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Substance P receptor expression in healthy and inflamed human pulp tissue

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Abstract

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Aim To use radioreceptor analysis for comparing substance P (SP) receptor expression in human pulp tissue samples collected from teeth having a clinical diagnosis of acute irreversible pulpitis, healthy pulps and teeth with induced inflammation.

Methodology Five pulp samples were obtained from teeth having a clinical diagnosis of acute irreversible pulpitis. Another 10 pulp samples were obtained from healthy premolars where extraction was indicated for orthodontic purposes. In five of these premolars inflammation was induced prior to pulp collection. All of the samples were processed and labelled with ^{125}I-SP. Binding sites were identified by ^{125}I-SP and standard SP competition assays. Kruskal–Wallis and Mann–Whitney (*post-hoc*) tests were used to establish statistically significant differences between the groups.

Results Substance P receptor expression was found in all human pulp tissue samples. Most receptors were found in the group of pulps from teeth having a clinical diagnosis of acute irreversible pulpitis, followed by the group of pulps having induced inflammation. The least number of receptors was expressed in the group of healthy pulps. Statistical analysis revealed significant differences between the group of healthy pulp and both inflamed pulp groups ($P < 0.01$).

Conclusion Substance P receptor expression in human pulp tissue is significantly increased during inflammatory phenomena such as acute irreversible pulpitis.

Keywords: human dental pulp, neurogenic inflammation, substance P, radioreceptor assay.

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Introduction

Dental pulp inflammation is a complex process involving a variety of nervous and vascular reactions, which are key components of the neurogenic phenomenon that could lead to pulp necrosis (Byers *et al.* 1990, Kim 1990). Neuropeptides play an active role in homeostatic regulation under normal conditions and during neurogenic inflammation of the pulp, controlling its blood flow and regulating later stages of inflammation and repairing processes (Olgart 1996). These neuropeptides include substance P (SP), calcitonin gene-related peptide (CGRP), neurokinin A (NKA), vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) amongst others (Casasco *et al.* 1990, Wakisaka 1990).

Substance P is produced in trigeminal cell bodies and transported via axonal flow to nerve terminals in the pulp, where it is co-stored with other sensory neuropeptides (Wakisaka & Akai 1989). These nerve terminals are mainly C-type fibres, which closely follow pulp microcirculation. When stimulated, neuropeptides are released (Awawdeh *et al.* 2002).

Substance P interacts with a great variety of cells, including mastocytes, macrophages, lymphocytes and endothelial cells, inducing the release of inflammatory mediators such as histamine, cytokines, prostaglandins

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and thromboxanes, which causes an increase in vascular permeability and consequently, local blood flow and tissue pressure rise (Payan 1989, Hargreaves *et al.* 1994, O'Connor *et al.* 2004). It has been demonstrated that pulp SP levels are significantly higher in patients with acute irreversible pulpitis or in the presence of pulpal irritants, such as deep cavity preparation and high intensity thermal/chemical stimuli (Takamori 2000, Bowles *et al.* 2003, Caviedes-Bucheli *et al.* 2005a).

It has been suggested that SP also plays a significant role in the tissue homeostatic regulation by maintaining vascular tone, ensuring smooth flow and the consistent supply of nutrients to the tissue, regulating the interstitial pulpal pressure and stimulating growth of pulpal cells, such as fibroblasts and odontoblast-like cells (Trantor *et al.* 1995, Patel *et al.* 2003, Park *et al.* 2004).

Substance P exerts its biological actions by binding to a high-affinity G-protein-coupled receptor denoted NK1, located on most inflammatory cells, such as mast cells and macrophages, and on other connective-tissue cells (Takahashi *et al.* 1992). At higher SP concentrations, the neuropeptide can also interact with NK2 and NK3 receptors (Helke *et al.* 1990). G-protein-coupled receptor subunits have been identified in normal human dental pulp suggesting the presence of NK1 receptor (Pozo *et al.* 2000). However, such identification is not conclusive of the presence of SP receptor, as a variety of cell-surface receptors mediate their intracellular actions by a pathway that involves activation of G proteins (Strader *et al.* 1994).

Recent studies have shown the presence of NK1, NK2 and NK3 receptors in rat oral soft tissues, and in bone and dental hard tissue cells (Fristad *et al.* 2003). NK1 receptors have also been found in the mature dental pulp of rats (Fristad *et al.* 1999). However, there is no evidence of the behaviour of NK1 receptor expression during normal and inflamed stages in human dental pulp. This study was thus aimed at comparing SP receptor expression in human pulp tissue samples collected from teeth having a clinical diagnosis of acute irreversible pulpitis, healthy pulps and teeth with induced inflammation.

Materials and methods

A descriptive comparative study was performed according to the Colombian Ministry of Health recommendations regarding ethical issues in research with human tissues. Written informed consent was obtained from each patient in the study. Pulp samples were obtained from 15 different human adult donors (18–38 years old) of both sexes, non-smokers and systemically healthy, following previously described procedures (Caviedes-Bucheli *et al.* 2004a, 2005b).

Five pulp samples were obtained from posterior teeth having a clinical diagnosis of acute irreversible pulpitis. These patients were suffering moderate to severe spontaneous pain of approximately 24 h duration. None of them were taking anti-inflammatory drugs. Teeth were anaesthetized (1.8 mL of 4% prilocaine by infiltration injection in the maxillary teeth and by inferior alveolar nerve block injection for mandibular teeth) and isolated with a rubber dam. Shortly after, cavity access was completed and pulp tissue was extracted with a sterile barbed broach.

Another 10 pulp samples were obtained from premolars extracted for orthodontic purposes. In five of these teeth, inflammation was induced prior to pulp collection. The remainders were used to establish normal neuropeptides expression. For the induced inflammation group, teeth were anaesthetized and isolated as described before. The inflammatory process was generated by mechanical exposure of the pulp using a No. 1 round carbide bur in a high-speed handpiece without irrigation. After a period of 10 min, the pulp tissue was extracted using a sterile barbed broach.

For the group of healthy pulps, the teeth were anaesthetized and extracted. Immediately after extraction, teeth were washed with 5.25% sodium hypochlorite to eliminate remnants of periodontal ligament that could contaminate the pulp sample. The teeth were then sectioned using a cylindrical diamond bur in a high-speed handpiece irrigated with saline solution. Pulp tissue was obtained using a sterile endodontic excavator.

All of the pulp samples were placed into a Eppendorf tube with 4% paraformaldehyde and kept frozen at −70 °C until use. After all the samples had been obtained, they were unfrozen at room temperature, added 200 μL of 4% saccharose solution in Tris–HCl buffer with protease inhibitors (0.1% EDTA, 0.1% PMFS) and 0.01% bovine serum albumin (BSA). Each sample was disaggregated in an ice bath at 0 °C using a tissue homogenizer (Wheaton Dounce, Milville, NJ, USA).

Samples were then centrifuged at 500 *g* for 5 min at 4 °C (IEC Centra GP6/GP6R; Thermo Electron Corporation, Waltham, MA, USA); obtaining precipitate P1 and supernatant S1. Five hundred microlitres of 4% saccharose buffer were added to precipitate P1; the

spun was repeated, obtaining precipitate P2 and supernatant S2. Another 500 μL of 4% saccharose buffer were added to precipitate P2 which was then subjected to 10 s pulses (10% intensity) using a Branson Digital Sonifier S-450D (Branson Ultrasonic Corporation, Danbury, CT, USA). Each sample was centrifuged (IEC Centra) at 500 *g*, obtaining precipitate P3 and supernatant S3. The mixture of supernatants S1, S2 and S3 was centrifuged at 51 400 *g* for 3 h at 4 °C (RC5C Centrifuge; Sorvall, Wilmington, DE, USA). Six hundred microlitres of Tris–HCl buffer (0.05 mol L^{-1} and pH 7.5) were added to precipitate obtained.

A 50 μL aliquot of suspension from each disaggregated sample was mixed with 150 μL detergent solution (5% CHAPS). This mixture was vigorously shaken in a vortex agitator (IKA Werke GmbH & Co., Staufen, Germany) to solubilize the proteins present in the tissue suspension, they were then centrifuged (Sorvall) at 29 300 *g* for 3 min at 4 °C. Twenty microlitres of phosphate-buffered saline (pH 7.4) and 100 μL reagent were added to an aliquot containing 80 μL supernatant for protein quantification (MicroBCA Protein Assay Reagent Kit; Pierce, Rockford, IL, USA). After being homogenized, the mixture was incubated at 37 °C for 2 h. Aliquot absorbency for each sample was read on a spectrophotometer (MultiSkan; Thermo Electron Corporation, Waltham, MA, USA) at 570 nm. Each sample's protein concentration was calculated by interpolating the absorbency read on a standard calibration curve (absorbency *cf* BSA concentration) previously done in the same conditions as the assay. Determinations were done in duplicate.

Radioreceptor assay

Standard SP and ^{125}I-SP were obtained from Phoenix Peptide Pharmaceutical Laboratories (Ref. 061-05 and T061-10, Belmont, CA, USA). Standard SP was reconstituted with distilled water and serially diluted with HSA/Tris–HCl buffer solution to obtain different reagent concentrations. ^{125}I-SP was reconstituted with distilled water and diluted until there were 10 000 counts per minute (cpm) in 100 μL reagent.

Each cell suspension sample was submitted to competition assays with ^{125}I-SP in the absence (total binding) or presence (non-specific binding) of standard SP and left incubated for 12 h at 4 °C. The reaction mixture was then passed through a 60 : 40 dibutylphthalate–dioctylphthalate cushion ($d = 1.015$ g mL^{-1}) to separate bound from free SP (Suarez *et al.* 2001). After centrifuging (Sorvall) at 28 400 *g* for 3 min, peptide bound to the cells was quantified by measuring the pellet radioactivity on a Gamma counter (Gamma Assay LS 5500; Beckman, Fullerton, CA, USA).

Free peptide (F) was quantified by measuring the difference between added cpm and bound peptide (B) at each point. The maximum quantity of bound peptide was extrapolated from the Rosenthal graph: B/F *cf* B.

Statistical analysis

The values obtained are expressed in pmol of bound peptide per 100 μg tissue protein. The mean and standard deviation were calculated for each group. The Kruskal–Wallis test was performed to establish statistically significant differences in values obtained from the three groups ($P < 0.05$). Mann–Whitney's *post-hoc* tests were done for comparing differences between different groups.

Results

Substance P receptor expression was found in all human pulp tissue samples. Most receptors were found in the group of pulps from teeth having a clinical diagnosis of acute irreversible pulpitis, followed by the group of pulps having induced inflammation. The least number of receptors was expressed in the group of healthy pulps.

Table 1 shows values obtained and mean receptor expression for the three groups, with their respective standard deviations.

The Kruskal–Wallis test revealed statistically significant differences between the three groups ($P < 0.01$). Mann–Whitney's *post-hoc* comparisons showed that the greatest difference in the amount of receptors expressed was between pulps having a diagnosis of acute

Table 1 Substance P receptor expression in healthy and inflamed human pulp tissue

Sample	Healthy pulp[a]	Pulp with induced inflammation[a]	Acute irreversible pulpitis[a]
1	5.85	9.50	13.13
2	7.01	13.17	22.28
3	8.76	8.55	14.60
4	6.70	12.96	11.71
5	8.69	10.48	17.45
Mean	7.40	10.93	15.83
SD	1.28	2.06	4.18

[a]Values are given in pmol of bound peptide per 100 μg of tissue protein.

irreversible pulpitis and healthy pulps ($P < 0.005$). The difference between the group of pulps having induced inflammation and healthy pulps was also significant ($P < 0.01$). The least difference found between the group of pulps having a diagnosis of acute irreversible pulpitis and pulps having induced inflammation was also statistically significant ($P < 0.05$).

Discussion

Neuropeptides and other biologically active peptides exhibit a high degree of functional diversity within the same group of peptides not only through the regulation of peptide production but also through peptide–receptor interaction. The mammalian tachykinin system represents a typical example; it comprises a group of multiple peptides and demonstrates a functional diversity at the levels of both peptide production and peptide reception (Nakanishi 1991). SP induces inflammatory reactions in peripheral tissues including the dental pulp, but its regulatory effects in target tissues are dependent on receptor signalling (Fristad *et al.* 1999). The objective of this study was, therefore, to compare SP receptor expression in pulp tissue having a clinical diagnosis of acute irreversible pulpitis with pulp having induced inflammation and healthy pulp.

Radioreceptor assay (RRA) measures a ligand interaction with its biological receptor by competition binding assay in which receptors function as binding proteins. RRA specificity completely depends on a ligand's affinity for its receptor (Chard 1990). This technique has been widely used for determining the presence of receptors, their affinity for their ligands and determining changes in their expression in different organs and pathological states (Caviedes-Bucheli *et al.* 2004b, 2005b).

Several studies have shown a significantly higher SP expression in the inflamed pulp, suggesting that SP plays an important role in the inflammatory process (Byers *et al.* 1990, Kim 1990, Awawdeh *et al.* 2002, Bowles *et al.* 2003, Caviedes-Bucheli *et al.* 2005a). Results from the present study correlate with previous statement, as there was a greater expression of SP receptors in both inflammatory conditions, indicative of SP active participation in pulpitis development.

Based on the results of a previous research (Caviedes-Bucheli *et al.* 2005b) where CGRP-receptor expression was measured under the same conditions of the present study, it was expected that SP receptor expression for the group of acute irreversible pulpitis be higher than the induced inflammation group. This could be explained by the evolution time of the inflammatory process, 10 min for the induced pulpitis group compared with at least 24 h in the acute irreversible pulpitis group, which allowed a greater number of inflammatory cells expressing the receptor being present in tissue.

It was not possible to identify which type of receptor (NK1, NK2 or NK3) was measured in the present study as a result of the nature of the assay, which relied exclusively on SP affinity for the receptor. However, according to literature, it is known that SP exerts its biological actions via the high-affinity receptor NK1, although at higher concentrations it may also interact with NK2 and NK3 receptors (Helke *et al.* 1990, Hokfelt *et al.* 2001). Based on SP affinity, it is most likely that receptors measured in this study are NK1, and probably some NK2 and NK3 in the acute irreversible pulpitis group, due to the higher SP concentration. Further studies are needed to ascertain this statement.

Animal studies have shown presence of NK1 receptor in rat dental pulp tissue. Odontoblasts and cells located adjacent to the odontoblast layer demonstrated a highly variable tachykinin (NK1 and NK2) receptor immunoreactivity (Fristad *et al.* 1999, 2003). It has been shown that SP exerts a stimulating effect on fibroblasts and other connective-tissue cells *in vitro* (Nilsson *et al.* 1985, Bongenhielm *et al.* 1995). Even though RRA does not allow the determination of the types of cell expressing SP receptors, it is conceivable that, besides inflammatory cells, connective-tissue cells in the subodontoblast area are also capable of expressing receptors for SP. These findings explain the presence of SP receptors in the healthy pulp group and are in accordance with the hypothesis that SP plays a role in homeostatic regulation of the pulp during normal conditions.

Local anaesthetic used in this study was 4% prilocaine without vasoconstrictor to prevent SP expression being attenuated by alpha-adrenergic agonists (e.g. vasoconstrictors) as stated by other authors (Pertl *et al.* 1997, Hargreaves *et al.* 2003). Only non-smoking human donors were included in this study, as it has been proved that the concentration of tachykinins is significantly higher in smokers (Awawdeh *et al.* 2002). It has also been demonstrated that nicotine triggers the release of SP, NKA and CGRP from C-fibres in the lungs and increases their expression in the pulmonary effluent (Lee *et al.* 1995).

Although the present findings cannot be extrapolated to resolve current clinical endodontic problems, these results have clinical significance, as they could be

useful for future research when proposing alternative modalities of biologic pulp therapy. Identifying SP receptor in human pulp, observing its regulating effect on immune reactions and verifying its inducing effect on fibroblastic proliferation is important for broadening knowledge regarding its biological action.

Conclusion

Substance P receptor is expressed in human pulp tissue with a significantly increased expression during clinical inflammatory phenomena. Its presence has clinical significance as it could play an important role in controlling pulpal blood flow and regulating later stages of inflammation and repair processes.

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