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Heat stress induces alkaline phosphatase activity and heat shock protein 25 expression in cultured pulp cells

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Abstract

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Aim To investigate the responses of cultured rat pulp cells to heat stress.

Methodology Pulp cells were obtained from rat incisors and cultured at 37 °C. The cells were cultured at 42 °C for 30 min and then cultured at 37 °C again. Morphology, alkaline phosphatase (ALP) activity and expression of heat shock protein 25 (HSP25) were investigated at 0, 1, 3, 5, 7, 10 and 14 days following stimulation. As a control, the cells were maintained at 37 °C.

Results Although there were few cells of apoptosis immediately after heat stress, there were mitotic cells from day 1 after heat stress. ALP activity in the heat stress group significantly increased at days 7 and 14 compared with the control group (about 1.7-fold, $P < 0.01$, Friedman test). HSP25 expression increased in both groups, with HSP25 in the heat stress group being expressed earlier than in the control group, and nuclear localization of HSP25 was observed at days 0 and 1 in heat-stressed cells.

Conclusion These results suggest that heat stress not only induces HSP25 but also enhances ALP activity in pulp cells.

Keywords: alkaline phosphatase activity, dental pulp, heat shock protein 25, heat stress.

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Introduction

Dental caries and various environmental stimuli such as mechanical, physical or chemical injuries can cause irritation in the dentine/pulp complex. In a clinical setting, heat is often produced during cavity preparation or laser irradiation. As a result, the temperature of the pulp rises. Heat stress modulates the degradation of numerous proteins and is known to induce death signals that lead to apoptosis (Schlesinger 1990, Sorger 1991). Furthermore, heat stress resulting from treatment of carious teeth is believed to be one of the major causes of damage to dental pulp tissue. Water spray during cavity preparation is therefore necessary to protect the pulp from substantial injury (Ohshima *et al.* 2003).

On the other hand, the heat shock response is the protective reaction of cells to a variety of environmental and pathological stimuli (Lindquist & Craig 1988). The most obvious feature of this response is that it leads to an increase in synthesis of heat shock proteins (HSPs) (Schlesinger 1990, Sorger 1991). Some studies have found that the pulp is capable of surviving various injuries, including those arising from heat stress (Kitamura *et al.* 2005, Amano *et al.* 2006) or hypoxia (Amemiya *et al.* 2003), suggesting that pulp cells have a recovery system to deal with environmental or pathological stresses. Amano *et al.* (2006) investigating the viability of pulp cells for up to 6 h following application of heat stimuli, and reported that they exhibited thermo-tolerance and that HSP restored their viability. On the other hand, Kitamura *et al.* (2005)

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noted that heat stress elicited apoptosis and phagocytosis in pulp cells. However, little information is available on the morphological or functional variation in pulp cells after direct heat stimulation.

In the present laboratory study, the morphological changes, alkaline phosphatase (ALP) activity and expression of HSP25 in rat dental pulp cells for 14 days following application of heat stress were investigated.

Materials and methods

Cell culture

All experiments were carried out according to the Guidelines for the Treatment of Animals established by Tokyo Dental College. Dental pulp cells were obtained from the incisors of young Sprague–Dawley rats ($n = 20$) weighing between 100 and 150 g each. Incisors were extracted under general anaesthesia and pulp tissue was cultured according to the method of Inoue *et al.* (1992), using the middle portion of the pulp to avoid odontogenic epithelium and tooth germ contamination. The cells were cultured for 4 weeks prior to the experiment in minimum essential medium (Invitrogen, Grand Island, NY, USA) containing 15% foetal bovine serum at 37 °C in a humidified incubator containing 5% CO_2. The cells were trypsinized and maintained for two to three passages.

Experimental design

The experimental design was similar to that of Amano *et al.* (2006) with modification. The cells were seeded at 1×10^4 cells per well into 35-mm plates and maintained at 37 °C. Heat stimulation was then carried out at 42 °C for 30 min in an incubator. Next, the cells were re-incubated at 37 °C in another incubator (time point 0). Samples were then harvested at 0, 1, 3, 5, 7, 10 and 14 days following stimulation. As a control, nonheat stimulation was carried out and the pulp cells were maintained at 37 °C in a humidified incubator.

Morphological observation

The heat-stressed and control cells ($n = 8$) were fixed in 4% paraformaldehyde in 0.1 mol L^{-1} phosphate buffer (PB) solution for 2 h and permeabilized in methanol/acetone at −80 °C for 20 min. After washing three times in phosphate buffer saline (PBS) for 5 min each, the cells were incubated with Alexa568-conjugated phalloidin (dilution 1 : 20; Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After washing in PBS, the cells were incubated in 4′,6-diamino-2-phenylindole (DAPI, 10 ng mL^{-1}; Molecular Probes) for 5 min and observed under a fluorescent microscope (Axiophot 2; Carl Zeiss, Oberkochen, Germany).

ALP activity

Alkaline phosphatase activity was measured using a colorimetric assay kit using *p*-mitrophemy/phosphate (*p*-NPP) as a substrate (ALPopt; Roche Diagnostics, Tokyo, Japan) according to the manufacturer's protocol. Briefly, the cells ($n = 7$) were washed with PBS and homogenized in distilled water. The homogenate was centrifuged at 10 000 rpm ($18400 \times \boldsymbol{g}$) for 5 min, and the supernatants assayed. One millilitre of pre-mixed solution containing 10 mmol L^{-1} *p*-nitrophenol substrate was added to 10 μL of supernatant. Absorbance at 405 nm was measured at 1, 2 and 3 min using a spectrophotometer (Shimazu Co. Ltd, Tokyo, Japan) and average absorbance for each time point was calculated.

Western blot

In order to examine HSP25 expression, Western blot was carried out according to the method of Amemiya *et al.* (2003). Cells were lysed in radio-immunoprecipitation assay buffer [1% Nonidet P-40, 150 mmol L^{-1} NaCl, 50 mmol L^{-1} Tris (pH = 7.4)] containing inhibitors ($n = 5$). Twenty-five micrograms of total lysate was subjected to 7.5% SDS-PAGE, and then transferred onto a polyvimylideme difluoride (PVDF) membrane (BioRad, Melvilla, NY, USA). The membranes were then incubated overnight with anti-HSP25 antibody (StressGen Biotechnologies, Victoria, BC, Canada; dilution 1 : 10 000) and anti-actin antibody (Sigma-Aldrich, St Louis, MO, USA; dilution 1 : 2500) at 4 °C. After washing, the membranes were incubated with horseradish peroxidase-conjugated rabbit IgG (1 : 1000; Amersham, Rochester, MI, USA) at room temperature for 1 h. Immunoreactive bands were detected using the ECL™ Western blot analysis system (Amersham).

Immunofluorescence

The heat-stressed and control cells ($n = 8$) were fixed in 4% paraformaldehyde in 0.1 mol L^{-1} PB solution

for 2 h and permeabilized in methanol/acetone at −80 °C for 20 min. After washing three times in PBS for 5 min each, nonspecific binding was blocked with 10% normal goat serum for 30 min at room temperature. The cells were then incubated with anti-HSP25 antibody (dilution 1 : 1000) for 1 h at room temperature. After incubation with the primary antibody, they were washed three times in PBS for 5 min each and then incubated with Alexa488-conjugated anti-rabbit antibody (dilution 1 : 200; Molecular Probes) for 1 h at room temperature. Specimens were observed under a fluorescent microscope (Axiophot 2; Carl Zeiss). As a negative control, immunofluorescence was also carried out without primary antibody.

Statistical analysis

Statistical significance of multiple comparisons was evaluated using the Friedman test. Statistical significance was set at $P < 0.01$.

Results

Morphological observation

Cultured pulp cells showed short processes and weak discontinuous actin filaments that were stained with phalloidin (red) immediately after heat stimulation (Fig. 1a), whilst control cells had long processes and strong continuous actin filaments (Fig. 1b). Rarely nuclear that was stained with DAPI (blue) showed fragmentation indicating signs of apoptosis immediately after heat stimulation (Fig. 1a). From day 1 after heat stress, thin elongated processes and strong actin filaments appeared in both heat-treated and control pulp cells (Fig. 1c,d), and mitotic finding became frequent in heat-treated cells (Fig. 1c).

ALP activity

The results revealed statistically significant differences (about 1.7-fold) in ALP activity at 7 and 14 days

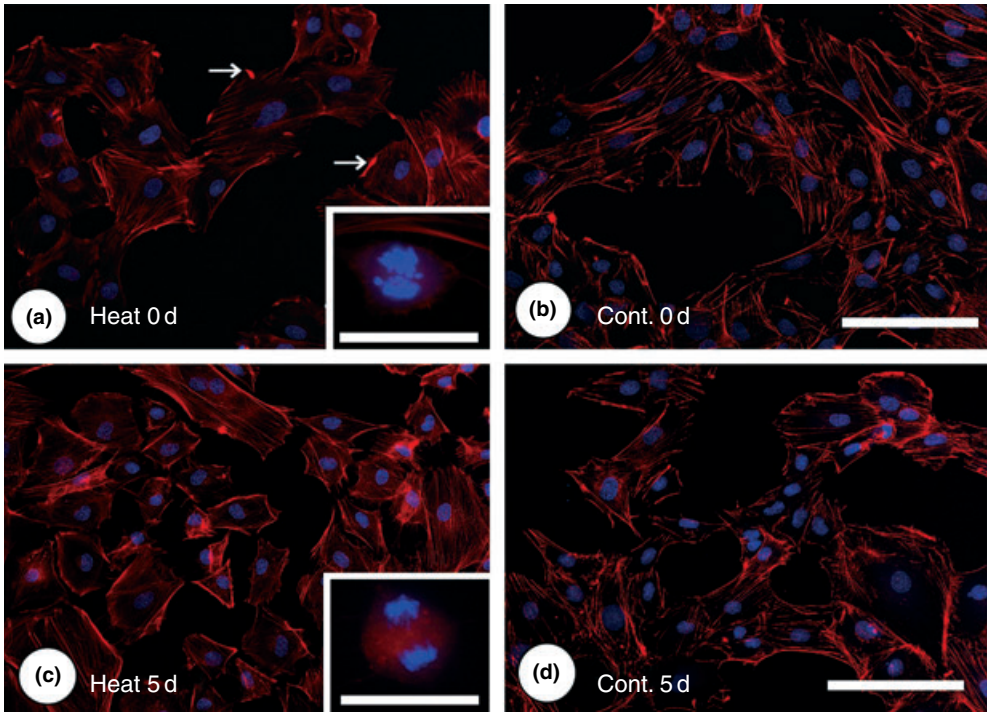

Figure 1 Morphology of heat-stressed and control cells: heat-treated pulp cells show short processes and weak spotty discontinuous actin filaments (arrows) immediately after heat stimulation (a). Nuclear fragmentation indicating apoptotic finding is rarely seen in heat-stressed cells (a, inset). Thin elongated processes and strong actin filaments are observed (c), along with frequent signs of mitosis (c, inset) at day 5 after heat stress. Control (non-heat-stressed) cells at days 0 and 5 are shown in (b) and (d), respectively. (a, c: bars = 50 μm, b, d: bars = 100 μm).

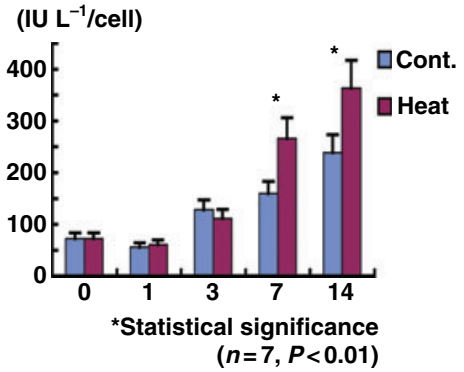

Figure 2 Alkaline phosphatase (ALP) activity: ALP activity shows clear differences (about 1.7-fold) at days 7 and 14 between heat-stressed and nonstressed (control) groups. Statistically significant differences were observed at days 7 and 14 between heat-stressed and control groups ($P < 0.01$).

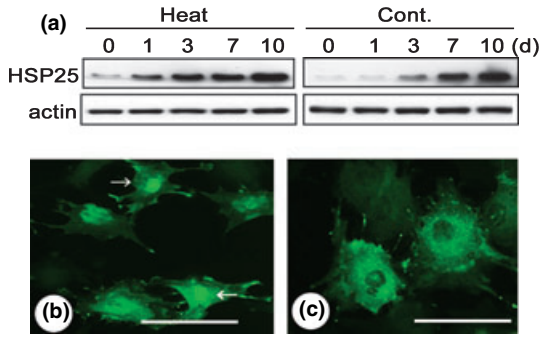

Figure 3 Expression of heat shock protein 25 (HSP25): (a) HSP25 expression increased over time in both heat-stressed and control groups according to Western blot analysis. Drastic change is seen in heat-stressed group. Higher expression of HSP25 was seen at days 1 and 3 in heat-stressed group than in control group. No change is seen between days 0 and 1 in control group. (b) Immunofluorescent analysis reveals nuclear localization of HSP25 (arrows) at day 1 in heat-stressed cells. (c) No nuclear localization is seen at day 5 in control (nonstressed) group. (b: bar = 60 μm, c: bar = 30 μm).

between the heat-stressed and nonstressed groups ($P < 0.01$), but no significant differences between 1 and 3 days (Fig. 2).

Expression of HSP25

Western blot analysis revealed that HSP25 was expressed in the pulp cells at all time points. HSP25 expression increased over time in both the heat-stressed group and the control group. Whilst no change was seen between days 0 and 1 in the control group, a drastic change was seen in the heat-stressed group. Higher expression of HSP25 was seen at days 1 and 3 in the heat-stressed group than in the control group (Fig. 3a).

Immunofluorescent analysis revealed nuclear localization of HSP25 at days 0 and 1 in heat-stressed cells (Fig. 3b), whereas the nonstressed group had no nuclear localization (Fig. 3c). The number of the cells that revealed nuclear localization of HSP25 decreased daily in the heat-stressed group.

Discussion

Heat stress modulates the degradation of numerous proteins (Lindquist & Craig 1988, Schlesinger 1990, Sorger 1991). Heat stress has been found to induce morphological change, including apoptosis in pulp cells (Kitamura *et al.* 2005). There may be important links between the heat shock response and mechanisms of protein folding and degradation. HSPs are believed to function in the protection and recovery of cells from environmental or pathological stress (Schlesinger 1990, Sorger 1991). HSP25 has been found to exert an anti-apoptotic effect (Schlesinger 1990, Mehlen *et al.* 1997) and promote cell survival (Lavoie *et al.* 1993). Expression of HSP25 has been addressed in pulp cells under stressful conditions including heat and cavity preparation (Ohshima *et al.* 2001, Ohshima *et al.* 2003, Kitamura *et al.* 2005, Amano *et al.* 2006). Apoptotic findings were rarely observed immediately after heat stress and disappeared from day 1 onwards in this study. Furthermore, HSP25 was expressed earlier in the heat-stressed group than in the control group in the present study. This indicates that earlier expression of HSP25 after heat stress is associated with protection and recovery of cells from environmental or pathological stress in cultured dental pulp.

Alkaline phosphatase is an enzyme expressed in the early stage of mineralization and is a marker of pulp cell viability (Inoue *et al.* 1992). However, the effect of heat stimulation on mineralization is not evident in dental pulp cells *in vitro*. Although the effect of heat on pulp cells from 1 to 6 h was investigated (Amano *et al.* 2006), no studies have investigated changes occurring up to 14 days. One report found that mild heat shock induced ALP activity and mineralization in bone marrow stromal cells (Shui & Scutt 2001). Spoto *et al.* (2001) investigated ALP activity in normal and inflamed dental pulps and reported that the tissue with reversible pulpitis exhibited higher ALP activity than normal pulp tissues. In the present study, the heat-stressed group gave higher ALP activity than the control

group. This implies that heat enhances ALP activity. The results, taken together with those of previous reports, suggest that heat stimulation enhances viability, including ALP activity, in dental pulp cells.

In this study, localization of HSP25 in the cell nuclei after heat stress but cytoplasmic localization under nonstressed conditions was found. Adhikari *et al.* (2004) investigated heat-stress-induced localization of HSPs in mouse myoblasts and also observed nuclear localization of HSPs in stressed cells. However, little is known about nuclear translocation as a response to stress. Such translocation from the cytoplasm to the nucleus implies a functional role for HSPs, including in the maintenance of stressed cells.

Conclusion

(i) Heat stress induced higher ALP activity in pulp cells and (ii) heat stress induced earlier expression and nuclear translocation of HSP25. These results suggest that heat stress not only induces HSP25 expression but also enhances ALP activity in pulp cells, and that earlier expression and nuclear location of HSP25 may play a role in the damage recovery response of pulp cells to heat stress.

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