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# Substance P influenced gelatinolytic activity via reactive oxygen species in human pulp cells

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## Abstract

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**Aim** To investigate the effects of substance P (SP) on gelatinolytic activity of matrix metalloproteinases (MMPs) in human pulp cells.

**Methodology** Human dental pulp cells were isolated and cultured. Subconfluent cells, between the third and sixth passages, were maintained under serum deprivation for 18 h followed by the treatment of varying doses of SP (1 pmol $L^{-1}$, 100 pmol $L^{-1}$, 10 nmol $L^{-1}$, 1 μmol $L^{-1}$ and 100 μmol $L^{-1}$). Conditioned media were then subjected to gelatin zymography using 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis minigels containing 1.5 g $L^{-1}$ gelatin. The effect of SP on intracellular reactive oxygen species (ROS) was also examined by confocal microscopy. ROS scavenger *N*-Acetyl-L-cysteine (NAC, 5 mmol $L^{-1}$) was utilized to evaluate the roles of ROS pathway in mediating the impact of SP on cellular gelatinolytic activity. Data were analysed using analysis of variance with Bonferroni correction for multiple comparisons or an unpaired Student's *t*-test.

**Results** Substance P, at levels above 1 μmol $L^{-1}$, remarkably enhanced MMP-2 activity reflected by the band migrating at 66 kDa ($P < 0.05$). A gelatinolytic band at approximately 44 kDa appeared to be intensified in a SP dose-dependent manner. In addition, it was demonstrated that SP could induce ROS production in pulp cells and ROS scavenger NAC was further found to significantly reduce MMP-2 activity ($P < 0.05$), as well as other bands of gelatinolytic proteinases.

**Conclusion** Substance P can influence gelatinolytic activity in human pulp cells via ROS pathway.

**Keywords:** gelatinolytic activity, human pulp cells, matrix metalloproteinases, reactive oxygen species, substance P.

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## Introduction

Dental pulp inflammation is a complex process, which is thought to be neurogenically mediated, involving a variety of nervous and vascular reactions that could lead to pulp necrosis (Byers *et al.* 1990, Kim 1990). A number of different neuropeptides, including substance P (SP), are known to be present in the nerve fibres of dental pulp (Casasco *et al.* 1990). Pulp cells were also reported to have the ability to release SP when stimulated by bacterial virulence factor RgpB (Tancharoen *et al.* 2005). In painful pulpitis, tissue level of SP was found to be increased (Awawdeh *et al.* 2002, Bowles *et al.* 2003). SP receptor expression in pulp tissue is also significantly increased during inflammatory phenomena such as acute irreversible pulpitis (Caviedes-Bucheli *et al.* 2007). Moreover, SP is released following dental procedures (e.g. cavity preparation), and its expression may contribute to inflammation and pain. (Caviedes-Bucheli *et al.* 2005).

Recently, SP has been documented to induce the production of inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-alpha in human pulp cell cultures (Patel *et al.* 2003, Park *et al.*

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2004, Yamaguchi *et al.* 2004). SP can also enhance expression of lipopolysaccharide-induced inflammatory factors in pulp cells (Tokuda *et al.* 2004). These cytokines are thought to be important in the pathogenesis of pulpitis. Therefore, SP may initiate, exacerbate or maintain the inflammatory processes in pulpitis.

A notable feature of suppurative pulpitis is the degradation of the extracellular matrix (ECM) (Gusman *et al.* 2002, Shin *et al.* 2002, Wahlgren *et al.* 2002, Tsai *et al.* 2005). Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases responsible for degrading structural proteins of ECM and for cleaving other non-ECM molecules ranging from growth factor precursors, cytokines and binding proteins, to cell surface receptors (Sternlicht & Werb 2001). In pulp cell cultures, stimulation of both inflammatory cytokines and bacterial extracts may induce production of MMPs and cell mediated collagen degradation (Panagakos *et al.* 1996, O'Boskey & Panagakos 1998, Lin *et al.* 2001, Chang *et al.* 2002, Wisithphrom & Windsor 2006, Wisithphrom *et al.* 2006). However, little information is currently known regarding the effects of SP on MMPs activity of pulp cells.

Reactive oxygen species (ROS) have emerged as essential signalling molecules in the regulation of inflammation processes (Springer *et al.* 2005, McCubrey *et al.* 2006, Nakano *et al.* 2006). Meantime, intracellular ROS can translocate into the ECM and oxidize latent MMP-2 complexes (Wang *et al.* 2005). To date, SP has been shown to induce ROS generation in rat microglia (Block *et al.* 2006) and A549 airway epithelial cells (Springer *et al.* 2005). The purpose of this study was to investigate the effects of SP on gelatinolytic activity of MMPs in human pulp cells and the possibility of ROS involvement.

## Materials and methods

### Isolation and culture of human pulp cells

Normal human impacted third molars were obtained from healthy donors, with informed consent, at the West China Stomatology Hospital of Sichuan University. The study was reviewed and approved by the ethical board of the hospital. Coronal pulp tissue was gently separated and minced with scalpels. Dental pulp cells were enzymatically released as previously described (Wang *et al.* 2006), then cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% foetal calf serum (Hyclone, South Logan, UT, USA), 2 mmol $L^{-1}$ L-glutamine, 100 units $mL^{-1}$ penicillin and 100 μg $mL^{-1}$ streptomycin. Confluent cells were detached with 0.20% trypsin and 0.02% ethylenediaminetetraacetate (EDTA). Immunocytochemical staining using anti-vimentin antibody was performed for tissue origin characterization. Fibroblast-like pulp cells between the third and sixth passages were used in the following. Cells were pre-incubated with serum-free DMEM for 18 h before the addition of other factors.

### Detection of intracellular ROS by confocal microscopy

The formation of intracellular ROS was determined by the 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence method (Wang *et al.* 2007). For experiments to determine the generation of ROS induced by SP, cells ($6 \times 10^4$ per dish) were plated and allowed to adhere on 35-mm glass-bottomed culture dishes (WPI Inc., Berlin, Germany) for 24 h. After the starvation, the serum-deprived cells were stabilized in serum-free DMEM without phenol red for at least 30 min. Then cells were loaded with a cell-permeant probe $H_2$DCFDA (Sigma, St. Louis, MO, USA) at 10 μmol $L^{-1}$ for 30 min. Afterwards, the medium was aspirated, and the cells were washed twice in media followed by the treatment with or without (control) 1 μmol $L^{-1}$ SP for 30 min. Where indicated, the cells were treated with 5 mmol $L^{-1}$ *N*-Acetyl-L-cysteine (NAC) (Alexis, San Diego, CA, USA) for 1 h before the treatment of SP. To assess ROS-mediated oxidation of $H_2$DCFDA to the fluorescent compound DCF, cells were immediately observed with a laser scanning confocal microscope (Leica TCS SP2, Leica, Wetzlar, Germany). Images (20 × objective) were recorded at an excitation wavelength of 488 nm and an emission wavelength 530 nm.

### Gelatin zymography

Cells were seeded at a density of $5 \times 10^4$ per well in 24-well plates (Corning, Corning, NY, USA) and grown to confluence. After the starvation, cells was washed once in fresh serum-free media and treated different doses of SP (1 pmol $L^{-1}$, 100 pmol $L^{-1}$, 10 nmol $L^{-1}$, 1 μmol $L^{-1}$ and 100 μmol $L^{-1}$) for 48 h. Where indicated, the cells were treated with 5 mmol $L^{-1}$ NAC for 1 h before the treatment of SP. Then conditioned media were collected and stored at −20 °C until assayed.

The gelatinolytic activity of the conditioned media was assayed by gelatin zymography (Gusman *et al.* 2002) using 8% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis minigels containing 1.5 g $L^{-1}$ gelatin (Merck, Darmstadt, Germany). The samples (each 20 μL) were mixed 4 : 1 with 5 × nonreducing sample buffer containing 0.25 mol $L^{-1}$ Tris–HCl (pH 6.8), 50% glycerol, 10% SDS and 0.005% bromophenol blue, before loaded onto the gels. After electrophoresis, gels were washed twice for 20 min in 2.5% Triton X-100 on a rotary shaker to remove SDS and to allow proteins to renature. The gels were then incubated in a buffer containing 50 mmol $L^{-1}$ Tris–HCl (pH 7.5), 100 mmol $L^{-1}$ NaCl, 5 mmol $L^{-1}$ $CaCl_2$, $ZnCl_2$ and 0.002% $NaN_3$ for 24 h at 37 °C. Zymographic gels were stained with 0.1% Coomassie Brilliant Blue R-250 and de-stained. Gels were photographed with the Gel Doc 2000 system (Bio-Rad, Hercules, CA, USA) and analysed with Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

### Statistical analysis

All assays were repeated at least three times to ensure reproducibility. Values in zymography assay were expressed as mean values and standard deviations of integrated optical density (per cent of control). All values obtained were analysed using analysis of variance (ANOVA) with correction for multiple comparisons (Bonferroni) or an unpaired Student's *t*-test ($n = 6$), with a significance level of $P < 0.05$.

## Results

### SP increased levels of ROS

The basic levels of ROS were very low (Fig. 1a). As shown in Fig. 1b, it was obvious that the intracellular ROS levels increased after the stimulation of SP. Conversely, significant inhibition of ROS generation was observed when the cells were pre-treated by the antioxidant NAC before the exposure of SP (Fig. 1c).

### SP influenced gelatinolytic activity of pulp cells

The gelatin zymography indicated that there were several kinds of gelatinolytic proteinase in the conditioned medium of pulp cells (Fig. 2). The major proteinase evident in the gels migrated at approximately 66 kDa, which corresponded to the active form of MMP-2. Interestingly, a less evident gelatinolytic band was visualized at about 116 kDa. A band with molecular masses between 66 and 97 kDa, presenting MMP-9, was very weak.

Following 1 or 100 μmol $L^{-1}$ SP stimulation, MMP-2 activity was significantly enhanced (ANOVA, $P < 0.05$) (Fig. 3). In this experiment, a gelatinolytic band at approximately 44 kDa was detected for the first time. Its intensity appeared to be SP dose dependent (Fig. 2). As shown in Figs 2 and 3, following the pre-treatment of cells with antioxidant NAC, MMP-2 activity was significantly inhibited (Student's *t*-test, $P < 0.05$) and the latent form of MMP-2 was evident compared with other lane (Fig. 2). In addition to MMP-2, other gelatinolytic bands also turned weaker after NAC treatment (Fig. 2).

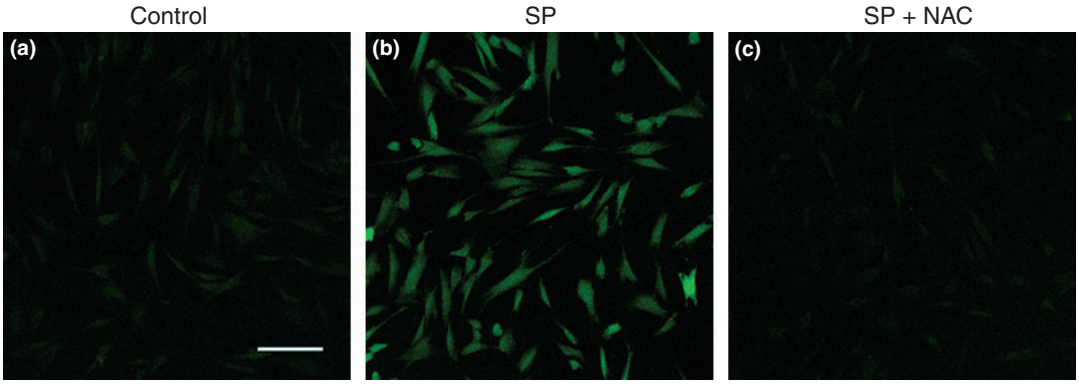


**Figure 1** Substance P (SP) increased intracellular levels of reactive oxygen species (ROS) in human pulp cells. Human pulp cells cultured on glass-bottomed dishes were loaded with 10 μmol $L^{-1}$ 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) for 30 min and then stimulated without (a) or with (b) 1 μmol $L^{-1}$ SP for 30 min. Pre-treatment with 5 mmol $L^{-1}$ *N*-Acetyl-L-cysteine (NAC) for 1 h before the exposure of SP inhibited ROS generation (c). Cells were imaged by confocal microscopy using 20 × objective. Bar, 150 μmol $L^{-1}$. Results were representative of three independent experiments.

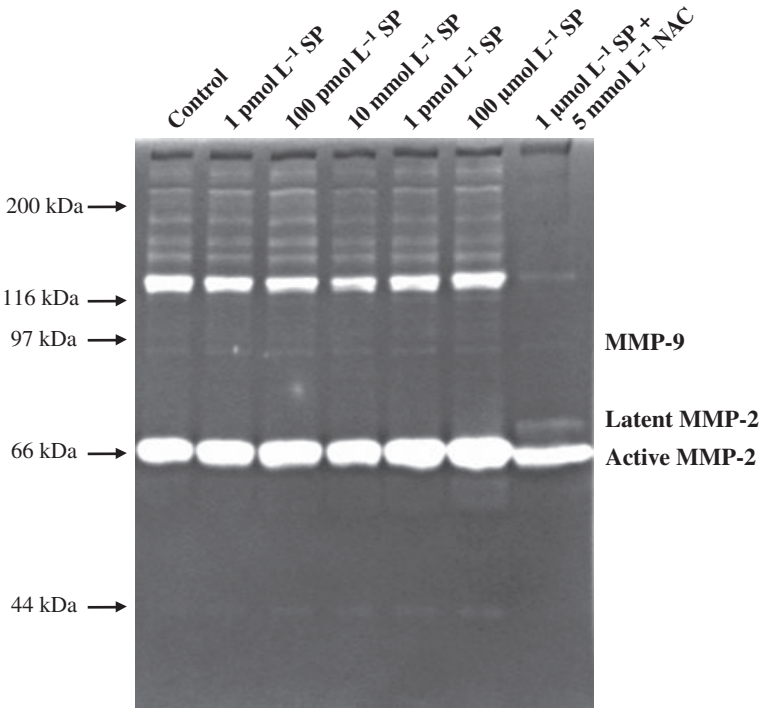


**Figure 2** Gelatin zymography of conditioned media from pulp cells incubated for 48 h with no treatment (control) and different doses of substance P (SP) (1 pmol $L^{-1}$, 100 pmol $L^{-1}$, 10 nmol $L^{-1}$, 1 μmol $L^{-1}$ and 100 μmol $L^{-1}$); 5 nmol $L^{-1}$ *N*-Acetyl-L-cysteine (NAC) was also used as antioxidant to assess the role of reactive oxygen species (ROS) in gelatinolytic activity. Results were representative of three independent experiments.

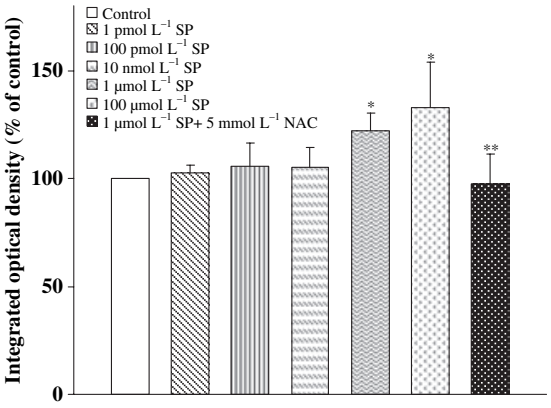


**Figure 3** Matrix metalloproteinase (MMP)-2 activity of conditioned media from pulp cells in response to 48 h treatment of substance P (SP) or in the presence of 5 mmol $L^{-1}$ *N*-Acetyl-L-cysteine (NAC). Values were expressed as means + SD of integrated optical density (IOD) (per cent of control). Statistically significant: $^{*}P < 0.05$, compared with control; $^{**}P < 0.05$, compared with treatment of 1 μmol $L^{-1}$ SP.

## Discussion

Toothache is the most common orofacial pain and often involves pulpitis. However, the pathological mechanisms of painful pulpitis still remain to be elucidated. It is known that dental pulp tissue is innervated with a subpopulation of sensory neurons containing neuropeptides, such as SP. These neuropeptides are associated with the development of neurogenic inflammation. The present study, therefore, was designed to investigate the mechanisms of ECM degradation in the aspect of SP.

In this study, intracellular ROS levels were measured using the fluorescence dye $H_2$DCFDA, which is a nonpolar compound that is converted into a nonfluorescent polar derivative ($H_2$DCF) by cellular esterases after incorporation into cells. $H_2$DCF is membrane impermeable and rapidly oxidized to the highly fluorescent DCF in the presence of intracellular ROS (Gomes *et al.* 2005). It was shown that after SP stimulation, ROS generation was induced in human pulp cells (Fig. 1b). ROS scavenger NAC significantly reduced the intracellular ROS level of pulp cells in response to SP (Fig. 1c). These findings suggest that ROS may mediate SP signalling, which is consistent with previous studies using other cell lines (Springer *et al.* 2005, Block *et al.* 2006).

Gelatin zymography has been extensively used to examine gelatinolytic activity of pulp cells. The results suggest that in the conditioned medium from pulp cells, MMP-2 is the major gelatinase and present mostly in active form migrating at 66 kDa whilst, only minimal

amounts of MMP-9 could be detectable. These data are in agreement with several previous studies examining conditioned medium using gelatin zymography (Panagakos *et al.* 1996, O'Boskey & Panagakos 1998, Ueda & Matsushima 2001). When cells were exposed to SP at levels above 1 μmol $L^{-1}$, MMP-2 activity was significantly enhanced. However, SP at levels below 1 μmol $L^{-1}$ did not influence MMP-2 activity significantly, although these levels of SP can activate p38 and ERK signalling in cultured pulp cells (Tokuda *et al.* 2005). This phenomenon might be due to high basic levels of active MMP-2 in pulp cells cultured *ex vivo*. However, the levels of immunoreactive SP in inflamed pulp are around 0.1 nmol $L^{-1}$ (Bowles *et al.* 2003). It means that native pulp cells might be more sensitive to SP and the supra-physiological concentration range of SP (>1 μmol $L^{-1}$), which has been selected in many experiments, is not suitable to clinical pulp inflammation. In the pathogenesis of clinical pulpitis, MMPs can be secreted by infiltrating cells such as polymorphonuclear leukocytes and macrophages (Gusman *et al.* 2002, Shin *et al.* 2002, Wahlgren *et al.* 2002). Complicated interaction between those infiltrating cells and pulp cells might affect the sensitivity of pulp cells to SP regarding to their MMPs production. As *ex vivo* culture models can mimick the natural environment, the present results at least indicated that the production of MMP-2 in pulp cells might be affected by tissue SP levels. Recently, it has been indicated that inflammatory cytokines stimulate the production of elevated levels of MMP-2 in pulp cells (O'Boskey & Panagakos 1998, Chang *et al.* 2001, Wisithphrom & Windsor 2006). As SP has been shown to up-regulate inflammatory cytokines (Patel *et al.* 2003, Park *et al.* 2004, Yamaguchi *et al.* 2004), one may speculate that cytokines might directly or indirectly mediate the enhancement of MMP-2 activity. Additionally, it is interesting to note that a band migrating at 44 kDa appeared to be intensified in a SP-dose-dependent manner (Fig. 2). Detailed information of the corresponding proteinase needs further investigation.

According to the gelatin zymography results, inhibition of ROS production significantly reduced MMP-2 activity of pulp cells exposed to SP. The band migrating at 44 kDa as well as those bands between 116 and 200 kDa were all attenuated by NAC treatment. It was notable that NAC pre-treatment caused appearance of latent MMP-2 in the conditioned medium, which suggests that ROS might be essential for the activation of latent MMP-2. Interestingly, previous study also reported that ROS was required for conversion of latent MMP-2 to its active form (Wang *et al.* 2005). As is suggested, ROS work through disturbance of the cysteine–zinc interaction of the cysteine switch in inactive zymogens (Hannas *et al.* 2007). Taken together, the present results imply that ROS play an important role in ECM degradation by proteinase secreted from pulp cells. Therefore, it can be proposed that the reduction of ROS levels by using antioxidants might be of potential value in preventing ECM degradation during pulpitis.

## Conclusions

In summary, neuropeptide SP can influence gelatinolytic activity in pulp cells, which is mediated by a ROS-dependent pathway. Nevertheless, the present study only examined effects of SP on gelatinolytic activity in conditioned medium from pulp cells. Except for gelatinases, MMPs also include collagenases, stromelysins, membrane-type MMPs, minimal-domain MMPs and others. *In vivo* MMPs activities are always regulated by their interaction with the tissue inhibitors of matrix metalloproteinases (TIMPs). Thus, future investigation should be undertaken to clarify whether SP also influence other MMPs activities as well as TIMPs.

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