Calcitonin gene-related peptide receptor expression in healthy and inflamed human pulp tissue

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Abstract

Caviedes-Bucheli J, Arenas N, Guiza O, Moncada NA, Moreno GC, Diaz E, Munoz HR. Calcitonin gene-related peptide receptor expression in healthy and inflamed human pulp tissue. *International Endodontic Journal*, **38**, 712–717, 2005.

Aim To use radioreceptor analysis for comparing calcitonin gene-related peptide (CGRP) 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 Six pulp samples were obtained from teeth having a clinical diagnosis of acute irreversible pulpitis. Another eight pulp samples were obtained from healthy premolars where extraction was indicated for orthodontic purposes. In four of these premolars, inflammation was induced prior to pulp collection. All the samples were processed and labelled with ^{125}I-CGRP. Binding sites were identified by ^{125}I-CGRP and standard CGRP competition assays.

Results CGRP 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. The Kruskal–Wallis and Mann–Whitney (*post-hoc*) tests showed statistically significant differences between the groups ($P < 0.05$).

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

Keywords: calcitonin gene-related peptide receptor, human pulp, neurogenic inflammation, radioreceptor assay.

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Introduction

Dental pulp inflammation is a complex process involving a great variety of nervous and vascular reactions, which are key components of the neurogenic phenomenon that could lead to pulp necrosis (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 process (Olgart 1996). These neuropeptides include substance P, calcitonin gene-related peptide (CGRP), neurokinin A, vasoactive intestinal peptide and neuropeptide Y (Wakisaka 1990).

CGRP is produced in the trigeminal cell bodies and is transported via axonal flow to the nerve terminals in the pulp, where it is co-stored with other sensory neuropeptides (Gazelius *et al.* 1987, Wakisaka & Akai 1989). These nerve terminals are mainly C-type fibres, which closely follow the pulp microcirculation. When stimulated, neuropeptides are released (Awawdeh *et al.* 2002). CGRP is a powerful vasodilator agent, which causes an increase in local blood flow and consequently, pulpal tissue pressure increases (Olgart *et al.* 1991). In addition to this effect, CGRP exerts stimulatory effects on the growth of pulpal cells, such as fibroblasts and odontoblast-like cells (Trantor *et al.*

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1995). It also increases the *in vitro* expression of bone morphogenetic protein-2 transcripts in human pulp cells, leading to odontoblast-like cell differentiation (Calland *et al.* 1997).

Release of CGRP plays an important role in the clinical inflammatory phenomenon of reversible and irreversible pulpitis, where its action is probably mediated by a specific receptor (Uddman *et al.* 1999). Flow cytometry analysis of human pulp tissue has shown higher CGRP expression in clinical stages of acute irreversible pulpitis when compared with healthy pulps (Caviedes-Bucheli *et al.* 2004a).

There are two known types of CGRP receptor. The CGRP1 receptor is a highly sensitive protein for CGRP; it has seven transmembrane domains and is a member of the G II protein receptor superfamily. This receptor has three components: G II protein calcitonin receptor, calcitonin-receptor-like receptor (CRLR) and a single-stage transmembrane receptor protein (RAM-1), which is presented as a heterodimer (Uddman *et al.* 1999). Evidence of the existence of a CGRP2 receptor has been presented, but its structure is still not well understood (van Rossum *et al.* 1997).

CGRP 1 receptor has been found on the periphery of blood vessels, such as superior mesenteric, femoral and coronary arteries; its presence has also been found in cerebral arteries, smooth muscular tissue and the trigeminal ganglion (Wimalawansa *et al.* 1987). Animal experiments have shown CGRP1 receptor expression in dental pulp of rats and ferrets (Berggreen & Heyeraas 2000, Fristad *et al.* 2003).

Although CRLR mRNA protein has been identified in human dental pulp and trigeminal neurons by reverse transcriptase-polymerase chain reaction (RT-PCR) (Edvinsson *et al.* 1997, Uddman *et al.* 1999), the CGRP1 receptor has not been localized or identified in human dental pulp. Identifying this receptor in human pulp, observing its regulating effect on immune reactions and verifying its inducing effect on fibroblastic proliferation is important for broadening the knowledge regarding its biological action.

The purpose of this study was to use radioreceptor analysis for comparing CGRP receptor expression in human pulp tissue having a clinical diagnosis of acute irreversible pulpitis, healthy pulp and induced pulpitis.

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 14 different human adult donors (19–36 years old) following previously described procedures (Caviedes-Bucheli *et al.* 2004a).

Six 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 evolution. 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, the pulp tissue was extracted with a sterile barbed broach file.

Another eight pulp samples were obtained from healthy premolars extracted for orthodontic purposes. In four of these bicuspids, inflammation was induced prior to pulp collection. The rest was used to establish normal CGRP receptor expression. For the induced inflammation group, teeth were anaesthetized and isolated as described before. The inflammatory process was generated by mechanical exposure of the buccal pulp horn using a no.1 round carbide bur in a high-speed handpiece without irrigation. After a period of 5–10 min, the pulp tissue was extracted using a sterile barbed broach file.

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 remains 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.

After all the samples had been obtained, they were ultrasonically disaggregated (Ultrasonic Processor S-2028-130; ISC Bio-Express, Kaysville, UT, USA) for their homogenization. The disaggregated tissue was spun at 400 *g* for 10 min (GS-6KR Centrifuge; Beckman, Fullerton, CA, USA). The supernatants were transferred to another tube, centrifuged at 11 000 *g* for 30 min (RC5C Centrifuge; Sorvall, Wilmington, DE, USA), and again the supernatants were transferred to another tube. Then 0.025 mL $NaCl/MgSO_4$ solution was added to unbind the membranes from the rest of the cell structures and then spun at 15 000 *g* for 1 h (Sorvall). The pellets were suspended with 10 mL 0.05 mol L^{-1} Tris–HCl buffer solution and centrifuged

again at 15 000 *g* for 40 min (Sorvall) to obtain a pellet containing just cell membrane. This was suspended with 1 mL 0.05 mol L^{-1} Tris–HCl buffer solution. The amount of protein was recorded for each cell membrane suspension (Bio Rad Kit SOP 3003-01; Bio Rad, Hercules, CA, USA).

Radioreceptor assay

Standard human CGRP and ^{125}I-CGRP were obtained from Phoenix Peptide Pharmaceutical, Belmont, CA, USA (Ref. RK-015-02 and RK-015-02E). Standard CGRP was reconstituted with distilled water and serially diluted with human serum albumin (HSA)/Tris–HCl buffer solution to obtain different reagent concentrations. ^{125}I-CGRP was reconstituted with distilled water and diluted until there were 10 000 counts per min (c.p.m.) in 100 μL reagent.

One hundred microlitres of each cell membrane suspension was submitted to competition binding studies with 100 μL ^{125}I-CGRP and 100 μL of different standard CGRP concentrations. Each sample was processed in duplicate in two different radio-immunochemical assays for precise intra- and inter-analysis interpretation. After 24 h incubation, the suspensions were spun at 14 000 *g* for 15 min (Beckman) to precipitate the bound fractions. One microlitre polyethylene glycol 8000 (Sigma-Aldrich, Ref. 81268, St Louis, MO, USA) was added to facilitate precipitation. The supernatants were decanted and pellet radioactivity was read on a Gamma Counter (Gamma Assay LS 5500; Beckman). Scatchard analysis of the binding data assessed the number of CGRP receptors in every sample.

Statistical analysis

The values obtained were expressed as the number of binding sites (CGRP receptors) per μg membrane protein. The mean and the 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 performed for comparing differences between different groups.

Results

Calcitonin gene-related peptide 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 Number of binding sites (calcitonin gene-related peptide receptors) per μg human pulp tissue cell membrane protein

Pulp sample	Acute irreversible pulpitis	Pulp with induced inflammation	Healthy pulp
1	2048.08	1979.26	985.11
2	2629.79	1751.30	1003.60
3	2868.02	2230.07	894.04
4	2212.83	1933.09	837.20
5	2263.11		
6	3457.95		
Mean	2579.96	1973.43	929.99
SD	524.29	197.37	78.23

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 ($P < 0.001$). Mann–Whitney's *post-hoc* test comparisons showed that the greatest difference in the amount of receptors expressed was between pulps having a diagnosis of acute irreversible pulpitis and healthy pulps ($P = 0.000$). The difference between the group of pulps having induced inflammation and healthy pulps was also significant ($P < 0.001$). 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

For a cell to be able to respond to a neuropeptide, it must express an appropriate receptor protein on its cell membrane. This receptor performs two basic functions: binding the natural agonist when this is present in the extracellular environment and generating a signal regulating various functions within a cell. It is unknown how many receptors each cell expresses for each molecule of neuropeptide. It is thought that the number of receptors is equal to the number of neuropeptide molecules; however, it has been established that there are multiple receptors on cell membrane for a single neuropeptide. Those receptors, which are not occupied by ligand, acquire an inactive conformation (Haorah 2002). The objective of this

study was, therefore, to compare CGRP receptor expression in pulp tissue having a clinical diagnosis of acute irreversible pulpitis with pulp having induced inflammation and healthy pulp.

Pioneering studies in CGRP physiology have shown sprouting of CGRP immunoreactive nerve fibres in response to dentine injury in rat molars and in the presence of inflammation (Taylor *et al.* 1988). Results from the present study correlate with the previous statement as there was a significant increase in CGRP receptor expression in both inflammatory phenomena.

Radioreceptor analysis (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. 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 (Haro 1984, Shimanda *et al.* 2002, Caviedes-Bucheli *et al.* 2004b).

Even though RRA does not permit to determine the types of cell expressing CGRP receptors, it allowed to establish that there were a greater number of CGRP binding sites in acute irreversible pulpitis and pulp having induced inflammation than in healthy pulp. This finding could be explained by the evolution time of the inflammatory process. In the induced pulpitis group, pulps were obtained 5–10 min after the stimulus was applied whilst inflammation in pulp tissue having a clinical diagnosis of acute irreversible pulpitis had approximately 24 h evolution, leading to a greater number of inflammatory cells expressing the receptor being present in tissue (Caviedes-Bucheli *et al.* 2004a). The 5–10 min waiting period before collecting pulp tissue was chosen taking into consideration that the mean life of CGRP in tissue is 10 min before it occupies the receptor or is degraded by endogenous peptidases (Kraenzlin *et al.* 1985).

On the contrary, more time was needed for the receptor to become expressed because receptor activity modifying protein-1 (RAMP-1) co-expression with CRLR is required for the CGRP receptor to be expressed, because RAMP-1 seems to promote increased CRLR production on cell surface. Intracellular retention was found with both CRLR and RAMP when expressed alone suggesting that an interaction between the two proteins is essential for their expression on cell surface. Neither RAMP-1 nor CRLR induced a significant response to CGRP when transferred alone, but the expression of both produced cells responding to CGRP by increasing intracellular cAMP levels (McLatchie *et al.* 1998).

Mechanical pulp exposure was successful to induce inflammation as stated in previous studies, where it has been demonstrated that high-speed drilling and mechanical pulp exposure are effective stimulus to release CGRP in dental pulp (Buck *et al.* 1999, Takamori 2000). Local anaesthetic used in this study was 4% prilocaine without vasoconstrictor to prevent CGRP expression to be attenuated by alpha-adrenergic agonists (e.g. vasoconstrictors) as stated by other authors (Pertl *et al.* 1997, Hargreaves *et al.* 2003).

Previous studies (McGillis & Humphreys 1991, Hargreaves *et al.* 1994, Levite & Cahalon 1998, Fernandez *et al.* 2001) have reported that CGRP interacts with mastocytes, inducing the release of histamine, thereby causing elevated vascular permeability and an increase in blood pressure in tissue. It also interacts with other inflammatory cells such as macrophages and lymphocytes altering its functions and inducing these cells to release inflammatory mediators such as cytokines, prostaglandins and thromboxanes having a direct effect on pulp tissue. This is why it can be supposed that CGRP receptors detected in both experimental groups are expressed on these types of cells; whilst receptors present in control group may be expressed mainly in blood vessels. However, further studies are required to ascertain this.

Animal studies have shown presence of CGRP receptor protein in rat dental pulp tissue. Odontoblasts including their cell processes demonstrated a highly variable tachykinin (NK1 and NK2) receptor immunoreactivity. However, CGRP1 receptor could not be identified on odontoblasts in the mature rat molar, suggesting that its presence is associated mainly with blood vessels (Fristad *et al.* 2003).

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 biological pulp therapy, such as blocking CGRP receptor to reduce the inflammatory process. The use of CGRP antagonist should also be investigated, as in migraine, where this type of therapy has shown optimistic results (Dennis *et al.* 1990, Edvinsson *et al.* 1997, 1998).

Conclusion

Calcitonin gene-related peptide receptor is expressed in human pulp tissue having 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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