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Sensory pulpal nerve fibres and trigeminal ganglion neurons express IL-1RI: a potential mechanism for development of inflammatory hyperalgesia

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Abstract

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Aim To localize interleukin-1 receptor type I (IL-1RI) in rat dental pulp and trigeminal ganglion (TG) and to test the hypothesis that pulpal inflammation increases neuronal expression of IL-1RI.

Methodology Female Wistar rats were subjected to unilateral pulp exposures in the maxillary and mandibular first molars, whereas the contralateral jaws served as untreated controls. Seven days later the animals were transcardiacally perfused and the jaws and the TGs were removed and prepared for immunohistochemistry. Immunoreactivity for IL-1RI was examined alone (DAB) and together with calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), CD31 or CD34 by multiple-labelling immunofluorescence. Quantification of IL-1RI-immunoreactive (-IR) cells in the maxillary and mandibular division of the ganglion was performed in parasagittal immunoreacted sections of the right and left TGs. Data were analysed with Mann–Whitney Rank Sum test ($P < 0.05$).

Results Interleukin-1 receptor type I was found on sensory (CGRP-IR) and sympathetic (NPY-IR) nerve fibres and on blood vessels (CD31- and CD34-IR) in the dental pulp. It was also localized on sensory neurons and axons in the TG. Pulpal inflammation significantly increased the expression of IL-1RI in the TG ($P < 0.001$).

Conclusions The localization of IL-1RI on sensory nerve fibres and its up-regulation in TG neurons during pulpal inflammation may imply a direct effect of IL-1 in pulpal nociception. The presence of IL-1RI on sympathetic nerve fibres and on blood vessels may indicate a vasoactive role of the same cytokine in the pulp.

Keywords: blood vessels, CGRP, immunohistochemistry, pain, pulpitis, receptor.

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Introduction

Pulpitis is a common inflammatory condition that occurs within rigid dentinal walls, usually after bacterial infection, and is often extremely painful. In the acute phase, pulpitis is characterized by vasodilatation and increased vascular permeability (Kerezoudis *et al.* 1993, Heyeraas *et al.* 1994), as well as production of inflammatory mediators with the pro-inflammatory cytokine interleukin-1 (IL-1) being a key component (Tani-Ishii *et al.* 1995, Kawashima *et al.* 2005, Bletsa *et al.* 2006b).

Traditionally, the cytokines of the IL-1 family play major roles in inflammatory responses (Dinarello 1996) and the dental pulp is not an exception. This family includes two agonists, IL-1α and IL-1β and an IL-1 receptor antagonist (IL-1Ra), which bind to two subtypes of receptors, type I and type II [interleukin-1

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receptor type I (IL-1RI) and interleukin-1 receptor type II (IL-1RII) respectively]. The two types of receptors have been cloned and characterized biochemically and it has been established that IL-1 signalling activity is mediated exclusively via the IL-1RI (80-kDa) whilst the IL-1RII (60-kDa) acts as a 'decoy' receptor for IL-1, inhibiting its activity by preventing IL-1 from binding to the signalling IL-1RI (Cunningham & De Souza 1993, Sims *et al.* 1993, Evans *et al.* 1995, Liege *et al.* 2000).

Interluekin-1 is a multipotent cytokine and amongst its diverse activities are induction of increased vascular permeability (Rossi *et al.* 1985, Bussolino *et al.* 1986, Martin *et al.* 1988) and inflammatory hyperalgesia (Bianchi *et al.* 1998, Wolf *et al.* 2003). Yet, the underlying mechanisms for the above biological functions remain elusive. Increased vascular permeability can be induced by IL-1 either directly at the endothelial cells (Martin *et al.* 1988) or indirectly by inducing other inflammatory mediators that affect the vascular endothelium (Rossi *et al.* 1985, Bussolino *et al.* 1986). IL-1 was found to evoke hyperalgesia indirectly through induction of prostaglandins (Schweizer *et al.* 1988, Follenfant *et al.* 1989), bradykinin (Ferreira *et al.* 1993, Davis & Perkins 1994), and nerve growth factor (NGF) (Safieh-Garabedian *et al.* 1995), all known mediators of inflammatory pain. However, the expression of IL-1RI on both endothelial cells and smooth muscle cells in the vascular walls (Boraschi *et al.* 1991), as well as on soma and nerve endings of nociceptive sensory neurons (Liu *et al.* 2006) led to the thought of a direct involvement of IL-1 on vascular changes and inflammatory pain.

When it comes to dental pulp, there is a body of evidence on up-regulation of IL-1 during pulpitis, but there is no information on the exact sites of action of this cytokine. The purpose of this study was to investigate the localization of IL-1RI under normal conditions and during inflammation in the pulp. When preliminary results showed co-localization of IL-1RI with sensory nerve fibres in the pulp, the hypothesis that pulpal inflammation induces increased expression of IL-1RI in the trigeminal ganglion (TG) was investigated further.

Materials and methods

Animal preparation

The material comprised of six female Wistar rats (Mol:WIST Han) weighing 175–230 g. All rats were housed in polycarbonate cages (2–3 rats per cage) and fed a standard pellet diet (801157W Expanded Pellets, Stepfield, Witham, UK) with tap water *ad libitum*. The experimental protocol was approved by the Regional committee for Animal Research Ethics, University of Bergen, under the supervision of the Norwegian Experimental Animal Board.

After a one week acclimatization period, the rats were anaesthetized with a mixture of medetomidine (Domitor® 1 mg mL^{-1}; Orion Pharma, Espoo, Finland), 0.4 mg kg^{-1} body weight and ketamine (Ketalar® 50 mg mL^{-1}; Pfizer AB, Sollentuna, Sweden), 60 mg kg^{-1} body weight, given intramuscularly. Unilateral pulp exposures were performed under a stereomicroscope on the first right maxillary and mandibular molars with round burs (number ½). At the end of the procedure, atipamezole (Antisedan® 5 mg mL^{-1}; Orion Pharma), 4 mg kg^{-1} body weight, was given intramuscularly, for reversal of the anaesthesia. All animals exhibited normal feeding habits and gained weight during the experimental period.

Seven days after pulp exposures, the rats were deeply re-anaesthetized with sodium pentobarbital (Mebumal® 50 mg mL^{-1}; Svaneapoteket, Bergen, Norway), 50 mg kg^{-1} body weight given intraperitoneally, and were transcardially perfused through the aorta with heparinized saline followed by 10 % (w/v) EDTA. The maxillae and the mandibles were dissected out and decalcified in 10 % (w/v) EDTA for approximately 4 weeks. After demineralization, the jaws were rinsed in 0.1 mol L^{-1} phosphate buffer, soaked in 30% (w/v) sucrose overnight and stored at −80 °C until sectioning. The TG were also dissected out carefully under a stereomicroscope, embedded in mounting compound (Tissue-Tek OCT; Sakura, Zoeterwoude, the Netherlands) and immediately frozen in liquid nitrogen precooled isopentane and stored at −80 °C until sectioning. Parasagittal sections of the jaws and TG, 30 μm thick, were cut in a freezing (−20 °C) slide microtome, with special care to tissue orientation.

Immunohistochemistry

The immunoreactions were performed on pre-coated glass slides (SuperFrost Plus, MenzelGläser, Braunschweig, Germany). The tissues were fixed on the glass with ice-cold acetone for 5–8 min before the immunohistochemical procedures.

Single immunohistochemical staining for IL-1RI and IL-1β (selective TG sections) was performed according to a previously described protocol (Bletsa *et al.* 2006a).

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Briefly, 2.5% (v/v) normal goat serum (Vector Laboratories Inc., Burlingam, CA, USA) was used as blocking step, followed by 72 h incubation with anti-rat IL-1RI (dilution 1 : 300; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-rat IL-1β (dilution 1 : 400; Santa Cruz Biotechnology) polyclonal antibodies raised in rabbit, at 4 °C. Antigen–antibody complexes were detected by the avidin–biotin peroxidase (ABC) method, using a commercially available kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) and visualized by 3,3'-diaminobenzidine (DAB; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in the presence of 0.2% (w/v) $(NH_4)_2Ni(SO_4)_26H_2O$ to enhance the immunostaining. Finally, the sections were counterstained with methylene blue/azure II, dehydrated in graded alcohol series, cleared in xylene and cover slipped with Eukitt (O. Kindler, Freiburg, Germany). The sections were evaluated in a photomicroscope (Nikon Eclipse E600; Nikon Instruments, Kanagawa, Japan) connected to a digital camera using Lucia imaging software (Lucia, v. 480; Laboratory Imaging, Hostivař, Czech Republic).

To identify the structures that showed IL-1RI-immunoreactivity, double immunofluorescence labelling of IL-1RI was performed together with calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY) and CD31/or CD34, the respective markers of sensory nerves, sympathetic nerves and blood vessels (Table 1). Briefly, the sections were blocked with 6% (v/v in PBS containing 0.3% Triton X-100) normal goat or donkey serum (Chemicon International Inc., Temecula, CA, USA) before incubating with primary antibodies overnight at 4 °C. After washing, samples were incubated with corresponding secondary antibodies Cy™2- and Cy™3-conjugated IgGs (dilution 1 : 300; Jackson ImmunoResearch, West Grove, PA, USA) (Table 1) for 1 h at room temperature. The sections were coverslipped with Vectashield (Vector) mounting medium and viewed with a fluorescence microscope (Axio Imager, Carl Zeiss MicroImaging, Göttingen, Germany). The images were captured with AxioCam MRm camera (Carl Zeiss) and the AxioVision 4.4 (Carl Zeiss) imaging system was used for analyses. The specificity of all immune reactions was tested by omission of the primary antibodies or substitution with isotype controls.

Quantification of IL-1RI-Immunoreactivity in Trigeminal Ganglion

Sections of the right (pulp exposure) and the left (control) trigeminal ganglia of three rats were used for quantification of IL-1RI staining under light microscopy using computer-assisted image analysis (Lucia imaging software). Every fifth serial section of the ganglia was used for evaluation; at least 20 sections were analysed per ganglion. First, the mandibular and/or maxillary divisions of the TG were identified under low magnification (4× and 10×) and then photographs were taken with higher magnification (20×). All images were coded and analysed at a later time point by one operator, making the measurements blind. The areas of interest were selected by means of the freehand outline tool and the numbers of IL-1RI+ cells were counted within the circumscribed area. The ratio of IL-1RI+ cells per μm^2 of area was calculated. Data were analysed using the statistical program SIGMASTAT, v 3.1, (Systat Software Inc., San Jose, CA, USA). Nonparametric statistical analysis (Mann–Whitney Rank Sum test) was used to test differences between right and left sides. Data are presented as mean ± SD. $P < 0.05$ was considered as statistically significant.

Results

The immunoreactivity was characterized specific for the primary antibodies used, as no labelling was observed upon omission of the primary antibodies or substitution with isotype controls.

Table 1 Antibodies used in double immunofluorescence labelling

Primary antibody	Dilution	Source	Secondary antibody*
Rabbit polyclonal IL-1RI	1 : 100	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Cy™2 donkey anti-rabbit IgG or Cy™3 goat anti-rabbit IgG
Goat polyclonal CGRP	1 : 100	Abcam, Cambridge, UK	Cy™3 donkey anti-goat IgG
Guinea pig polyclonal NPY	1 : 1000	Abcam	Cy™3 donkey anti-guinea pig IgG
Mouse monoclonal CD31	1 : 50 1 : 100	AbD-Serotec, Oxford, UK and Abcam	Cy™2 goat anti-mouse IgG
Goat polyclonal CD34	1 : 100	AbD-Serotec	Cy™3 donkey anti-goat IgG

*The corresponding secondary antibodies were all from Jackson ImmunoResearch, West Grove, PA, USA and they were used in dilution 1 : 300.

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Pulp

Under normal conditions, immunoreactivity for IL-1RI was seen in long structures resembling nerve and/or blood vessels centrally in both coronal and root pulp (Fig. 1a,b). One week after pulp exposures, strong immunoreactivity for IL-1RI was observed along the inflamed root pulp (Fig. 1c), as well as in the periapical area (Fig. 1d).

To identify the structures that showed immunoreactivity for IL-1RI, double immunofluorescence was performed. IL-1RI was localized on sensory CGRP-immunoreactive (IR) and sympathetic NPY-immunoreactive nerve fibres (Fig. 2a,b respectively) in normal pulp. Furthermore, IL-1RI was also seen on the wall of CD31-labelled blood vessels (Fig. 2c).

During inflammation, there was also co-localization of IL-1RI with CGRP-immunoreactive nerve fibres both in inflamed root pulp and periapical area (Fig. 3a,b) and partly with CD34-immunoreactive vessel walls (Fig. 3c, arrow). Double immunofluorescence with NPY showed the same staining pattern as in normal pulp, namely co-localization of IL-1RI on NPY-labelled nerve fibres that seemed to surround vessels (not shown).

Trigeminal ganglia

Double immunofluorescence revealed that IL-1RI was localized on CGRP-stained neurons and their axons both under normal conditions and during inflammation (Fig. 4a). However, the IL-1RI immunoreactivity was stronger after pulp exposure, in the V_{II} (maxillary) and V_{III} (mandibular) division of the TG (Fig. 4b). The ratio of IL-1RI$^+$ cells per μm^2 of area was significantly higher in the right (pulp exposure) side compared with the left (control) side (0.000474 ± 0.000166 vs. 0.000239 ± 0.0000964, $n = 90$ counted areas, $P < 0.001$; Fig. 4c). Up-regulation of IL-1RI seemed to take place mostly in small sized neurons in the exposed side TG. In addition, staining for IL-1RI/or IL-1β of consecutive sections from right TG (exposed side) showed that IL-1β$^+$ cells were in the vicinity of IL-1RI-immunoreactive neurons and that IL-1β$^+$ cells seemed to be both neurons and glial-like cells (data not shown).

Discussion

This study is the first report describing the distribution of IL-1RI in the dental pulp. The presence of IL-1RI on the blood vessels and on sensory and sympathetic nerve

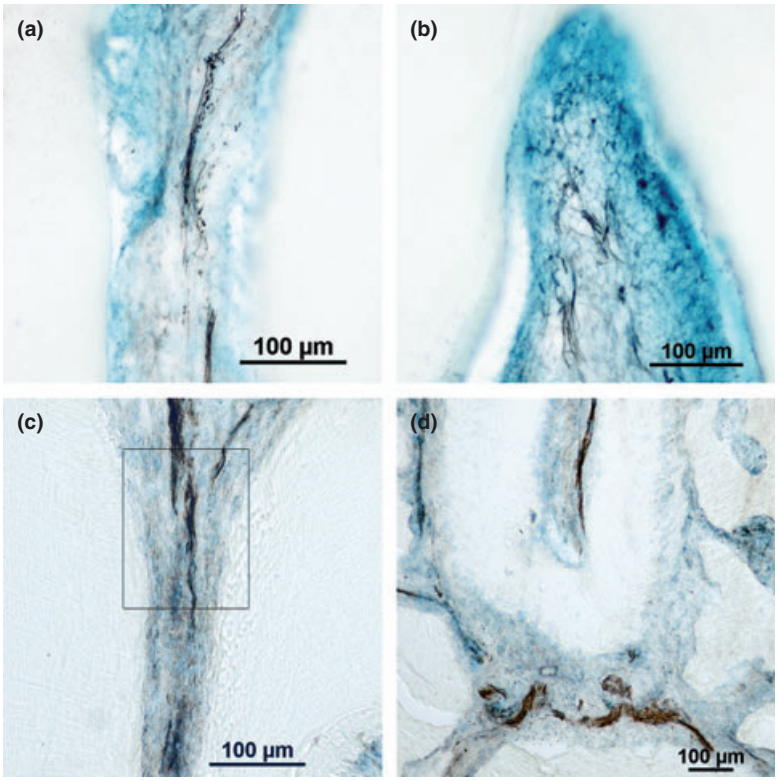

Figure 1 Immunohistochemical microphotographs of rat pulp and periapical area stained for interleukin-1 receptor type I (IL-1RI). Long structures stained for IL-1RI were seen centrally in the root (a) and coronal (b) pulp under normal conditions, and were not extended to the odontoblast layer. One week after pulp exposure, heavy staining for IL-1RI was observed in the inflamed pulp (c) and periapical area (d). Boxed area in (c) is shown is Fig. 3a.

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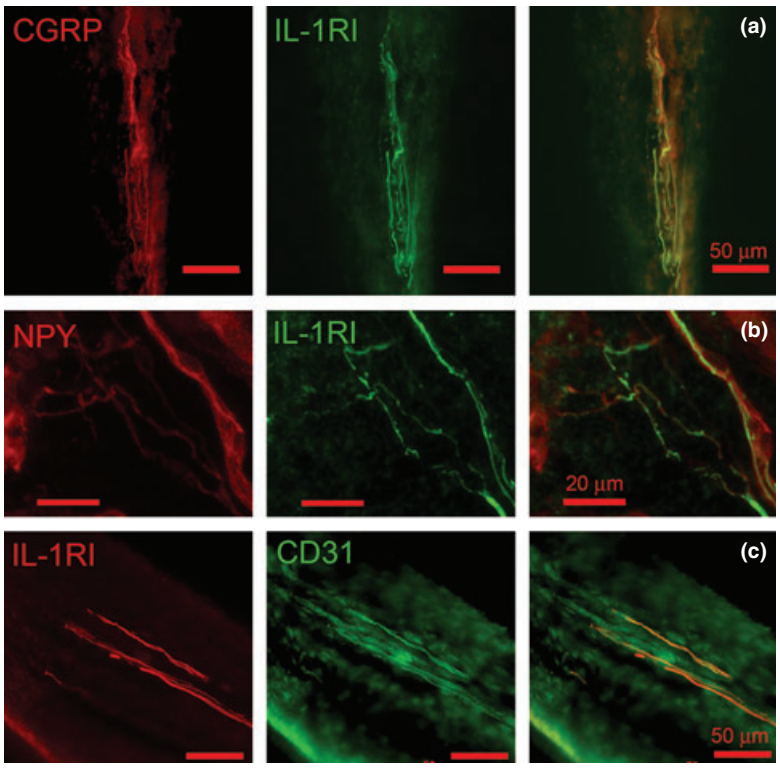

Figure 2 Localization of interleukin-1 receptor type I (IL-1RI) in normal rat pulp. Double immunofluorescence with IL-1RI and calcitonin gene-related peptide (CGRP) (a) or neuropeptide Y (NPY) (b) or CD31 (c) revealed that IL-1RI is localized on sensory and sympathetic nerve fibres, as well as, on blood vessels of the normal pulp. Right images in all panels are merged.

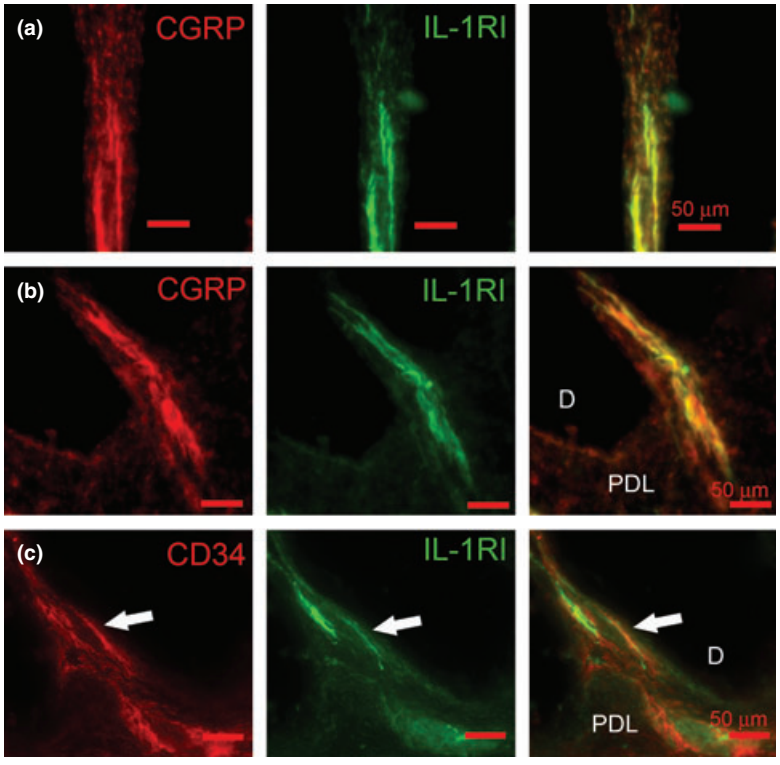

Figure 3 Localization of interleukin-1 receptor type I (IL-1RI) in inflamed rat pulp. During pulpal inflammation, strong immunoreactivity for IL-1RI was seen on sensory nerve fibres in the root pulp (a) and periapical area (b) of exposed pulps. IL-1RI was partly localized on the wall of CD34-immunoreactive blood vessels (c, arrow). D: dentine; PDL: periodontal ligament. Right images in all panels are merged.

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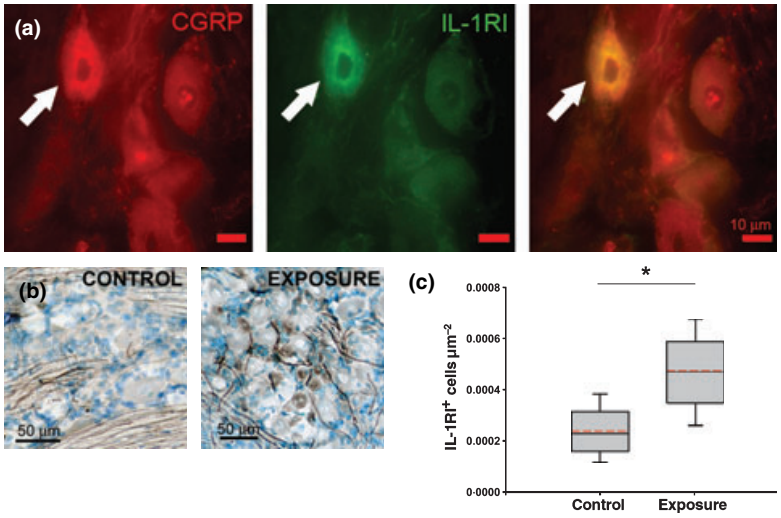

Figure 4 Interleukin-1 receptor type I (IL-1RI) expression in rat trigeminal ganglion (TG) changes during inflammation. Double immunofluorescence staining with IL-1RI and calcitonin gene-related peptide (CGRP) revealed that sensory neurons in the TG are also immunoreactive with IL-1RI (a). However, increased immunoreactivity for IL-1RI was observed in the TG of the side that pulp exposures were performed (b). The ratio of IL-1RI+ cells per μm^2 of TG was doubled upon inflammation (Mann–Whitney Rank Sum test) (c). Data were expressed as mean (dotted line) ± SD, $n = 90$ counted areas,*$P < 0.001$.

fibres indicates a direct effect of the pro-inflammatory cytokine IL-1 on the above structures.

As pointed out earlier, vascular cells are an important target for IL-1. Change in the structural integrity of the vascular wall is one mechanism for oedema formation, and widespread pulpitis with increased fluid filtration in the encapsulated pulp tissue may lead to circulatory failure (Bletsa *et al.* 2006b). In a recent study in which an animal model of retrograde pulpitis was used, an increased amount of IL-1 in the interstitial fluid (IF) of incisor pulp was found, which was shown to be produced locally (Bletsa *et al.* 2006b). In the same study, measurements of colloid osmotic pressure in plasma and pulp IF led to the conclusion that the permeability of pulp vessels was increased during pulpitis. Taken together with the present findings of IL-1RI on blood vessel walls, this study complements and is in agreement with earlier studies where IL-1 was showed to have a vasoactive effect (Martin *et al.* 1988, Sedgwick *et al.* 2002).

Furthermore, the action of IL-1 as a vasoactive agent can also be supported by the co-localization of IL-1RI on NPY-immunoreactive nerve fibres. Sympathetic fibres are mainly associated with larger blood vessels in the root pulp and deeper parts of the pulp tissue proper (Fristad *et al.* 1994, Zhang *et al.* 1998) and IL-1RI was clearly seen on NPY-labelled nerve fibres surrounding blood vessels. The IL-1RI/NPY co-localization may also be indicative of IL-1-induced sympathetic hyperalgesia. This phenomenon has been first attributed to IL-8, as IL-8 was the first endogenous mediator to be identified as evoking hyperalgesia involving the sympathetic nervous system (Cunha

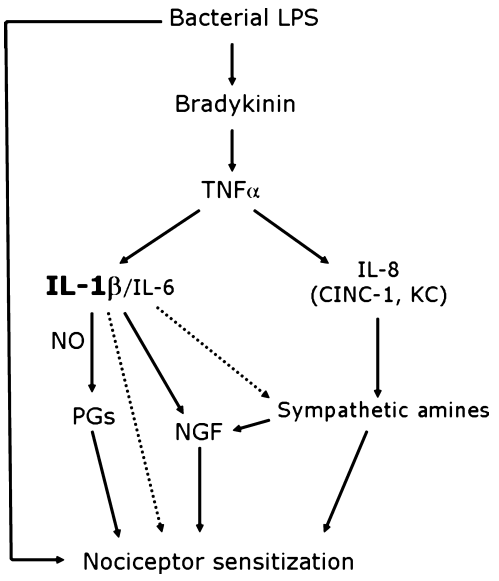

Figure 5 Signalling cascade in the development of inflammatory hyperalgesia. There are many inflammatory mediators that contribute to nociceptor sensitization during inflammation in a rather complex manner. Dotted arrows indicate alternative pathways for IL-1β suggested from the findings of the current study. CINC-1: cytokine-induced neutrophil chemoattractant-1 (rat analogue to IL-8); IL: interleukin; KC: keratinocyte-derived chemokine (mouse analogue to IL-8); LPS: lipopolysaccharide; NGF: nerve growth factor; NO: nitric oxide; PGs: prostaglandins; TNFα: tumour necrosis factor alpha.

et al. 1991), but in the light of the current findings, the contribution of IL-1 cannot be excluded (Fig. 5). Sympathetic neurons have been found to synthesize

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and release IL-1 (Freidin *et al.* 1992) and to express functional IL-1RI (Bai & Hart 1998) *in vitro*, indicating that IL-1 may have a role in the secretory pathway of sympathetic amines and neuropeptides.

In the context of inflammatory pain, an interesting key finding of this work was the localization of IL-1RI on CGRP-immunoreactive nerve fibres. IL-1β has been characterized as a mediator of inflammatory pain (Fukuoka *et al.* 1994, Bianchi *et al.* 1998, Wolf *et al.* 2003), with indirect algogenic action through other inflammatory mediators. Inflammatory pain is characterized by hyperalgesia because of the sensitization of primary sensory nociceptive neurons. The dominant concept in the generation of inflammatory hyperalgesia involves a well defined sequential release of cytokines initiated by production of bradykinin and leading to the synthesis of prostaglandins and release of sympathetic amines (Poole *et al.* 1999) (Fig. 5). Under this concept, the release of cytokines constitutes a link between cellular injury and the release of the (final) hyperalgesic mediators. Confirming this notion, inflammatory agents, such as lipopolysaccharide (LPS), evoked mechanical hypersensitivity in rats by initiating a cascade of events started with bradykinin release, followed by TNFα and in turn, production of IL-1β/IL-6, and of IL-8 (Ferreira *et al.* 1993). It has been demonstrated that IL-1β and IL-6 stimulate the production of eicosanoids (Ferreira *et al.* 1988, Cunha *et al.* 1992) whereas IL-8 and its homologues, rat cytokine-induced neutrophil chemoattractant-1 and mouse keratinocyte-derived chemokine, stimulates the production of sympathomimetic amines (Cunha *et al.* 1991, Lorenzetti *et al.* 2002) (Fig. 5). Furthermore, IL-1β/IL-6 induces NGF, another potent mediator of inflammatory hyperalgesia (Yoshida & Gage 1992, Lewin *et al.* 1994, Sweitzer *et al.* 2001) (Fig. 5). Besides the above indirect mechanisms of nociceptor sensitization, there is recent evidence of a direct LPS-induced pathway as pointed out by the co-localization of Toll-like receptor-4 and CD14 on sensory nerve fibres in pulp and in nociceptive TG neurons (Wadachi & Hargreaves 2006) (Fig. 5).

The presence of IL-1RI on sensory nerve fibres is a strong indication of direct involvement of IL-1 in nociceptor sensitization and this is one of the few reports on the localization of IL-1RI on sensory nerve fibres. The co-localization of IL-1RI with CGRP-immunoreactive nerve fibres was seen in normal pulp and during inflammation in the time frame where sprouting of CGRP-labelled nerve fibres in inflamed pulp and periapical area takes place (Khayat *et al.* 1988, Byers *et al.* 1990). Moreover, this study revealed that IL-1RI is expressed in the somata of the trigeminal neurons and this expression was increased when pulpitis was induced. The above findings are in accordance with a recent study (Liu *et al.* 2006) where IL-1RI was not only found to be expressed in the TG, but also in the peripheral trigeminal nerve endings of the snout skin in rats. They reported that the majority of the IL-1RI$^+$ trigeminal neurons were of small diameter (i.e. <30 μm), which indicates the presence of IL-1RI on nociceptor neurons (Takeda *et al.* 2005, 2007). The size of IL-1RI-immunoreactive neurons in the TG was not measured in this study, but the up-regulation was mainly observed in the small diameter neurons during inflammation. Previously, expression of IL-1RI has been detected in dorsal root ganglia neurons (Copray *et al.* 2001, Obreja *et al.* 2002), suggesting a possible autocrine or paracrine influence of IL-1 on sensory processing.

The findings from the TG are in line with a recent study in which inflammatory hyperalgesia in rat whisker pad led to increased IL-1RI immunoreactivity in the TG neurons innervating the facial skin (Takeda *et al.* 2007). The authors proposed that activation of satellite glial cells within the TG modulates the excitability of small-diameter TG neurons via IL-1β following inflammation. Activation of satellite glial cells within the TG is known to occur after tooth pulp injury (Stephenson & Byers 1995) and IL-1β is synthesized and released not only by immune cells but also by neuronal and glial cells under pathological conditions (Braddock & Quinn 2004). This is in line with the present findings from selective sections of right (pulp exposure) TG that showed IL-1β$^+$ cells in the vicinity of IL-1RI-immunoreactive neurons. Taken together, the above findings suggest that inflammation induces IL-1β production by glial cells and/or neuronal cells in the TG, which in turn leads to up-regulation of IL-1RI in small-diameter nociceptive TG neurons with a paracrine manner. Alternatively, binding of IL-1 on the peripheral nerve endings may result in up-regulation of IL-1RI in small diameter TG neurons. Thus, IL-1 receptor blockers may be potential therapeutic agents for inflammatory hyperalgesia.

Conclusions

Summarizing, the well-established algogenic role of IL-1 (IL-1β) during inflammation has previously been attributed to induction of a variety of other hyperalgesic mediators. This study provides evidence of a more

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direct action of IL-1 on sensory nerves in dental pulp and TG neurons, which may be another mechanism for inflammatory hyperalgesia. In addition, IL-1 may directly increase pulp vessel permeability. Thus, targeting IL-1 may provide new opportunities for therapeutic intervention during pulpitis.

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