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Neuropeptide Y Y1 receptor in human dental pulp cells of noncarious and carious teeth

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Abstract

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Aim To determine the distribution of the NPY Y1 receptor in carious and noncarious human dental pulp tissue using immunohistochemistry. A subsidiary aim was to confirm the presence of the NPY Y1 protein product in membrane fractions of dental pulp tissue from carious and noncarious teeth using western blotting.

Methodology Twenty two dental pulp samples were collected from carious and noncarious extracted teeth. Ten samples were processed for immunohistochemistry using a specific antibody to the NPY Y1 receptor. Twelve samples were used to obtain membrane extracts which were electrophoresed, blotted onto nitrocellulose and probed with NPY Y1 receptor antibody. Kruskal–Wallis one-way analysis of variance was employed to test for overall statistical differences between NPY Y1 levels in noncarious, moderately carious and grossly carious teeth.

Results Neuropeptide Y Y1 receptor immunoreactivity was detected on the walls of blood vessels in pulp tissue from noncarious teeth. In carious teeth NPY Y1 immunoreactvity was observed on nerve fibres, blood vessels and inflammatory cells. Western blotting indicated the presence and confirmed the variability of NPY Y1 receptor protein expression in solubilised membrane preparations of human dental pulp tissue from carious and noncarious teeth.

Conclusions Neuropeptide Y Y1 is expressed in human dental pulp tissue with evidence of increased expression in carious compared with noncarious teeth, suggesting a role for NPY Y1 in modulation of caries induced pulpal inflammation.

Keywords: caries, dental pulp, human, NPY, NPY Y1, receptor.

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Introduction

Data is emerging that supports the view that neuropeptides have a role in the pulpal response to caries. It has been shown that the levels of neuropeptides such as substance P (SP), neurokinin A (NKA), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP) and neuropeptide Y (NPY) are elevated in the dental pulps of carious compared with noncarious teeth (Rodd & Boissonade 2000, Awawdeh *et al.* 2002, El Karim *et al.* 2006a,b). However, neuropeptides cannot cross cell membranes and therefore to exert their biological effects they must bind to selected receptors on target cell membranes. More than 80% of neuropeptide receptors are G protein coupled receptors, so-called because they bind guanosine triphosphate and act as intermediaries between the receptor and several second messenger systems. Neuropeptides usually have several different iso-receptors or receptor subtypes that can be distinguished by specific agonists, antagonists or antibodies. The concept of multiple types of receptors explains subtle differences in neuropeptide effects within various tissues.

Neuropeptide Y exerts its actions through at least five different receptors subtypes (Michel 1991). To date the most important and widely studied NPY receptor

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subtypes are NPY Y1 and NPY Y2. Both NPY Y1 and NPY Y2 have been reported to mediate the vascular effects of NPY (Nilsson *et al.* 1996, Rump *et al.* 1997) and NPY Y1 is known to have a widespread distribution in the peripheral circulation (Uddman *et al.* 2002). In addition NPY Y1 receptors are believed to be involved in the pain modulatory effects of NPY (Zhang *et al.* 2000, Wang *et al.* 2001).

Neuropeptide Y receptor subtypes have been demonstrated in a variety of different tissues, where they have been localised to blood vessel walls, nerve fibres, epithelium and endocrine-like cells (Jackerott & Larsson 1997, Mannon *et al.* 1999, Matsuda *et al.* 2002, Uddman *et al.* 2002). Although mRNA encoding the NPY Y1 receptor has been demonstrated in human dental pulp (Uddman *et al.* 1998), evidence for the expressed NPY Y1 protein product and its cellular localisation remain to be reported.

The aim of this study was to determine the distribution of the NPY Y1 receptor in carious and noncarious human dental pulp tissue using immunohistochemistry. A subsidiary aim was to confirm the presence of the NPY Y1 protein product in membrane fractions prepared from dental pulp tissue of carious and noncarious teeth using western blotting.

Materials and methods

Clinical samples

The study was approved by the local Research Ethics Committee, Queen's University Belfast, UK and all subjects (n = 22) gave their informed written consent. Carious teeth (range from superficial to deep caries) were collected from patients requiring permanent tooth extraction for various therapeutic reasons. Exclusion criteria for carious teeth were teeth from patients with clinically significant medical problems, teeth that had undergone any form of endodontic therapy, teeth with associated pathosis other than dental caries and teeth with periapical pathosis suggestive of pulp necrosis. Control teeth with no caries were obtained from patients having extractions for orthodontic reasons or having wisdom teeth removed.

All extractions were performed under local anaesthesia (2% lignocaine 1 : 80 000 epinephrine) and were uncomplicated, as only fully erupted teeth were included in the study. Immediately following extraction, each tooth was split in a vice fitted with a cutting edge (Lilja 1979) and the pulp tissue was removed with fine tweezers within 1–2 min. Pulp tissue required for immunohistochemistry was immediately fixed in 4% paraformaldehyde in phosphate buffered saline (PBS). The pulp tissue required for western blotting was placed in a pre-weighed Eppendorf tube, immediately frozen in liquid nitrogen and stored at −70 °C. The split halves of the teeth were used to visually assess the extent of caries using a dissecting microscope at 20× magnification. Each tooth was categorised as: (1) noncarious, i.e. no colour change indicative of caries within dentine; (2) mild/moderate caries, i.e. the colour change did not extend beyond half the dentine thickness; (3) advanced/gross caries, i.e. the colour change extended beyond half the dentine thickness.

Immunohistochemistry

Pulp tissue from carious (n = 5) and non carious (n = 5) teeth was processed for immunohistochemistry. Pulp tissue which had been fixed in 4% paraformaldehyde in PBS for 4 h at 4 °C, was washed in 5% sucrose for 20 min and cryoprotected in 30% sucrose until submerged. Specimens were frozen onto stubs using embedding media (O.C.T. compound) and sections (10 μm) were cut using a cryostat and collected onto 3-aminopropyltriethoxysilane (APES) coated slides. Specimens were then processed for immunohistochemical staining for NPY Y1 receptor using the Dako Envision system (Dako Co., Carpinteria, CA, USA). After rehydration in PBS for 15 min, sections were incubated in 10% normal goat serum for 30 min, permeablised in 0.2% Triton X-100 for 1 h and then treated with peroxidase block reagent (0.03% hydrogen peroxidase containing sodium azide) for 5 min. The sections were washed in PBS and incubated with polyclonal rabbit anti-human NPY Y1 (DiaSorin Ltd, Wokingham, UK) at a dilution of 1/100 in PBS overnight at 4 °C. Following a washing step in PBS, specimens were incubated with secondary anti-rabbit peroxidase-labelled polymer (peroxidase labelled polymer conjugated to goat anti-rabbit immunoglobulins in Tris-HCl buffer) for 1 h at room temperature. The substrate diaminobenzidine (DAB) was added to visualise bound secondary antibody. The slides were incubated with DAB substrate solution for 2–3 min and then washed in distilled water before being counterstained with Harris' haematoxylin (BDH, Poole, UK) for 1 min. Sections were dehydrated in degrading alcohols, cleared in xylene and mounted in

DPX (Searle Diagnostic, UK). Pre-immune rabbit serum was used as a negative control in place of the primary antibody.

Western blotting

Since the NPY Y1 receptor is an integral membrane protein, it was necessary to prepare membrane extracts from the dental pulp tissue prior to electrophoresis. Membrane extracts were prepared from the dental pulps of noncarious ($n = 4$), moderately carious ($n = 4$) and grossly carious teeth ($n = 4$) using the Mem-PER eukaryotic membrane protein extraction reagent kit (Pierce, Rockford, IL, USA). The hydrophobic membrane protein phase obtained from the membrane extraction kit was further prepared for electrophoresis using PAGEprep clean up and enrichment kit (Pierce, Rockford, IL, USA). Equal volumes of samples (15 μL) were electrophoresed and blotted onto nitrocellulose as previously described (Lundy & Wisdom 1992). The blot was blocked for 2 h in a solution of 5% non fat milk in tris-buffered saline (TBS; 0.02 mol L^{-1} Tris-HCL buffer, pH 7.4, containing 0.15 mol L^{-1} NaCl). After washing in TBS the blot was incubated with polyclonal rabbit anti-human NPY Y1 (Abcam, Cambridge, UK) at a dilution of 1/2000 overnight at room temperature. Subsequent detection of bound primary antibody was achieved by incubation for 2 h with anti-rabbit immunoglobulin-alkaline phosphatase conjugate (Sigma Chemical Co., Gillingham, UK) at a dilution of 1/1000. Bound alkaline phosphatase was detected as previously described (Lundy & Wisdom 1992). Samples which were electrophoresed, blotted onto nitrocellulose and probed as described above in the absence of primary antibody served as controls for nonspecific binding. The intensity of the immunoreactive bands was measured using a BioRad GS-670 Imaging Densitometer. Each band was scanned in triplicate and the intensity of the background was subtracted before the result was recorded.

Statistical analysis

Data were analyzed using the statistical package SPSS version 15.0. The level of statistical significance for all tests was set at $P < 0.05$. Kruskal–Wallis one-way analysis of variance was employed to test for overall statistical differences between NPY Y1 levels in noncarious, moderately carious and grossly carious teeth.

Results

Immunohistochemistry

Neuropeptide Y Y1-immunoreactivity (Ir) was detected in the dental pulp of both noncarious and carious teeth. In the dental pulp of noncarious teeth NPY Y1-Ir was demonstrated in the walls of small and medium sized vessels in the coronal, subodontoblastic and central pulp regions. The endothelial cells of many small blood vessels were intensely stained (Fig. 1a). In the dental pulp of carious teeth there was variable expression of NPY Y1-Ir, where NPY Y1-Ir was detected in walls of blood vessels as well as in a subpopulation of nerve fibres scattered throughout the subodontoblastic layer and pulp region proper (Fig. 1b,c). In some of the grossly carious teeth positive staining was also observed in inflammatory cells with large and /or multi-lobed nuclei resembling lymphocytes and polymorphonuclear leukocytes (Fig. 1d). The specificity of the antibody-antigen interaction was confirmed by absence of immunoreactivity in samples incubated with pre-immune rabbit serum (results not shown).

Western blotting

The presence of NPY Y1 receptor proteins in solubilised membrane preparations of human dental pulp tissue was confirmed by western blotting. A major immunoreactive band was detected at approximately 55 kDa in all samples derived from both intact and carious teeth. There was considerable variability in the density of the immunoblots within the groups of healthy or carious teeth (Fig. 2). There was a trend for the mean blot density to increase from a low value of 0.06 (SD 0.04) in healthy through an intermediate value in moderately carious (0.11 SD 0.05) to the highest value in membrane extracts of pulp tissue from grossly carious teeth (0.17 SD 0.11) (Fig. 3). These differences, however, were not statistically significant. Control blots incubated in the absence of primary antibody showed no immunoreactive bands (results not shown).

Discussion

The immunohistochemical localisation and distribution of NPY Y1 receptors has previously been reported in various tissues but to date, not in the dental pulp. The present study demonstrated that NPY Y1 receptor protein is present in human dental pulp and its expression varies in health and disease. NPY Y1-Ir

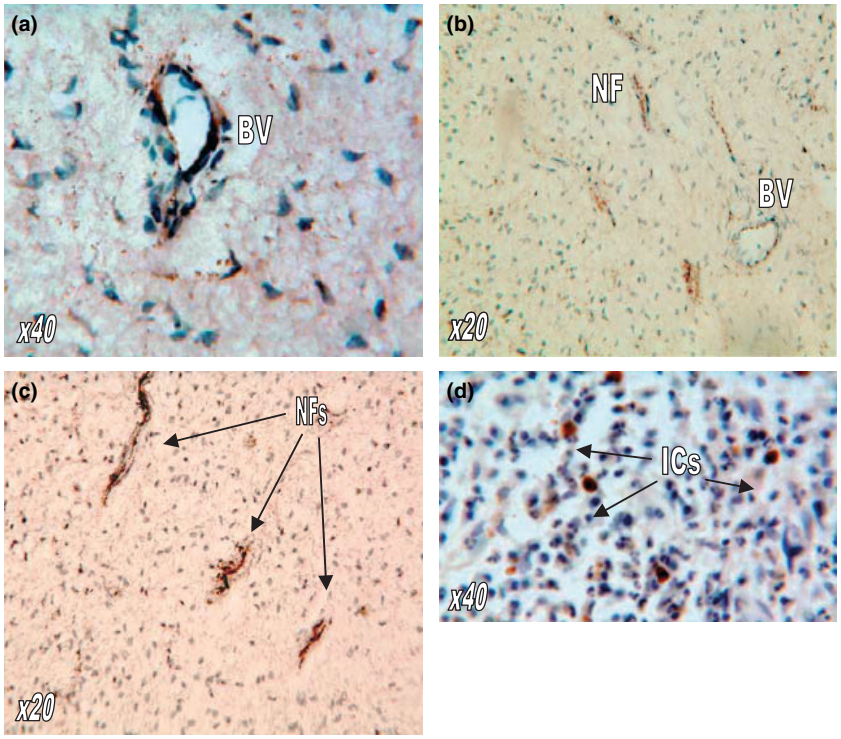

Figure 1 NPY Y1 receptor expression in (a) a small blood vessel [BV] in noncarious pulp tissue, (b) a blood vessel [BV] and nerve fibres [NFs] in moderately carious pulp tissue, (c) nerve fibres [NFs] in grossly carious pulp tissue, (d) inflammatory cells [ICs] in grossly carious pulp tissue.

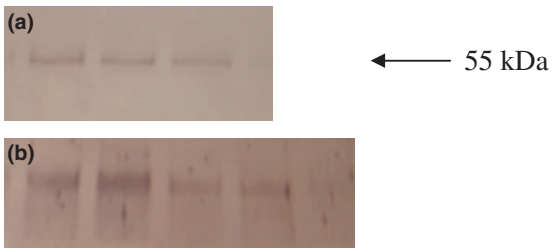

Figure 2 Representative western blots of NPY Y1 receptor proteins in membrane extracts prepared from human dental pulp of carious and noncarious teeth. (a) noncarious pulp samples. (b) Carious pulp samples.

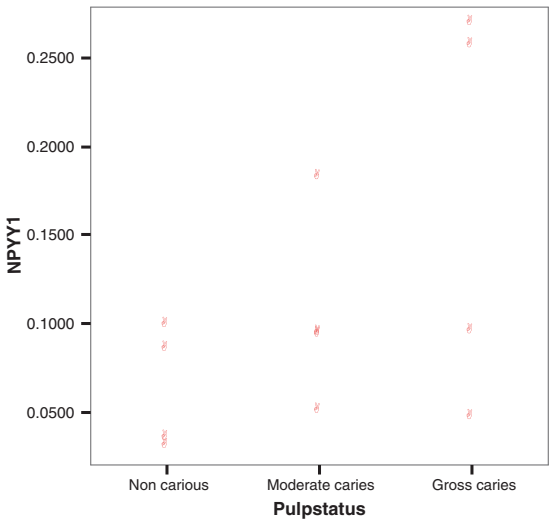

Figure 3 Scattered plot showing intensity of NPY Y1-Ir band staining according to pulp status.

was shown to be localised in the smooth muscles cells of smaller arteries as compared to large arteries and veins suggesting an important role for NPY in small resistance vessels (Matsuda *et al.* 2002, Uddman *et al.* 2002). The fact that the largest vessels found in the human dental pulp are arterioles, together with the currently reported localisation of NPY Y1 in pulpal blood vessels and the previous evidence for an association of NPY fibres with pulpal blood vessels (El Karim *et al.* 2006a) support an important role for NPY acting via Y1 receptors in pulpal haemoregulation. In carious teeth NPY Y1 receptor was found to be localised not

only to the walls of blood vessels but to be distributed more widely in nerves fibres and in grossly carious teeth in inflammatory cells as previously described in other tissues (Mannon *et al.* 1999, Uddman *et al.* 2002).

Neuropeptide Y Y1 has been shown to play an essential role in mediating the inhibitory effects of NPY in neurogenic inflammation. Using Y(1)-deficient mice an essential role for NPY Y1 in mediating plasma extravasation and antinociception has been reported (Naveilhan *et al.* 2001). Deletion of NPY Y1 receptors has also been shown to be associated with increased release of SP and CGRP (Shi *et al.* 2006) as did the use of NPY Y1 antagonists (Gibbs *et al.* 2006). It has been shown previously that NPY co-localises with SP in nerves in the dental pulp of carious teeth (El Karim *et al.* 2006a) and the present localisation of NPY Y1 to the nerve fibres reported in present study lends support to the hypothesis that NPY acting via NPY Y1 receptors in the dental pulp of carious teeth may could mediate inhibition of SP and CGRP release and subsequent inhibition of neurogenic inflammation and pain.

In the present study there was a trend for increased NPY Y1 receptor expression with the degree of caries, however there was considerable sample variation and this trend did not reach statistical significant. It has been reported previously that increased expression of NPY occurs in the dental pulp of carious teeth (El Karim *et al.* 2006a) and that there is a tendency for increased NPY expression in moderately carious teeth. This would fit with an increased role for NPY acting through the Y1 receptor in the suppression of pulpal inflammation resulting from dental caries. In the present study there was variability in receptor expression which was particularly evident within the grossly carious group, which had not only the highest levels of NPY Y1 but also one very low value similar to that recorded from the healthy pulp samples. This may reflect the state of the pulp in the grossly carious teeth. Inflammation of the dental pulp commences early during the caries process, as reversible pulpitis. During a neurogenic inflammatory response in a low compliance environment such as the dental pulp, increased plasma extravasation and oedema formation associated with SP and CGRP release, can lead to irreversible inflammation and pulpal necrosis. As inflammation increases then so does NPY Y1 expression. The pulp tissue harvested from grossly carious teeth may in some cases be partly necrotic and therefore immunoblots derived from membrane extracts could as a result have low density.

Another proposed mechanism for NPY Y1-mediated inhibition of inflammation in immune cells has been reported. In Y(1)-deficient mice Wheway *et al.* (2005) demonstrated a bimodal role for Y1 in immune cells. Whereas its presence is essential for the function of the antigen presenting cells, NPY Y1 was also found to inhibit T cell response. Thus, signalling via the Y1 receptor is necessary for the proper functioning of antigen presenting cells as priming elements for T cells and at the same time it appears to be protective against excessive inflammation by inhibiting hyperresponsiveness of T cells.

Conclusion

Neuropeptide Y Y1 was expressed in human dental pulp with evidence of increased expression in carious compared with noncarious teeth. NPY Y1 receptors were localised to nerve fibres and inflammatory cells in the dental pulp of carious teeth. Taken together with the prevailing evidence from animal studies on the function of NPY in pain and inflammation these results are consistent with a role for this neuropeptide acting through the NPY Y1 receptor in the modulation of pulpal inflammation. Further studies are warranted to improve understanding of the functional role of NPY acting through the NPY Y1 receptor in pulpal pain and inflammation.

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