Heat shock induces the synthesis of the inflammatory mediator leukotriene B_4 in human pulp cells

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Abstract

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Aim To measure the synthesis of leukotriene B_4 (LTB_4) in cultures of human dental pulp cells induced by heat shock.

Methodology Primary pulp cells (PC) and dental pulp stem cells (DPSC) were cultivated under appropriate conditions. For the characterization of PC the expression of dentine sialophosphoprotein (DSPP) was evaluated by reverse transcription-polymerase chain reaction. Thermal stimulation of cell cultures was performed at temperatures of 37, 38, 39, 40, 42 and 45 °C for stimulation times of 5 and 30 s. LTB_4 was quantified by reversed-phase high-performance chromatography and differences between the LTB_4 concentrations of controls and heat stimulated cells were analysed with Friedman analysis of variances by ranks and multiple comparisons ($P < 0.05$).

Results Both cell cultures expressed DSPP under the conditions of the present experiment. The analysis revealed significantly enhanced LTB_4 synthesis following thermal stimulations at 38, 39, 40, 42 and 45 °C compared with unstimulated controls for both PC and DPSC.

Conclusion The present study demonstrated the capability of pulp cells to synthesize the arachidonic acid mediator LTB_4 in response to heat shock. LTB_4 has the capacity to induce inflammatory reactions and to sensitise afferent nociceptive nerve endings. LTB_4 synthesis is induced by minor temperature changes, which are relevant for various clinical situations.

Keywords: arachidonic acid, heat shock, leukotriene, pulp cells.

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Introduction

Various treatments in dental practice lead to a rise in temperature within the dental hard and soft tissues. Initial research in this field was related to the elevated temperatures in pulp tissue because of the effects of rotary instruments on tooth tissues (Stüben & Hoppe 1964). In modern dental practice increased pulpal temperatures induced by rotary instruments are not considered harmful if adequate water-cooling is provided. However, numerous procedures have evolved that call attention to research related to thermal effects in pulp tissues. In fact, recent studies have reported a substantial increase in the temperature of pulp tissues by light polymerization (Hannig & Bott 1999, Yap *et al.* 2004), the intraoral placement of temporary crowns (Moulding & Teplitsky 1990), in combination with laser therapy (Oelgiesser *et al.* 2003) and after bleaching procedures (Baik *et al.* 2001).

The temperature within pulp tissues varied between 36.5 and 45 °C depending on the dental application used (Moulding & Teplitsky 1990, Hannig & Bott 1999,

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Oelgiesser *et al.* 2003, Ozturk *et al.* 2004). Temperature changes in pulp tissue may induce inflammatory reactions, hypersensitivity or even lead to pulpal necrosis requiring root canal treatment (Zach & Cohen 1965). However, the extent of thermal injury that can be tolerated by the dental pulp and the mechanisms of injury are unknown (Baldissara *et al.* 1997, Stewardson *et al.* 2004). Most studies evaluate the changes within pulp tissues caused by thermal stimulation using histological techniques (Zach & Cohen 1965, Klaiber *et al.* 1985, Ozturk *et al.* 2004). These studies could only demonstrate visible changes of the cells but were not designed to reveal early inflammatory or metabolic changes within them. For the evaluation of cellular changes that could be induced by heat shock the evaluation of inflammatory mediators synthesized by cells of pulpal origin might be a valuable tool. Increased levels of such mediators in human tissues may cause humoral or cellular inflammatory reactions leading to cellular necrosis and may be accompanied by common clinical symptoms and therapeutic consequences. Leukotriene B_4 (LTB_4) for example is a potent proinflammatory mediator of the arachidonic acid cascade. Through its high chemotactic capacity to polymorphonuclear granulocytes (Ford-Hutchinson *et al.* 1980, Mensing & Czarnetzki 1984, Czarnetzki & Mertensmeier 1985) and its capacity to increase the permeability of blood vessels (Higgs *et al.* 1981) LTB_4 is involved in early inflammatory reactions. For a review of the multifaceted role of eiconasoide mediators in inflammation and its sequelae such as pain, swelling and bone resorption and their possible role in the pathogenesis of pulpal and periapical diseases see the reviews by Torabinejad *et al.* (1991) and Funk (2001).

The aim of the present study was to measure the synthesis of the arachidonic acid mediator LTB_4 induced by heat shock in cultures of human dental pulp cells (PC).

Materials and methods

Pulp cell cultures

Primary PC were cultivated from six healthy third molars that had been removed during routine oral surgery procedures. The teeth had been extracted for orthodontic reasons and were obtained from patients (age 18–32 years) who consented to their use for research. The hard tissues were removed and the pulp tissues were cultivated in medium (Alpha medium; Biochrom, Berlin, Germany) substituted with 10% foetal calf serum (FCS). The cells were grown at 37 °C in an atmosphere of 5% carbon dioxide. Subcultures of the cells were harvested and the cells were used between the second and fifth passage for stimulation experiments.

Dental pulp stem cells (DPSC) were kindly provided by Dr S. Shi, Bethesda, MA, USA (Gronthos *et al.* 2000). The cells were cultivated under the appropriate conditions (37 °C, 5% CO_2) in Alpha-Eagle medium substituted with 20% FCS, glutamine (10 μL mL^{-1}), penicillin (10 μg mL^{-1}), amphotericin B (10 mg mL^{-1}) and ascorbic acid (30 μg mL^{-1}). The cells were subcultivated not more than five times before stimulation.

Expression of DSPP

For the characterization of the PC under study the expression of dentine sialophosphoprotein (DSPP) was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) methods with the appropriate primers and conditions: 5′-TCA CAA GGG AGA AGG GAA TGG-3′ (forward), 5′-CTT GGA CAA CAG CGA CAT CCT-3′ (reverse); 95 °C per 5 min, 95 °C per 30 s, 63 °C per 30 s, 72 °C per 30 s, 44×, using TaqPolymerase III (Invitrogen, Karlsruhe, Germany). The mRNA was extracted from the cells using a commercially available kit (Rnase mini kit; Qiagen, Hilden, Germany) and cDNA was synthesized by reverse transcription (Superscript III; Invitrogen). A 370 bp fragment of β-actin: 5′-CAT-GGATGATGATATCGCCGCG-3′ (forward), 5′-ACA-TGATCTGGGTCATCTTCTCG-3′ (reverse); 95 °C per 5 min, 95 °C per 15 s, 63 °C per 30 s, 72 °C per 1 min, 30×, was used as an internal standard. Cloned fragments of DSPP and β-actin were used as positive controls. Water served as negative control. The PCR products were separated on a 2% agarose gel.

Thermal stimulation

Thermal stimulation of the cell cultures was performed at temperatures of 37, 38, 39, 40, 42 and 45 °C for stimulation times of 5 and 30 s. For control purposes the cells were cultivated at 36 °C. Each experiment was repeated four times. The experimental setup included pre-heated water baths for a temperature constant environment of the cell culture wells as well as for the medium that was used for thermal stimulation. The temperature was controlled by a digital thermometer with a precision of 0.1 °C. Numerous preliminary experiments demonstrated that the experimental conditions were able to be maintained at a pre-selected temperature within the cell culture chambers with a

variation of ±0.2 °C over a period of up to 30 s. After thermal stimulation the cells were cultivated for 2 h at 37 °C in an atmosphere of 5% CO_2. Subsequently, the cells were incubated for 5 min at 37 °C with arachidonic acid at an end concentration of 1.5 μmol L^{-1}. After this step the cells were washed twice with phosphate buffered saline, harvested from the culture wells and then frozen at −80 °C until further preparation. Each stimulation was accompanied by cell counting procedures in a Neubauer cell counting chamber.

RP-HPLC analysis of LTB_4

Eicosanoids were extracted from cells, separated and quantified by reversed-phase high-performance chromatography (RP-HPLC) as described by Eberhard *et al.* (2000). After homogenization, the reaction mixture was centrifuged at 1500 *g* at 0 °C for 10 min. The lipids were extracted by addition of 200 μL ice-cold methanol and stored for 10 min at −20 °C. The reaction mixture was centrifuged at 1500 *g* at 0 °C for 10 min and 200 μL of the supernatants were acidified with trifluoracetic acid and loaded to a RP-HPLC system as reported previously. Briefly, the HPLC consisted of a tertiary gradient system equipped with a photodiode array detector (Gynkotek, Germering, Germany). Chromatography was performed at room temperature and the flow rate was 0.5 mL min^{-1}. RP-HPLC was carried out with an analytical column (125.0 × 4.6 mm) of HyPURITY™ Elite C18 (5 μm particle size). A guard column (20 × 4.5 mm) of the same material HyPURITY™ Elite-HCT Guards C18 (5 μm particle size, ThermoHypersil, Cheshire, UK) was used. Two continually degassed solvents were used: (i) 0.002% (v/v) trifluoroacetic acid (TFA) in 25% water and 75% acetonitrile (v/v) and (ii) 0.002% (v/v) TFA in 7.5% water, 38.5% acetonitrile and 54% methanol (v/v). The eluate was monitored for LTB_4 at 280 nm absorption.

For calibration, standards of LTB_4 were diluted in 0.002% (v/v) TFA, in 25% water, and 75% acetonitrile and analysed by RP-HPLC. Their purity was verified by HPLC (>95% of dry weight). Serial dilutions of LTB_4 between 1 and 250 pg were analysed as external standards to demonstrate their linear responses and recoveries.

Analysis of data

For descriptive statistics the mean concentrations of LTB_4 measured by HPLC were calculated to 10^6 cells and were presented as bar graphs. Obvious extreme values or outliers were not observed. Nevertheless, normal distribution was not assumed in the data for statistical testing, because for four repeats in each experimental group any test for normal distribution has only small power. Differences between the LTB_4 concentrations of controls and heat stimulated cells were analysed with Friedman analysis of variances by ranks and multiple comparisons. All calculations were performed with the statistical program SPSS 11 (SPSS, Munich, Germany). Effects were regarded as statistically significant for $P < 0.05$.

Results

Detection of DSPP

Figure 1 displays the RT-PCR products with the specified primers for DSPP and β-actin separated on a 2% agarose gel. The gel indicated that both cell cultures expressed the odontoblast-specific marker under the conditions of the present experiment.

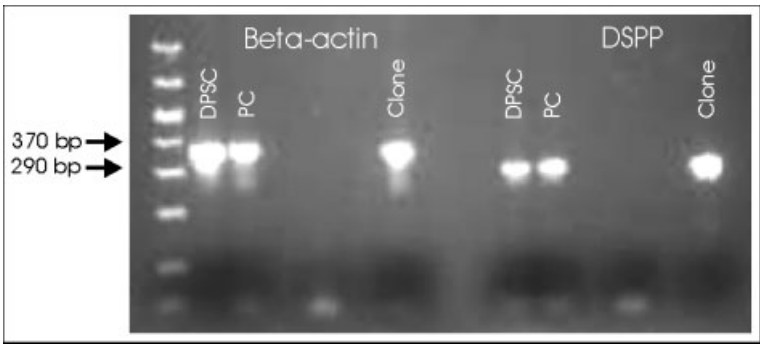

Figure 1 Dentine sialophosphoprotein (DSPP) mRNA expression by primary pulp cells (PC) and dental pulp stem cells (DSPC).

LTB_4 synthesis after thermal stimulation

At 36 °C primary PC cultures and DPSC produced basal amounts of LTB_4 (Figs 2 and 3). Stimulation of these cells with cell culture mediums of temperatures between 37 and 45 °C led to elevated LTB_4 levels within the cell cultures. The synthesis of LTB_4 reached a maximum between 39 and 40 °C and was reduced for temperatures of 42 and 45 °C. The statistical analysis by the Friedman test of variances by ranks revealed no statistically significant differences between a stimulation for 5 or 30 s for the primary PC ($P = 0.346$) and for the dentinal pulp stem cells ($P = 0.059$). These data were pooled for the statistical analysis of differences between the control treatment and stimulations by heat shock. Statistically significant differences between the control treatment and thermal stimulations with 38, 39, 40, 42 and 45 °C were observed for primary cell cultures as well as for the DPSC (Table 1).

Discussion

To our knowledge this is the first report on the synthesis of an inflammatory mediator by PC induced by heat shock. The present experiments provide quantitative data of an early cellular response induced by thermal stimuli that could not be generated by histological studies. These data provide information of cellular reactions in pulp tissues that could be induced by various dental treatments in dental practice.

The experimental setup established in the present study was suitable to stimulate PC in an accurate and reproducible fashion for time periods of 5–30 s, which represent time periods of thermal stimulation that

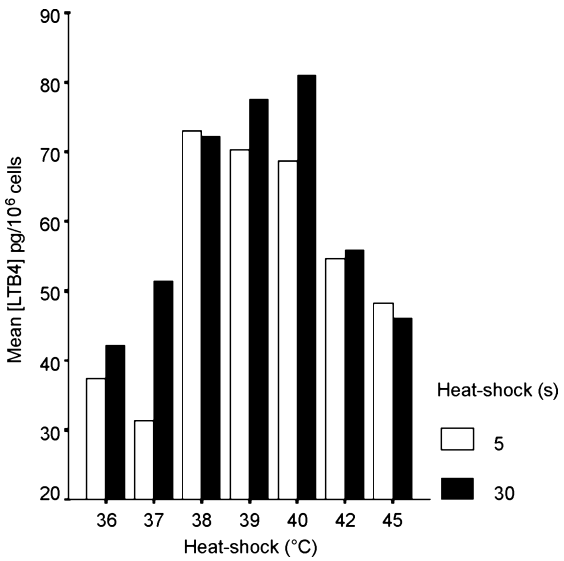

Figure 2 Leukotriene B_4 synthesis in cultures of human pulp cells after thermal stimulation with temperatures between 37 and 45 °C. Statistical analysis by the Friedman test revealed no significant differences between different stimulation times.

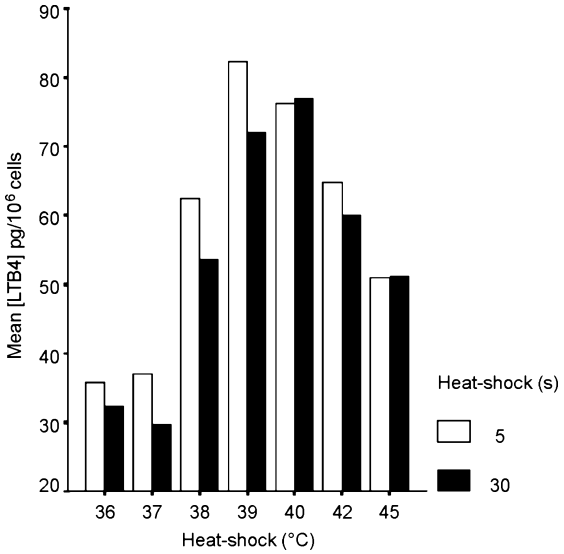

Figure 3 Leukotriene B_4 synthesis in cultures of dental pulp stem cells after thermal stimulation with temperatures between 37 and 45 °C. Statistical analysis by the Friedman test revealed no significant differences between different stimulation times. 150 × 153 mm (96 × 96 DPI).

Table 1 Concentrations of LTB4 synthesized by pulp cells measured by RP-HPLC

	Leukotriene B_4 (pg/10^6 cells)			
	Primary pulp cells		Dental pulp stem cells	
	Mean	SD	Mean	SD
Stimulatory temperature (°C)				
Control	39.8	6.1	34.0	6.2
37	41.4	11.3	33.4	6.1
38	72.7*	12.1	58.1*	6.8
39	74.0*	11.3	77.2*	13.3
40	75.0*	11.4	76.7*	2.4
42	55.3*	4.3	62.5*	9.4
45	47.2*	2.2	51.2*	5.9

*Significant differences between unstimulated controls and heat-shock stimulated pulp cells (Friedman analysis of variance followed by multiple comparisons $P < 0.05$)

could be experienced during clinical situations, for example by light curing. The temperatures within the dental pulp tissues that resulted from dental procedures ranged between 37 and 40 °C for hard tissue preparations (Cavalcanti *et al.* 2002, Ozturk *et al.* 2004), between 40 and 45 °C for light curing of composite materials (Hannig & Bott 1999), between 38 and 41 °C for laser irradiation (Oelgiesser *et al.* 2003) and accumulated to 44 °C for the manufacturing of temporary crowns (Moulding & Teplitsky 1990). These clinically relevant temperatures were used in the present study.

Primary PC as wells as DPSC were used for the heat shock experiments. The odontoblast-like character of the cells was confirmed by the mRNA expression of DSPP. It has been considered that DSPP is synthesized and secreted by odontoblasts and is found exclusively in the extracts of dentine extracellular matrix (Weinstock & Leblond 1973, Butler 1998). Recently, it has been demonstrated that DSPP was expressed in osteoblasts and bone as well (Qin *et al.* 2002), but at much lower levels in osteoblasts than in odontoblasts (Qin *et al.* 2003). Using qualitative RT-PCR we were not able to quantify the expression of DSPP mRNA but with respect to the growing conditions and used media it has to be assumed that the odontoblast-like cells were compounded with fibroblasts as well. By Northern blot analysis DPSC were found to be negative for DSPP, which is suggestive of an undifferentiated phenotype (Gronthos *et al.* 2000). The observed expression of DSPP mRNA in DPSC in the present study, leads to the conclusion that either differentiation processes have occurred during cultivation or that regulatory mechanisms governing the DSPP transcription are also involved. Long-term cultivation under the appropriate conditions demonstrated the differentiation potential of DPSC line that was also accompanied by the expression of DSPP in transplant experiments (Gronthos *et al.* 2000).

For the quantification of LTB_4 synthesis in PC an RP-HPLC approach was employed that had been used successfully in earlier studies (Eberhard *et al.* 2000). LTB_4 belongs to the arachidonic acid meditors of the 5-lipoxygenase cascade, which are related to the cyclo-oxygenase products prostaglandins. LTB_4 is a main product of granulocytes (Samuelsson 1972) and has been shown in keratinocytes (Black *et al.* 1985) and gingival cells (Eberhard *et al.* 2002). Okiji *et al.* (1992) demonstrated that inflamed pulpal tissues were capable of producing LTB_4 after stimulation with the calcium ionophore A23187. In gingival epithelial cells bacteria could increase the synthesis of LTB_4 and it could be assumed that LTB_4 synthesis may be an early defense mechanism, because of the high chemotactic activity of the mediator to immune cells (Ford-Hutchinson *et al.* 1980). In addition the capacity of LTB_4 to increase leukocyte adhesion to endothelial tissues also suggests that this mediator is active in early inflammatory reactions.

In the present study, it has been demonstrated that a heat shock of 38–45 °C significantly increased the synthesis of LTB_4 compared with unstimulated controls. Exogenous arachidonic acid is an inductor of LTB_4 synthesis and led to the observed LTB_4 synthesis in unstimulated cells. In agreement with the present findings it has been reported that even minor thermal injuries of 37 °C activated the human heat-shock factor expressed in Drosophila cells (Clos *et al.* 1993). Heat shock between 42 and 45 °C induced the synthesis of prostaglandins and LTB_4 in mammalian cells (Calderwood *et al.* 1989). In human polymorphonuclear leukocytes the activation of 5-lipoxygenase by heat shock of up to 45 °C for 15–45 min led to the formation of LTB_4 as well (Werz *et al.* 2002). These results clearly demonstrated that slight thermal changes are capable of stimulating PC and may induce inflammatory reactions and the infiltration of inflammatory cells in pulp tissues.

The question arises whether there is a biological role in addition to inflammatory events that can be attributed to the observed LTB_4 synthesis in PC. In fact, Martin *et al.* (1987) reported that LTB_4 sensitized cutaneous C- and A-delta mechanonociceptors in the hairy skin of rat hindlimbs. Madison *et al.* (1992) studied the effects of LTB_4 on the nerve activity from primary afferents innervating the dentine of canines in adult cats. They demonstrated that in teeth treated with LTB_4 the stimulus-evoked intradental nerve activity was significantly enhanced. These results provide evidence that LTB_4 is able to sensitize nociceptors and may be a long-lasting hyperalgesic factor, which may contribute to pain of pulpal origin. It can be suggested that this action of LTB_4 may be relevant to the observed synthesis of the mediator in PC induced by heat shock. The signalling pathways and receptor capacities of PC involved in the induction of LTB_4 by heat shock and in relation to clinical pain perception have yet to be studied.

Conclusions

The present study demonstrated the capability of PC to synthesize the arachidonic acid mediator LTB_4 in

response to heat shock. LTB_4 has the capacity to induce inflammatory reactions and to sensitize afferent nociceptive nerve endings. LTB_4 synthesis is induced by minor temperature changes, which are relevant for various clinical situations.

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