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Effects of Sequential Exposure to Lipopolysaccharide and Heat Stress on Dental Pulp Cells

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Abstract In the present study, we examined the effects of sequential exposure to bacterial lipopolysaccharide (LPS) and heat stress on dental pulp cells. LPS induced the proliferation of pulp cells through the activation of p38 MAPK. HSP27 was expressed in cells with or without LPS during the entire period of heat stress, while transiently phosphorylated by short-term heat stress. In LPS-treated cells, short-term heat stress also induced the phosphorylation of HSF1. The immediate phosphorylation of HSF1 and HSP27 in LPS-treated cells by short-term heat stress occurred dependent on the activation of p38 MAPK. However, with long-term heat stress, the activation of HSF1 and induction of HSP27 occurred independent of p38 MAPK. Further, full activation of Akt in LPS-treated cells was immediately induced by short-term heat stress and lasted during the entire period of heat stress. IκBα was induced and phosphorylated throughout sequential exposure to LPS and heat stress. These results suggest that LPS has the unique effects on the cytoprotection and the cell death of pulp cells during heat stress through the modification and the activation of heat stress responsive molecules, HSF1 and HSP27, and cell survival molecules, Akt and NF-κB/IκBα. J. Cell. Biochem. 99: 797–806, 2006.

Key words: dental pulp cells; thermoresistance; heat stress; lipopolysaccharide

In both physiological and pathological conditions, cells are exposed to several types of stress, including that associated with heat. Heat stress results in a rapid production of heat shock proteins (HSPs), and the expression and phosphorylation of HSPs contribute to the repair of damaged proteins and cell survival. HSP27 is known to be an anti-apoptotic molecule [Garrido et al., 1999], and mainly regulated by heat shock transcription factors (HSFs), especially HSF1 [Frohli et al., 1993], and also by post-translational modification such as phosphorylation. Phosphorylated HSP27 interacts with proapoptotic molecules to prevent apoptosis, though it is not well known how the phosphorylation of HSP27 influences such cellular responses in thermoresistance [Landry et al., 1992; Knauf et al., 1994; Lavoie et al., 1995]. It has also been indicated that HSP27 forms a signaling module with p38 mitogen-activated protein kinase (MAPK), MAPK-activated protein kinase-2 (MAPKAPK-2), and Akt (protein kinase B) [Rane et al., 2003].

Lipopolysaccharide (LPS) is a component of the outer membrane of gram-negative bacteria that activates a number of cellular responses [Ulevitch and Tobias, 1995; Itoh et al., 2003]. LPS interacts with the LPS-sensing cluster consisting of the core molecular complex (CD14, Toll-like receptor 4, and MD2) and other receptor molecules such as CD11/CD18 [Triantafilou and Triantafilou, 2005], and the activation of LPS-sensing clusters results in the activation of a variety of signaling pathways, including MyD88-dependent [Muzio et al.,

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1997; Medzhitov et al., 1998], TIR domain adaptor inducing interferon-β (TRIF)-dependent [Hoebe et al., 2003; Yamamoto et al., 2003], MAPKs [Nick et al., 1996; Hull et al., 2002], and phosphoinositide 3 kinase (PI3-kinase)-Akt [Li et al., 2003; Vivarelli et al., 2004] pathways.

Dental pulp cells are exposed by the bacteria following caries infection of teeth, and insults by bacterial LPS occur directly or indirectly through dentinal tubules [Nakane et al., 1995; Matsushita et al., 1999]. Pathological changes of dental pulp caused by LPS give rise to painful tooth symptoms, and can adversely affect the response of pulp cells to restorative procedures. In the process of restoration on carious infected teeth, heat stress is produced during cavity preparation, and is believed to be one of major causes of damage to dental pulp cells [Mjör, 2002]. Our previous studies found that apoptosis is induced in dental pulp cells during wound healing and regeneration, and that HSPs, c-Jun, and c-Jun N-terminal kinase may play roles during pulp apoptosis and regeneration [Kitamura et al., 2001, 2003]. Further, we also demonstrated that pulp cells show the reduction of viability with apoptosis induction by long-term heat stress and the recovery of the viability after the removal of heat stress [Kitamura et al., 2005]. However in the clinical and physiological contexts, pulp cells are generally pre-exposed to bacterial LPS before the exposure to heat stress produced by cavity preparation, and little is known about the responses of pulp cells during sequential exposure to LPS and heat stress.

It is generally known that the phenotypic responses of cells to heat stress is critically linked to the state of the cells. Several studies have reported that exposure to LPS, followed by heat stress, accelerated cell death by apoptosis, whereas heat stress followed by exposure to LPS, induced cytoprotection in cells [Buchman et al., 1993; Xu et al., 1996]. The paradoxical ability of heat stress to induce the cytoprotection and the cytotoxicity is called the heat shock paradox, which is closely related with cytoprotective and cytotoxic functions of NF-κB/IκBα (called the NFκB paradox) [DeMeester et al., 2001]. However, the molecular mechanisms in the heat shock paradox during the cell responses against the combination of heat stress and other stresses such as LPS are poorly understood.

In the present study, we examined the effects of sequential exposure to LPS and heat stress on pulp cells, as well as the roles of HSF1, HSP27, p38 MAPK, Akt, and NF-κB/IκBα with the thermoresistance and the survival of pulp cells.

MATERIALS AND METHODS

Cell Cultures

Rat clonal dental pulp cells (RPC-C2A) [Kasugai et al., 1988] were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA) containing 10% heat-inactivated fetal calf serum (FCS), 100 μg/ml of streptomycin, and 100 U/ml of penicillin, and then incubated in a humidified atmosphere of 5% CO_2 at 37°C. First, the cells were subcultured for 24 h in DMEM containing 5% heat-inactivated FCS, 100 μg/ml of streptomycin, and 100 U/ml of penicillin, then treated with LPS from *Actinobacillus actinomycetemcomitans* Y4 dissolved in DMEM containing 1% heat-inactivated FCS, 100 μg/ml of streptomycin, and 100 U/ml of penicillin for 48 h, after which they were sequentially exposed to heat stress at 43°C for 5 and 10 min (short-term), and 45 min (long-term).

Cell Proliferation Assay

The proliferation of RPC-C2A cells after the exposure to LPS and heat stress was examined by determining 5-bromo-2′-deoxyuridine (BrdU) incorporation during DNA synthesis using Cell Proliferation Biotrak ELISA System, version 2 (Amersham Biosciences Corp., Piscataway, NJ) according to the manufacturer's instructions. RPC-C2A cells (3×10^3/well) were cultured in 96-well dishes in a humidified atmosphere of 5% CO_2 at 37°C for 24 h, then treated with several concentrations of LPS (0, 1, 10, and 50 μg/ml) for 48 h, after which BrdU (10 μM/well) was added to each well. RPC-C2A cells (3×10^3/well) were also cultured in 96-well dishes in a humidified atmosphere of 5% CO_2 at 37°C for 24 h, followed by treatment with LPS (50 μg/ml) for 48 h, and then sequentially exposed to heat stress at 43°C for 5, 10, and 45min. At each heat stress time point, BrdU (10 μM/well) was added to each well. After the incorporation of BrdU by incubation of RPC-C2A cells in a humidified atmosphere of 5% CO_2 at 37°C for 4 h, the cells were fixed with ethanol, and blocked with blocking buffer, then incubated with the peroxidase-labeled anti-BrdU

antibody for 90 min at room temperature. After washing with 1× phosphate buffered saline (PBS), 3,3′,5,5′-tetramethylbenzidine was added to each well as a substrate. The reaction was stopped with 1 M sulfuric acid after 5 min, and BrdU incorporation during DNA synthesis was analyzed. All experiments were performed in triplicate.

Cell Viability Assay

Inhibition of cell viability after the exposure to LPS and long-term heat stress (45 min) was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. RPC-C2A cells (1×10^4/well) were cultured in 96-well dishes in a humidified atmosphere of 5% CO_2 at 37°C for 24 h, treated with LPS (0 and 50 μg/ml) for 48 h, and then exposed to heat stress at 43°C for 45 min. In each time point following long-term heat stress, MTT (Sigma-Aldrich Co., St. Louis, MO) (20 μg/well) added to each well, and the dishes were incubated at 37°C for 4 h. After acid-isopropanol (100 μl/well) was added and mixed thoroughly, the cell viability in each time point was analyzed. The viability of RPC-C2A cells exposed to long-term heat stress followed by LPS-treatment was also analyzed by MTT assay. All experiments were performed in triplicate.

Western Blot Analysis

Following exposure to LPS and heat stress, RPC-C2A cells were lysed in buffer containing 3% sodium dodecyl sulfate (SDS) in 50 mM Tris/HCl, pH 6.8. Western blot analysis was performed using whole-cell lysates. Proteins from each lysate were separated by SDS-polyacrylamide gel electrophoresis, and blotted onto an Immobilon-P Transfer membrane (Millipore Corp., Bedford, MA). Blotted membranes were blocked in PBS containing 5% nonfat milk powder and incubated with the following primary antibodies: rabbit anti-HSF1, goat anti-HSP27, goat anti-HSP27 phosphorylated at serine 82 (Ser-82), goat anti-Akt1, rabbit anti-Akt phosphorylated at threonine 308 (Thr-308), rabbit anti-IκBα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and rabbit anti-Akt phosphorylated at serine 473 (Ser-473), rabbit anti-IκBα phosphorylated at serine 32 (Ser-32) (Cell Signaling Technology, Inc., Beverly, MA). Subsequently, the membranes were incubated with the following secondary antibodies: donkey anti-goat IgG, horseradish peroxidase-conjugated secondary antibody (Chemicon International, Temecula, CA), and donkey anti-rabbit IgG, peroxidase-linked secondary antibody (Amersham Biosciences Corp.). Immunoreactive bands were then visualized with ECL or ECL plus Western blotting detection system (Amersham Biosciences Corp.) according to the manufacturer's instructions. After exposure to film, the membranes were stained with Coomassie Brilliant Blue G-250 to confirm equal loading of proteins.

p38 MAPK Inhibition Assay

A p38 MAPK inhibitor, SB203580 (Calbiochem-Novabiochem Corp., San Diego, CA), was dissolved in dimethyl sulfoxide at a maximum concentration of 0.1%, which did not have an effect on the RPC-C2A cells in the present experiments (data not shown). RPC-C2A cells cultured in 96-well dishes were treated with LPS (50 μg/ml in each well) for 48 h in the presence of SB203580 (0, 5, 10, 20, and 30 μM/well), after which cell proliferation was analyzed for measurement of BrdU incorporation by the cells. The expression and phosphorylation of HSF1 and HSP27 in RPC-C2A cells exposed to LPS and heat stress, with or without the p38 inhibitor, were also analyzed by Western blotting.

Statistical Analysis

Statistical differences were determined using Student's *t*-test. All data are expressed as the mean ± SD.

RESULTS

Proliferation of Pulp Cells After the Exposure to LPS and Long-Term Heat Stress

To determine whether LPS influences the proliferation of pulp cells, RPC-C2A cells were exposed to several concentrations (0, 1, 10, and 50 μg/ml) of LPS for 48 h. The incorporation of BrdU into the cells was increased by LPS in a dose-dependent manner (Fig. 1A). The effects of long-term heat stress (45 min) on the survival of LPS-treated cells were shown in Figure 1B. When we compared the viability between cells before exposure to LPS and cells treated with or without LPS, the viability of cells without LPS decreased, whereas the viability of LPS-treated cells maintained but not increase. After exposure to heat stress, the viability of cells decreased at 12 h after long-term heat stress, thereafter

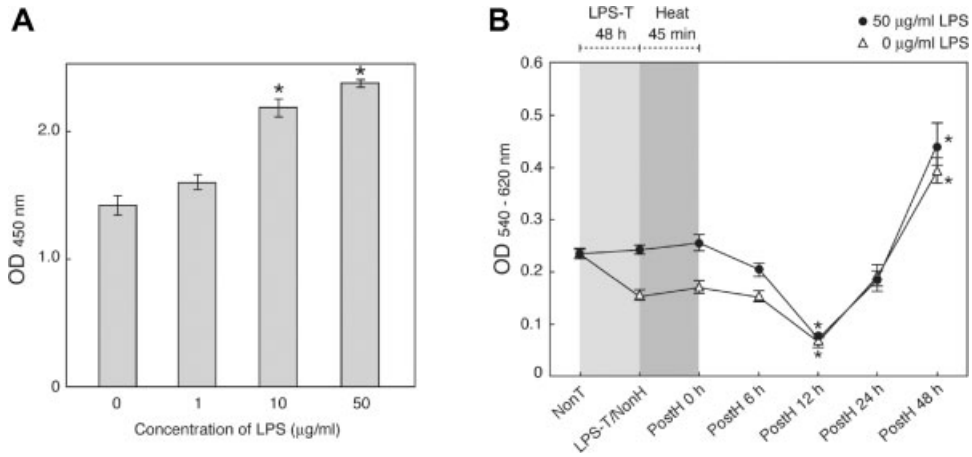

Fig. 1. **A**: Incorporation of BrdU into RPC-C2A cells after exposure to LPS (0, 1, 10, 50 μg/ml). Data are expressed as the mean ± SD of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. Statistical differences were determined between 0 μg/ml of LPS and other concentrations of LPS. *, $P < 0.01$ (Student's *t*-test). **B**: The viability of LPS-treated RPC-C2A cells after long-term heat stress. Data are expressed as the mean ± SD of triplicate cultures. Each experiment was performed three times, with similar results obtained in each experiment. Statistical differences were determined between cells without heat stress (LPS-T/NonH) and cells with heat stress in each LPS-treatment set (0 and 50 μg/ml of LPS). *, $P < 0.01$ (Student's *t*-test). NonT, non-treated; LPS-T, LPS-treated; NonH, non-heated; PostH, post heat stress time point.

the cell viability increased at 24 and 48 h, both in cells with or without pre-exposure to LPS. The viability of RPC-C2A cells exposed to long-term heat stress followed by LPS treatment was also analyzed. The change of the viability of cells exposed to heat stress followed by LPS treatment was almost same with that of cells pre-exposed to LPS followed by heat stress. After pre-exposure to heat stress, the viability of cells with or without LPS decreased at 12 h, thereafter the cell viability increased (data not shown).

Proliferation of LPS-Treated Pulp Cells During Heat Stress

The effects of short-term heat stress (5 and 10 min) on RPC-C2A cells were examined (Fig. 2A). The incorporation of BrdU into the

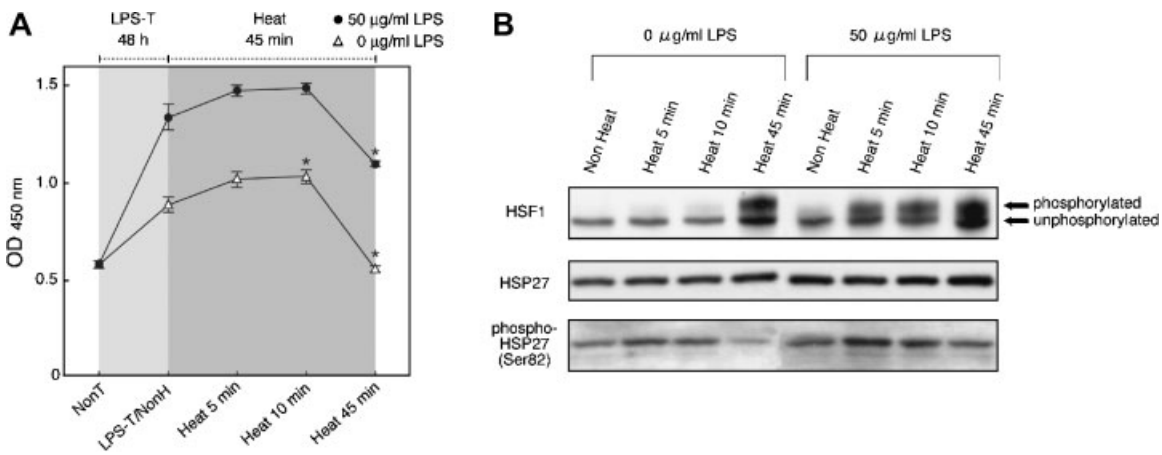

Fig. 2. **A**: BrdU incorporation into LPS-treated RPC-C2A cells during heat stress for short-term (5, 10 min) and long-term (45 min). Data are expressed as the mean ± SD of triplicate cultures. Each experiment was performed three times, with similar results obtained in each experiment. Statistical differences were determined between cells without heat stress (LPS-T/NonH) and cells with heat stress in each LPS-treatment set (0 and 50 μg/ml of LPS). *, $P < 0.01$ (Student's *t*-test). NonT, non-treated; LPS-T, LPS-treated; NonH, non-heated; PostH, post heat stress time point. **B**: Western blot analysis of HSF1, HSP27, and phosphorylated HSP27 at Ser-82 extracted from RPC-C2A cells sequentially exposed to LPS and heat stress. The membranes were stained with Coomassie Brilliant Blue G-250, and we confirmed equal loading of proteins (data not shown).

cells was increased by LPS, which was shown in Figure 1A. During short-term heat stress, BrdU incorporation was maintained and slightly increased, whereas it was significantly decreased by long-term heat stress, in cells with or without pre-exposure to LPS.

Expression and Phosphorylation of HSF1 and HSP27 in LPS-Treated Pulp Cells During Heat Stress

Next, we investigated the expression and phosphorylation of HSF1 and HSP27 to determine whether LPS has an effect on those molecules in RPC-C2A cells (Fig. 2B). Unphosphorylated HSF1, which was seen as a single band, was expressed in the cells with or without LPS before heat stress. Following the exposure of LPS-treated cells to heat stress, the phosphorylation of HSF1, detected as a slower migrating band, was immediately induced and lasted throughout the entire heat stress exposure period. However, in cells not exposed to LPS, HSF1 was phosphorylated only by long-term heat stress, not by short-term heat stress.

HSP27 was expressed in RPC-C2A cells with or without LPS before heat stress, which was independent of the phosphorylation of HSF1. Throughout the entire period of heat stress, HSP27 was stably expressed in the cells with or without pre-exposure to LPS. The phosphorylation of HSP27 at Ser-82 was slightly induced without heat stress, while strongly induced after 5 min of exposure to heat stress in both LPS-treated cells and cells without LPS. Thereafter, the level of phosphorylation was gradually decreased at 10 min, and markedly suppressed after 45 min of heat stress.

Effects of p38 MAPK Inhibitor on Proliferation of LPS-Treated Pulp Cells, and Expression and Phosphorylation of HSF1 and HSP27

We also examined the association of p38 MAPK with the proliferation of RPC-C2A cells induced by LPS (Fig. 3A). When the p38 MAPK inhibitor, SB203580, was added to the cells with LPS, cell proliferation was suppressed in a dose-dependent manner. Lower concentrations (5 and 10 μM) of SB203580 did not have an effect on the level of incorporated BrdU in the cells, however, when higher concentrations (20 and 30 μM) of SB203580 were added to the cells with LPS, BrdU incorporation was significantly suppressed.

The roles of p38 MAPK with the expression and phosphorylation of HSF1 and HSP27 in LPS-treated cells during heat stress were also examined (Fig. 3B). SB203580 did not have an effect on the expression of unphosphorylated HSF1 in LPS-treated cells before heat stress. The short-term heat stress induced phosphorylation of HSF1 in LPS-treated cells was completely suppressed by the addition of the

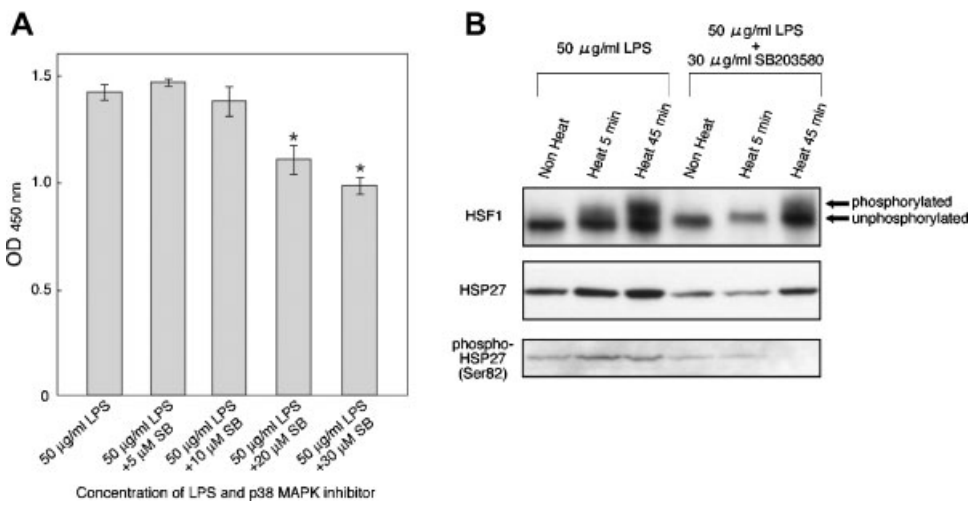

Fig. 3. Effects of p38 MAPK inhibitor on responses of pulp cells exposed to LPS and heat stress. **A**: Different concentrations (5, 10, 20, 30 μM) of SB203580 (SB) were added to RPC-C2A cells with LPS, and the incorporation of BrdU into cells was measured. Data are expressed as the mean ± SD of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. Statistical differences were determined between 50 μg/ml of LPS without SB and 50 μg/ml of LPS with SB. *, $P < 0.01$ (Student's *t*-test). **B**. Western blot analyses of HSF1, HSP27, and phosphorylated HSP27 at Ser-82 extracted from RPC-C2A cells sequentially exposed to LPS and heat stress in the absence and presence of SB203580. The membranes were stained with Coomassie Brilliant Blue G-250, and we confirmed equal loading of proteins (data not shown).

p38 MAPK inhibitor, however, HSF1 was phosphorylated by long-term heat stress in the presence of SB203580. Expression of HSP27 in the cells exposed to LPS and short-term heat stress was also suppressed by the addition of SB203580, whereas the expression level of HSP27 was increased with long-term heat stress in the presence of the inhibitor. In addition, the phosphorylation of HSP27 at Ser-82 in LPS-treated cells during heat stress was markedly suppressed by SB203580.

Expression and Phosphorylation of Akt and IκBα in Pulp Cells Exposed to LPS Following Heat Stress

Figure 4 shows the expression and phosphorylation of Akt and IκBα in RPC-C2A cells after sequential exposure to LPS and heat stress.

Akt1 was slightly expressed in the cells with or without LPS before heat stress, and expression gradually increased during heat stress. The phosphorylation of Akt at Thr-308 was induced by LPS and observed throughout the entire period of heat stress. In the cells without pre-exposure to LPS, the phosphorylation of Akt at Thr-308 was detected faintly under both non-heat and short-term heat stress conditions, while intensely detected after long-term heat stress. In contrast, phosphorylation at Ser-473 was not detected in the cells with or without LPS before heat stress. In LPS-treated cells, Ser-473 was immediately phosphorylated by short-term heat stress (5 min), which continued throughout the entire period of heat stress. On the other hand, without pre-exposure to LPS, the phosphorylation of Akt at Ser-473 was not detected before heat stress, and then faintly detected following short-term heat stress. With long-term heat stress, Ser-473 was strongly phosphorylated in cells without pre-exposure to LPS.

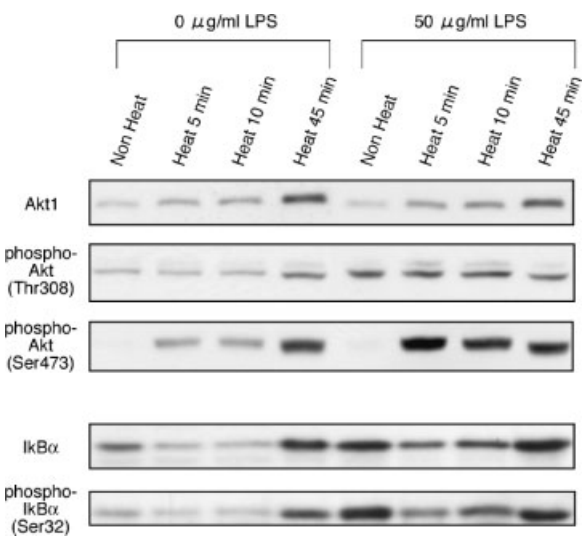

Fig. 4. Western blot analyses of Akt1, phosphorylated Akt at Thr-308, phosphorylated Akt at Ser-473, IκBα, and phosphorylated IκBα at Ser-32, extracted from RPC-C2A cells sequentially exposed to LPS and heat stress. The membranes were stained with Coomassie Brilliant Blue G-250, and we confirmed equal loading of proteins (data not shown).

IκBα was expressed in the cells with or without LPS before heat stress, and the intense signal of IκBα was observed in LPS-treated cells. In LPS-treated cells, IκBα slightly decreased by short-term heat stress, while markedly increased by long-term heat stress. In the cells without pre-exposure to LPS, IκBα was faintly detected during short-term heat stress and induced by long-term heat stress. The phosphorylation of IκBα at Ser-32 was intensely induced by LPS. In LPS-treated cells, the phosphorylation of IκBα at Ser-32 was suppressed by short-term heat stress, though the phosphorylation level was still higher than those of cells without LPS. By long-term heat stress, phosphorylated IκBα was markedly induced. In contrast, the phosphorylation of IκBα at Ser-32 in the cells without LPS was faintly detected under non-heat and short-term heat stress condition. Long-term heat stress induced the phosphorylation of IκBα at Ser-32 in the cells without the pre-exposure to LPS.

DISCUSSION

Dental pulp cells insulted by bacterial LPS are exposed to heat stress during the clinical procedures. We recently reported that pulp cells showed the reduction and the recovery of the viability in the post-period after long-term heat stress, indicating that pulp cells have the thermotolerance [Kitamura et al., 2005]. In the present study, we focused on the cytoprotective and cytotoxic effects of LPS on pulp cells during heat stress. We first examined the effect of LPS on the survival of pulp cells before heat stress, and found that LPS induces the proliferation of pulp cells. The viability reduction of cells without LPS may be the effect of low concentration of FCS. LPS-treated pulp cells showed the reduction and the recovery of the viability during the post period after long-term

heat stress. Pulp cells also show the thermotolerance when the cells were pre-exposed to long-term heat stress followed by LPS treatment. We next examined the effect of LPS on the thermoresistance of pulp cells during heat stress, and found that, regardless of pre-exposure to LPS, the survival of pulp cells maintained during short-term heat stress, while reduced by long-term heat stress. However, when we analyzed expression of heat stress related molecules (HSF1 and HSP27) and cell survival molecules (Akt and NF-κB/IκBα) in pulp cells by Western blotting, we found the specific effects of LPS on these molecules.

HSF1 was expressed in pulp cells with or without LPS before heat stress, while the immediate activation of HSF1 by heat stress was found only in LPS-treated pulp cells. These results suggest that LPS does not phosphorylate HSF1, but may have an ability to modify HSF1 to be easily phosphorylated by heat stress, and that immediately activated HSF1 plays cytoprotective role during heat stress. HSP27 was expressed in pulp cells with or without LPS, which was independent of the activation of HSF1. It has been reported that signal transducer and activator of transcription (STAT) 1 and 3 can regulate the expression of HSP27 [Yokota et al., 2003; Song et al., 2004]. Thus, the induction of HSP27 before heat stress may be regulated by transcriptional factors such as STAT1/3. HSP27 was expressed during the entire period of heat stress, while the phosphorylation of HSP27 at Ser-82 was transiently induced by short-term heat stress. It is known that the phosphorylation of HSP27 modulates several events such as anti-apoptotic events and thermoresistance [Lavoie et al., 1995; Charette et al., 2000], and that the Ser-82 residue is the major site of in vivo phosphorylation of HSP27 [Landry et al., 1992]. Our results suggest that phosphorylated HSP27 at Ser-82 may play a cytoprotective role during short-term heat stress. Also, HSP27 expressed by long-term heat stress may play a role in the survival of pulp cells in the post long-term heat stress period.

We focused on the regulation mechanism of HSF1 and HSP27 during sequential exposure to LPS and heat stress. It has been suggested that p38 MAPK can regulate expression and activation of HSP27 [Rouse et al., 1994; Huot et al., 1995; Geum et al., 2002]. SB203580, a p38 MAPK inhibitor, suppressed the expression of HSP27 in LPS-treated pulp cells, suggesting that HSP27 expression before heat stress may be regulated through p38 MAPK signaling pathway. LPS-induced proliferation of pulp cells also occurred through the activation of p38 MAPK, suggesting the association of HSP27 with the proliferation of LPS-treated pulp cells through p38 MAPK. It is also known that p38 MAPK is activated by cytotoxic and severe stress. In the present study, pulp cells were exposed to LPS in the medium with 1% FCS to minimize the effects of the serum. In this severe condition, LPS induced the proliferation of pulp cells through p38 MAPK activation, suggesting that p38 MAPK may play as a cytoprotective factor in the LPS-induced pulp cell survival. When LPS-treated pulp cells were exposed to short-term heat stress, the phosphorylation of HSF1 and HSP27, as well as the expression of HSP27, were suppressed by SB203580, suggesting that the activation of p38 MAPK is essential for the induction and activation of HSF1 and HSP27 during short-term heat stress. In contrast, with long-term heat stress, the phosphorylation of HSF1 and expression of HSP27 were induced even in the presence of the p38 MAPK inhibitor. These results suggest that HSF1 and HSP27 may associate with the pulp cell survival through LPS- and p38 MAPK-independent signaling pathway after long-term heat stress.

We also examined the roles of cell survival factors, Akt and NF-κB/IκBα in the thermoresistance of pulp cells. It is known that the phosphorylation of Thr-308 is essential for the activation of Akt, and the full activation of Akt requires phosphorylation of Ser-473 [Alessi et al., 1996]. In LPS-treated pulp cells, the phosphorylation of Akt at Thr-308 was induced by LPS and Ser-473 of Akt was immediately phosphorylated by short-term heat stress, suggesting that LPS modifies Akt by the phosphorylation of Thr-308 to accelerate the immediate activation of Akt by the phosphorylation of Ser-473, resulting in pulp cell thermoresistance against short-term heat stress. Our findings also indicated that Akt was fully activated at the same time as the phosphorylation of HSP27 in LPS-treated pulp cells. It has been indicated that Akt activation is important for the activation of HSP27 [Rane et al., 2003]. LPS may promote the activation of HSP27 through the activation of Akt during short-term heat stress. In long-term heat stress, expression and

activation of Akt were induced independently of pre-exposure to LPS, suggesting that LPS may not affect on the pulp cells survival in post-period after long-term heat stress.

The phosphorylation of IκBα at Ser-32 and expression of IκBα were induced by LPS and heat stress. It is known that NF-κB/IκBα plays cytoprotective and cytotoxic roles in the interaction between heat stress and inflammatory responses, and that the phosphorylation of IκBα at Ser-32 is essential for the release of the active NF-κB [Finco et al., 1994: DeMeester et al., 1997]. Our results suggest that LPS simultaneously induces cytoprotective and cytotoxic roles of NF-κB/IκBα, and the phosphorylation level of IκBα may regulate the function of NF-κB in LPS-treated pulp cells during heat stress. The transient decrease of IκBα by short-term heat stress may suggest the inhibitory effect of heat stress on LPS signaling to NF-κB/IκBα. Also, phosphorylation and expression of IκBα in the cells with or without LPS during long-term heat stress suggest that LPS-independent signaling may play a role in the cell fate decision after long-term heat stress.

Taken together, our results demonstrate unique effects of LPS on the thermoresistance of dental pulp cells through the modification and the activation of HSF1, HSP27, Akt, and NF-κB/IκBα (Fig. 5). The cytoprotective mechanism for the thermoresistance of LPS-treated pulp cells depends on the term of heat stress. LPS appears to mainly have effects on the cytoprotection of pulp cells against short-term heat stress. In long-term heat stress, major cytoprotective signaling may be switched from LPS- to heat stress-dependent pathway. Also, the pulp cell fate after sequential exposure to LPS and heat stress may depend on the balance of the induction and activation of HSF1, HSP27, Akt, and NF-κB/IκBα. It has been reported that Akt and HSP27 contribute to the phosphorylation and the degradation of IκBα

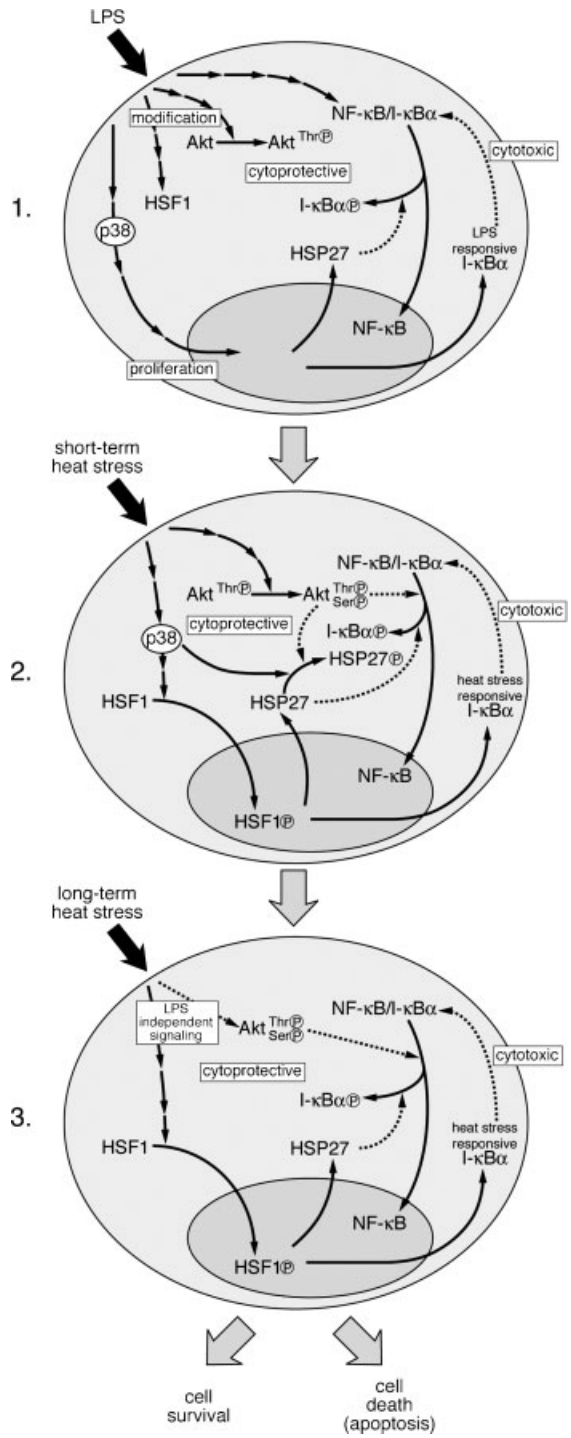

Fig. 5. Mechanistic scheme about the effects of sequential exposure to LPS and heat stress. **1**. LPS modifies Akt and HSF1, and activates NF-κB/IκBα signaling pathways (cytoprotective), as well as induces IκBα (cytotoxic). LPS also induces pulp cell proliferation through p38 MAPK. **2**. In LPS-treated cells, short-term heat stress immediately induces the activation of HSF1 and HSP27 through p38 MAPK, as well as the full activation of Akt (cytoprotective). Full-activated Akt may associate with the phosphorylation of HSP27 during short-term heat stress. NF-κB/IκBα signaling pathway of LPS-treated cells is also activated by short-term heat stress. **3.** Long-term heat stress induces the activation of HSF1 and expression of HSP27 through p38 MAPK independent signaling pathway (cytoprotective). NF-κB/IκBα signaling pathway is intensely activated by long-term heat stress (cytoprotective), as well as the induction of IκBα (cytotoxic). Induced HSP27 and active Akt may associate with the phosphorylation and degradation of IκBα, and the pulp cell fate may depend on the balance of these molecules induced by long-term heat stress.

[Das et al., 2003; Parcellier et al., 2003]. Activated Akt increases the activity of IκBα kinase, resulting in the phosphorylation of IκBα, and HSP27 maintains NF-κB activity by promoting the proteasome-dependent degradation of IκBα. It is also known that heat shock responsive element is found in the promoter of IκBα, and induced IκBα by heat stress suppresses NF-κB DNA binding activity, resulting in the cytotoxic effect [Wong et al., 1997]. The interaction of these molecules may modify the paradoxical ability of NF-κB/IκBα, which may leads to the heat shock paradox, and result in the pulp cell fate to survival or cell death during sequential exposure to LPS and heat stress.

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