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International Journal of Psychophysiology 52 (2004) 211–216
INTERNATIONAL JOURNAL OF PSYCHOPHYSIOLOGY
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Short communication

Photo-acoustic stimulation increases the amount of 70 kDa heat shock protein (Hsp70) in human whole saliva. A pilot study

Tibor Károly Fábián[a], Zsuzsanna Tóth[a], László Fejérdy[a], Borbála Kaán[a], Péter Csermely[b,*], Pál Fejérdy[a]

[a]*Faculty of Dentistry, Semmelweis University, Clinic of Prosthetic Dentistry H-1088 Budapest, Mikszáth Kálmán square 5, Budapest, Hungary*
[b]*Faculty of Medicine, Institute of Medical Chemistry, Semmelweis University, H-1444, Budapest 8 P.O. Box 260, Hungary*

Received 19 June 2003; received in revised form 3 July 2003; accepted 22 October 2003

Abstract

Long-term photo-acoustic stimulation leads to changes in the composition of saliva, which may have a key contribution to the effectivity of this technique in easing mucosal symptoms of psychosomatic patients. In the present study a significant ($P \leq 0.01$) increase in salivary 70 kDa heat shock protein, Hsp70 was demonstrated in human whole saliva after repeated photo-acoustic stimulation. Increased salivary chaperones may contribute to the effectivity of photo-acoustic stimulation, because of their cytoprotective extracellular actions.

Keywords: Chaperone; Hsp70; Mucosal; Saliva; Extracellular; Photo-acoustic stimulation; Human

1. Introduction

Previous studies demonstrated the strong effect of short run photo-acoustic stimulation on the central nervous system. Berger (1930) demonstrated a decreasing effect of photic stimulation on the amplitude of alpha waves on EEG. Similarly, an occipital decrease of alpha density (average of alpha wave's amplitude) has been found by Kawabata (1972) using a 10 Hz frequency flash light stimulus or a confluent stimulus for 2 s on closed eyes. These effects have been called 'light on effects'. The same effect have been found by him when he turned the 2 s light off. This effect has been called as 'light off effect'. Aranibar and Pfurtscheller (1978) published results about the positive correlation between the 'on' effect and the intensity of the stimulus, and about the specific sensor modality of the 'on' and 'off' effects. These effects were more effective in the occipital region of the brain in the case of photic stimulation, and in the centric region of the brain in the case of the acoustic one. All above data indicate an effective stimulation of the central nervous system by a short photo-acoustic treatment. In contrast, long lasting photo-acoustic stimulation leads to sleepiness, and to a mixed alpha–theta activity of the

*Corresponding author. Tel.: +36-1-266-2755x4102; fax: +36-1-266-7480.
E-mail address: csermely@puskin.sote.hu (P. Csermely).

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doi:10.1016/j.ijpsycho.2003.10.004

EEG (Williams and West, 1975). Similar results were found by Brauchli (1993), who compared the effect of long lasting photo-acoustic stimulation with that of relaxing music. He found a decreased EMG activity, decreased salivary cortrisol level, and increased skin resistance as well as an increased salivary secretory IgA level, together with a subjective 'warm' and 'calm' feeling in both cases. In our previous study (Fábián et al., 2002) beside the relaxing effect, a strong trance inductive ability, and clinical effectivity of photo-acoustic stimulation during supportive psychotherapy of psycho-somatic patients with oral and oro-facial symptoms were demonstrated. This method seems to be more powerful than relaxation in connection with breathing in and out, which we used previously in the treatment of such patients (Fábián and Fábián, 1998).

The effectivity of the photo-acoustic stimulation during supportive psychotherapy of psycho-somatic patients with mucosal symptoms (i.e. oropyrosis) was also indicated in our previous study (Fábián et al., 2002). Beside the strong general relaxing effect of photo-acoustic stimulation, salivary changes may have a crucial role in the local effectivity of this technique related to mucosal symptoms (Brauchli, 1993; Fábián et al., 2002). An increased level of salivary secretoric IgA after photo-acoustic stimulation was reported (Brauchli, 1993). However, there is no data in the literature about other salivary proteins like mucins (Schwartz et al., 1995), or salivary chaperones (e.g. the 70 kDa heat shock protein, Hsp70) (Fábián et al., 2003), which may play a role in the mucosal cell protection as well.

The presence of a key molecular chaperone (Hsp70) in the human whole saliva was first demonstrated in our previous study (Fábián et al., 2003). Chaperones are usually considered to be intracellular, with a function to prevent protein aggregation, and to refold damaged other proteins. However, there is increasing evidence demonstrating the presence of chaperones outside the cell with cytokine-like effects and modulating various immune functions (Henderson et al., 1996; Srivastava, 2002). Exogenous Hsp70 administration protects arterial smooth muscle cells from toxic effects through association with the cell surface (Johnson and Tytell, 1993). Exogenous Hsp70 administration also lowers the number of apoptotic and necrotic human promonocytic cells treated with tumour necrosis factor-alpha through cell surface interaction followed by internalisation (Guzhova et al., 1998). Hsp70 was also found in the human blood sera (Rea et al., 2001; Wright et al., 2000), and the presence of some other chaperones (cpn10 and cpn60) was reported in the pancreatic juice (Velez-Granell et al., 1994). The above findings suggest that salivary chaperones (like Hsp70) may play a role in the mucosal cell protection of the oral cavity and upper gastrointestinal tract besides their immune modulating effects in the environments.

In the current study the effect of photo-acoustic stimulation on the amount of salivary Hsp70 (a possible mucosal defence protein, see above) in the human whole saliva was studied. Some other basic secretoric parameters, like salivary secretory rate, total protein and amylase output of the whole saliva were also measured.

2. Materials and methods

Six young healthy individuals (three women and three men; age between 19 and 28 years) took part in the study. The participants were not taking any medications or contraceptives. There were no detectable oral inflammations, periodontal inflammations, periodontal pockets, caries, or dental plaque in their mouths.

Only adult subjects took part in the study, voluntarily, knowing all of the important circumstances and parameters in the study. Sample collection was completely non-invasive. Our experiments were planned in compliance with the Helsinki Declaration of the World Medical Association (World Medical Association, 1994).

Ten minutes of mixed (8 Hz mean frequency) photic and acoustic stimulation was used two times for stimulation. Resting saliva was collected before, during and after the stimulation phases for 10 min as described previously (Fábián et al., 2003). The stimulation was carried out with the use of a signaller ('David paradise XL'; Comptronic devices Ltd, Edmonton, Alberta, Canada) through closed eyes, in sitting position as previ-

ously (Fábián et al., 2002). The experiments were repeated three times.

Whole saliva was collected with the method described by Schwartz et al. (1995). The participants were told to swallow first, and then allow the resting (or stimulated) saliva to accumulate in their mouth for 10 min, and finally to transfer it into a collecting vessel.

The samples were precleared by centrifugation (20 000×*g*, 4 °C, 10 min), and sterile filtered using Millex-GV 0.22 μm pore size filters (Millipore). Salivary volume was measured with a small measuring cylinder. Salivary protein concentration was determined by the Lowry method (Lowry et al., 1951; Bio-Rad). Amylase activity was determined with the Phadebas Amylase Test (Pharmacia and Upjohn).

For detection of Hsp70 a semi-dry Western blot analysis was used on nitro-cellulose membrane, with a primary antibody against the inducible form of Hsp70 (72 kDa) (StressGen), as described in our previous study (Fábián et al., 2003). Briefly: 20 μl of whole saliva was solubilized in Laemmli sample buffer (Laemmli, 1970), and separated on a 12.5% SDS-PAGE gel using a Bio-Rad slab mini-gel system. An amount of low-flow-hypoxic rat liver homogenate (Zhong et al., 1996) containing 9 mg proteins solubilized in Laemmli sample buffer was used as a positive control for Hsp70. A semi-dry Western blot analysis on nitro-cellulose membrane was used to detect Hsp70, with a primary antibody against the inducible (72 kDa) form of Hsp70 (StressGen). Hsp70 bands were detected with a horseradish peroxidase-linked secondary antibody against mouse IgG (Amersham), and with the ECL-Western blotting analysis system (Amersham). Three percent blotting grade blocker non-fat dry milk (Bio-Rad) was used for blocking after the primary antibody at 25 °C for 1 h. X-Ray photographs were scanned (Epson Perfection 1640SU) and Image Master TotalLab soft-ware (version 1.11) was used for quantitative analysis of the detected bands as in our previous study (Fábián et al., 2003).

For statistical evaluation the Student's *t*-test for two samples was used, with the use of the Statistical Package for the Social Sciences SPSS/PC version 8.0.

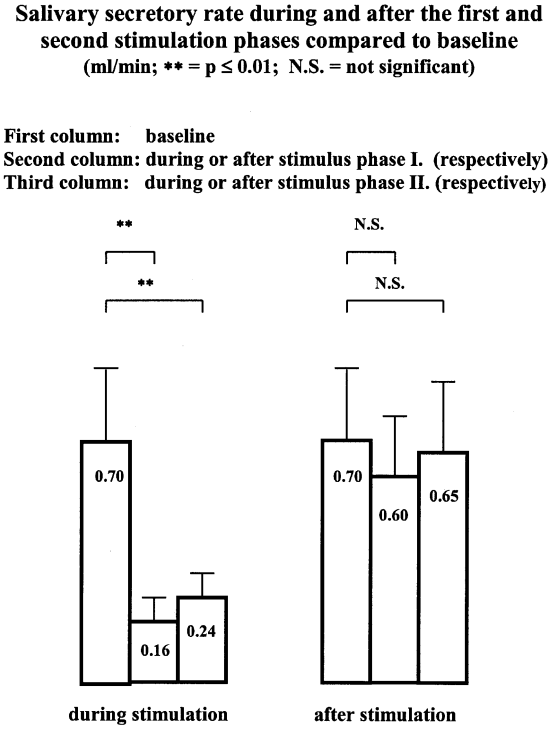

Fig. 1. The effect of repeated photo-acoustic stimulation on the secretory rate of human whole saliva (ml/min; $*P \leq 0.01$; N.S. = not significant).

3. Results

Photo-acoustic stimulation strongly decreased the secretory rate ($P \leq 0.01$) during the stimulation phases compared to baseline (starting control value), but secretory rate returned nearly to the baseline during the resting phases between and after the stimulation phases (Fig. 1).

As a consequence of the strong decrease ($P \leq 0.01$) of the secretory rate during the stimulation phases, there was a drastic decrease of total protein ($P \leq 0.01$) and amylase ($P \leq 0.01$) output in these phases (Fig. 2).

Interestingly, Hsp70 output changed differently. There was only a moderate decrease during the first ($P \leq 0.05$), and a non-significant decrease during the second stimulation phase if compared to baseline (Fig. 2) indicating a rather stable and independent excretion of Hsp70 if compared to

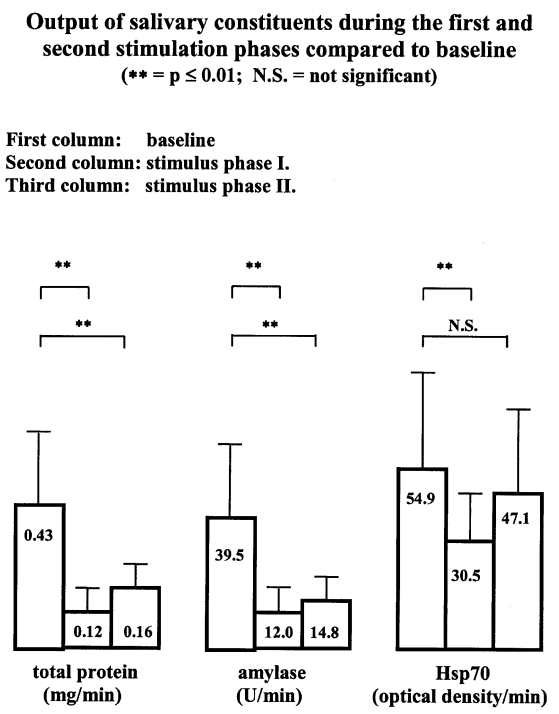

Fig. 2. Output of total protein, amylase and Hsp70 during the stimulation phases comparing to baseline (*$P \leq 0.05$; **$P \leq 0.01$; N.S. = not significant; OD = total optical density).

that of total protein and amylase, during the stimulation phases.

During the resting phases between and after the stimulation phases the secretory rate returned nearly to baseline as mentioned above (Fig. 1). In contrast, the output of salival constituents is increased (Fig. 3). The slight increase of total protein output is statistically not significant. However, the increase of amylase output proved to be significant in the second resting phase ($P \leq 0.05$) if compared to baseline (Fig. 3). Hsp70 output increased significantly ($P \leq 0.05$) already in the first resting phase, and it increased further ($P \leq 0.01$) in the second resting phase. The repeated stimulation seems to have a cumulative effect on all of the three constituents tested (Fig. 3).

4. Discussion

Our data confirm previous findings showing that photo-acoustic stimulation leads to significant changes of salivary secretion (Brauchli, 1993; Fábián et al., 2002). The most important new finding of the present study that photo-acoustic stimulation increases the secretion of salivary Hsp70, a possible factor of mucosal cell protection and immune modulation (Guzhova et al., 1998; Henderson et al., 1996; Johnson and Tytell, 1993; Rea et al., 2001; Srivastava, 2002, Velez-Granell et al., 1994; Wright et al., 2000).

The clinical consequences of these findings lie in the therapy of psychosomatic patients with mucosal symptoms. Beside the putative cytoprotective effects of salivary Hsp70, the increase of this salivary chaperone may lead to an enhanced mucosal immune defence, since molecular chaperones elicit a strong immune response when become accessible to immuncompetent effector cells (Multhoff and Botzler, 1998). Chaperones may protect and repair antibody and mucosal cell surface protein structures during the antibody cata-

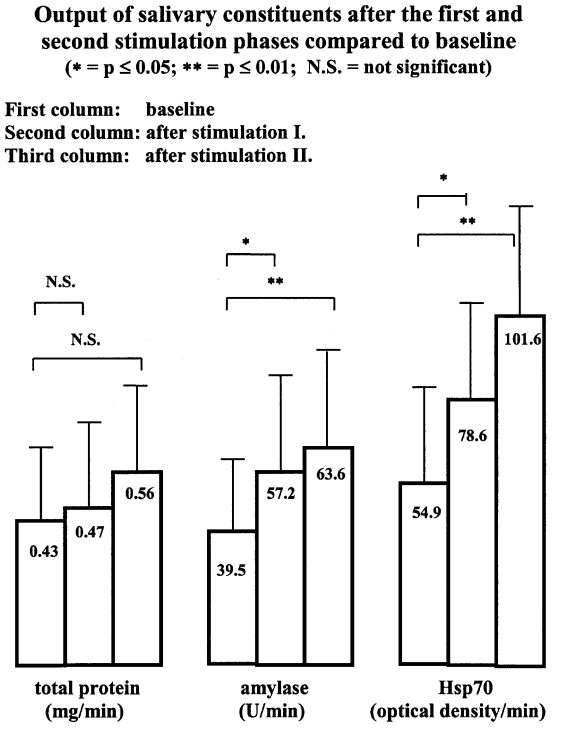

Fig. 3. Output of total protein, amylase and Hsp70 in the resting phases comparing to baseline (*$P \leq 0.05$; **$P \leq 0.01$; N.S. = not significant; OD = total optical density).

lysed ozone formation (Wentworth et al., 2002; Nathan, 2002), as well as during the proteotoxic immune response against bacteria. These effects may be highly important, since photo-acoustic stimulation increases not only Hsp70 but secretoric IgA level (Brauchli, 1993) of whole saliva, and ozone formation occurs regardless the source or antigen specificity of the antibody (Wentworth et al. 2002).

Beside the above important consequences, present data support our previous findings related to an indication of a rather independent transport of Hsp70 into the saliva. In our previous study (Fábián et al. 2003) we used a strong secretory stimulation (taste and chewing together), and the secretory parameters of Hsp70 showed a considerable independence from those of amylase (being the main secretory protein of salivary gland acinar cells), as well as a moderate independence from the secretory parameters of total salivary protein (a mixture containing proteins from acinar cells and from blood serum (Garret, 1981; Schwartz et al., 1995). In this study, using a rather different stimulation modality (photo-acoustic stimulation), we can see similar independence of Hsp70 excretion if compared to those of total protein or amylase especially during the stimulation phases (Fig. 2). Taken together these studies may indicate a rather independent transport of Hsp70 into the saliva.

In conclusion: our results suggest that photo-acoustic stimulation can lead to the increase of salivary Hsp70 output, a possible factor to preserve mucosal cell integrity and efficient immune response of the oral cavity and upper gastro-intestinal tract. Further investigations are needed to demonstrate the long run therapeutic effects of this technique related to Hsp70 concentration. A more detailed investigation is also needed to find out the mechanism of action of Hsp70 and possible other chaperones on mucosal cells and immune reactions as well as to elucidate the secretion mechanism leading to the appearance of Hsp70 (and possible other chaperones) in the saliva.

Acknowledgments

This work was supported by research grants from the Hungarian Science Foundation (OTKA-T37357), from the Hungarian Ministry of Social Welfare (ETT-32/03), from the International Centre for Genetic Engineering and Biotechnology (ICGEB, CRP/HUN 99-02), and from the EU 6th Framework program (FP6506850).

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