophilic antioxidant and plays a vital role in preventing oxidative stress. NAC is a cysteine derivative that is more stable than glutathione and protects cells against oxidative stress in various ways (39). In our study, the addition of NAC to cells blocked heat-induced IL-8 and Nrf2 target gene expression (Fig. 3), which is consistent with other studies (40). Combined, our results imply that ROS play an important role in proinflammatory cytokine production by heat stress from HDP cells.

In summary, this is the first study to show that heat stress activates proinflammatory chemokine and Nrf2-mediated antioxidant responses in HDP cells. Figure 4 is a schematic representation of the signaling pathway involved in Nrf2 activation, and the subsequent induction of antioxidant/detoxification enzymes protects the cells from heat-induced proinflammatory cytokine and oxidative damage in HDP cells. Further studies are necessary to understand the Nrf2-antioxidant pathway in inflamed HDP cells and periapical lesions.

References

1. Nicoll BK, Peters RJ. Heat generation during ultrasonic instrumentation of dentin as affected by different irrigation methods. J Periodontol 1998;69:884–8.
2. Cavalcanti BN, Lage-Marques JL, Rode SM. Pulpal temperature increases with Er:YAG laser and high-speed handpieces. J Prosthet Dent 2003;90:447–51.
3. Lee FS, Van Cura JE, BeGole E. A comparison of root surface temperatures using different obturation heat sources. J Endod 1998;24:617–20.
4. Türkmen C, Günday M, Karaçorlu M, et al. Effect of CO_2. Nd:YAG, and ArF excimer lasers on dentin morphology and pulp chamber temperature: an in vitro study. J Endod 2000;26:644–8.
5. Baik JW, Rueggeberg FA, Liewehr FR. Effect of light-enhanced bleaching on in vitro surface and intrapulpal temperature rise. J Esthet Restor Dent 2001;13:370–8.
6. Wang X, Zou Y, Wang Y, et al. Differential regulation of interleukin-12 and interleukin-10 by heat shock response in murine peritoneal macrophages. Biochem Biophys Res Commun 2001;287:1041–4.
7. Wang Y, Li C, Wang X, et al. Heat shock response inhibits IL-18 expression through the JNK pathway in murine peritoneal macrophages. Biochem Biophys Res Commun 2002;296:742–8.
8. Leon LR, Blaha MD, Dubose DA. Time course of cytokine, corticosterone and tissue injury response in mice during heat strain recovery. J Appl Physiol 2006;100:1400–9.
9. Lin MT, Kao TY, Jin YT, et al. Interleukin-1 receptor antagonist attenuates the heat stroke-induced neuronal damage by reducing the cerebral ischemia in rats. Brain Res Bull 1995;37:595–8.
10. Yang Q, Sun X, Pritts TA, et al. Induction of the stress response increases IL-6 production in intestinal mucosa of endotoxemic mice. Clin Sci 2000;99:489–96.
11. Parikh AA, Moon MR, Kane CD, et al. Interleukin-6 production in human intestinal epithelial cells increases in association with the heat shock response. J Surg Res 1998;77:40–4.
12. Eberhard J, Zahl A, Dommisch H, et al. Heat shock induces the synthesis of the inflammatory mediator leukotriene B4 in human pulp cells. Int Endod J 2005;38:882–8.
13. Kitamura C, Nishihara T, Ueno Y, et al. Thermotolerance of pulp cells and phagocytosis of apoptotic pulp cells by surviving pulp cells following heat stress. J Cell Biochem 2005;94:826–34.
14. Amano T, Muramatsu T, Amemiya K, et al. Responses of rat pulp cells to heat stress in vitro. J Dent Res 2006;85:432–5.
15. Lee MW, Muramatsu T, Uekusa T, et al. Heat stress induces alkaline phosphatase activity and heat shock protein 25 expression in cultured pulp cells. Int Endod J 2008;41:158–62.
16. Kitamura C, Nishihara T, Ueno Y, et al. Effects of sequential exposure to lipopolysaccharide and heat stress on dental pulp cells. J Cell Biochem 2006;99:797–806.
17. Ohshima H, Nakakura-Ohshima K, Yamamoto H, et al. Responses of odontoblasts to cavity preparation in rat molars as demonstrated by immunocytochemistry for heat shock protein (Hsp) 25. Arch Histol Cytol 2001;64:493–501.
18. Zhang DD, Hannink M. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol Cell Biol 2003;23:8137–51.
19. Steinkellner H, Rabot S, Kassie F, et al. Dietary induction of phase II enzymes: a promising strategy for protection against DNA-reactive intermediates in man? Adv Exp Med Biol 2001;500:629–33.
20. Min KS, Kwon YY, Lee HJ, et al. Effects of proinflammatory cytokines on the expression of mineralization markers and heme oygenase-1 in human pulp cells. J Endod 2006;32:39–43.
21. Min KS, Hwang YH, Ju HJ, et al. Heme oxygenase-1 mediates cytoprotection against nitric oxide-induced cytotoxicity via the cGMP pathway in human pulp cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;102:803–8.
22. Min KS, Lee HJ, Kim SH, et al. Hydrogen peroxide induces heme oxygenase-1 and dentin sialophosphoprotein mRNA in human pulp cells. J Endod 2008;34:983–9.
23. Lee SK, Min KS, Kim Y, et al. Mechanical stress activates proinflammatory cytokines and antioxidant defense enzymes in human dental pulp cells. J Endod 2008;34:1364–9.
24. Ishii T, Itoh K, Yamamoto M. Roles of Nrf2 in activation of antioxidant enzyme genes via antioxidant responsive elements. Meth Enzymol 2002;348:182–90.
25. Liu H, Colavitti R, Rovira II, et al. Redox-dependent transcriptional regulation. Circ Res 2005;97:967–74.
26. Chen ZH, Saito Y, Yoshida Y, et al. 4-Hydroxynonenal induces adaptive response and enhances PC12 cell tolerance primarily through induction of thioredoxin reductase 1 via activation of Nrf2. J Biol Chem 2005;280:41921–7.
27. Katschinski DM, Boos K, Schindler SG, et al. Pivotal role of reactive oxygen species as intracellular mediators of hyperthermia-induced apoptosis. J Biol Chem 2000;275:21094–8.
28. Davidson JF, Schiestl RH. Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in Saccharomyces cerevisiae. Mol Cell Biol 2001;21:8483–9.
29. Chen XL, Kunsch C. Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases. Curr Pharm Des 2004;10:879–91.
30. Trayner ID, Rayner AP, Freeamn GE, et al. Quantitative multiwell myeloid differentiation assay using dichlorodihydrofluorescein diacetate (H2DCFDA) or dihydrorhodamine 123 (H2R123). J Immunol Methods 1995;186:275–84.
31. Moulding MB, Teplitsky PE. Intrapulpal temperature during direct fabrication of provisional restorations. Int J Prosthodont 1990;3:299–304.
32. Hannig M, Bott B. In-vitro pulp chamber temperature rise during composite resin polymerization with various light-curing sources. Dent Mater 1999;15:275–81.
33. Oelgiesser D, Blasbalg J, Ben-Amar A. Cavity preparation by Er-YAG laser on pulpal temperature rise. Am J Dent 2003;16:96–8.
34. Lin P, Quamo S, Ho K, et al. Hyperthemia enhances the cytotoxic effects of reactive oxygen species to Chinese hamster cells and bovine endothelial cells in vitro. Radia Res 1991;126:43–51.
35. Jin ZG, Melaragno MG, Liao DF, et al. Cyclophilin A is a secreted growth factor induced by oxidative stress. Circ Res 2000;87:789–96.
36. Liao DF, Jin ZG, Bass AS, et al. Purification and identification of secreted oxidative stress-induced factors from vascular smooth muscle cells. J Biol Chem 2000;275:189–96.
37. Singh IS, Gupta A, Nagarsekar A, et al. Heat shock co-activates interleukin-8 transcription. Am J Respir Cell Mol Biol 2008;39:235–42.
38. Chen XL, Kunsch C. Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases. Curr Pharm Des 2004;10:879–91.
39. Matsuzaka K, Muramatsu T, Katakura A, et al. Changes in the homeostatic mechanism of dental pulp with age: expression of the core-binding factor alpha-1, dentin sialoprotein, vascular endothelial growth factor, and heat shock protein 27 messenger RNAs. J Endod 2008;34:818–21.
40. Hellsten Y, Nielsen JJ, Lykkesfeldt J, et al. Antioxidant supplementation enhances the exercise-induced increase in mitochondrial uncoupling protein 3 and endothelial nitric oxide synthase mRNA content in human skeletal muscle. Free Radic Biol Med 2007;43:353–61.
41. Min KS, Kim HI, Chang HS, et al. Involvement of mitogen-activated protein kinases and nuclear factor-kappa B activation in nitric oxide-induced interleukin-8 expression in human pulp cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008; 105. 654–-60.