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Norepinephrine stimulates calprotectin expression in human monocytic cells

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Background and objective: Calprotectin is composed of two proteins, S100A8 and S100A9, which are S100 family members, and is detected in gingival crevicular fluid and gingival tissue with inflammation. The release and production of calprotectin are regulated by lipopolysaccharides of periodontopathic bacteria and cytokines. Emotional or psychological stress, a risk factor of periodontal disease, is transmitted by stress modulators including norepinephrine and cortisol. The aim of the present study was to investigate the effect of stress on calprotectin expression using norepinephrine and cortisol.

Methods: U-937 cells, a human monocytic cell line, were incubated with norepinephrine in the presence or absence of β- or α-adrenergic receptor antagonists, or with cortisol. The expression of S100A8/S100A9 mRNAs was examined by northern blotting and the amount of calprotectin was measured by enzyme-linked immunosorbent assay (ELISA). The DNA binding activity of C/EBPα (CCAAT enhancing binding protein), a transcription factor, was examined by electrophoretic mobility shift assay.

Results: Norepinephrine stimulated the expression of S100A8/S100A9 mRNAs via β-adrenergic receptors in U-937 cells and significantly increased calprotectin production to about 3.6-fold that of the control. However, cortisol had no effect on calprotectin expression at the mRNA and protein levels. Norepinephrine elevated C/EBPα DNA binding activity, but cortisol did not increase the activity.

Conclusion: Norepinephrine, a stress modulator, stimulated calprotectin expression in human monocytic cells. Calprotectin expression may be regulated by stress in addition to inflammatory factors.

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Calprotectin is a calcium-binding protein produced by neutrophils, monocytes/macrophages and epithelial cells, and is composed of two subunits, S100A8 and S100A9 (1, 2). It is known that calprotectin increases in several inflammatory diseases, and is also detected in the infiltrated inflammatory cells and epithelial cells of gingival tissue from periodontitis patients (3–5). Previously, we reported that the calprotectin level in gingival crevicular fluid of periodontitis patients was significantly higher than that of healthy subjects and suggested that calprotectin is a useful marker for periodontitis (6, 7). Furthermore, we have recently found that the production and release of calprotectin are induced by a lipopolysaccharide of *Porphyromonas gingivalis* and inflammatory cytokines in human neutrophils and monocytes (5, 8–10). Calprotectin inhibits the growth of some microbes, including *P. gingivalis* (unpublished data) and *Candida* (11), via a zinc-chelating action, and suppresses *P. gingivalis* adhesion to epithelial cells (12), showing that calprotectin has antimicrobial action. This protein has chemotactic activity and regulates adhesion and migration of neutrophils or monocytes (2, 13). These reports and findings suggest that calprotectin acts on inflammatory processes and plays a role in host defense by innate immune responses in periodontal diseases.

Emotional or psychological stress is well known as a risk factor for

periodontal diseases and a predisposing factor of necrotizing ulcerative gingivitis, and financial and academic stresses were reported to aggravate the inflammation and degradation of periodontal tissues in periodontal disease (14–16). Emotional or psychological stress causes significant effects on immune responses via pathways involving the central nervous, neuroendocrine and immune systems. In immune responses induced by stress signals from the central nervous system (CNS), two pathways mainly function: sympathetic nerve fibers of the autonomic nervous system and the hypothalamic–pituitary–adrenal axis. Stress stimulates norepinephrine release from sympathetic nerve fibers and adrenal medulla cells, and norepinephrine regulates the differentiation and functions of lymphocytes, and cytokine production (14, 16), suggesting that stress-induced norepinephrine regulates cellular immune responses.

In the hypothalamic–pituitary–adrenal axis stimulated by stress, glucocorticoid (cortisol) is released from the adrenal cortex by the actions of endocrine hormones, reduces the number of inflammatory cells in the circulation or at inflammatory sites, and inhibits the physiological actions of these cells (14–16). Breivik *et al*. (14) and Genco *et al*. (15) reviewed studies showing that emotional or psychological stress regulated the cellular immune response of periodontal tissues via norepinephrine and glucocorticoid, and affected the progression of periodontal disease.

Monocytes infiltrate from blood vessels into gingival tissues with periodontal disease, and produce and release inflammatory cytokines in response to stimulation by periodontal virulence factors. Peripheral blood monocytes and monocytic cell lines express or release calprotectin in response to stimulation by several factors including tumor necrosis factor-α, interleukin-1β and vitamin D3 (5, 10, 17). Furthermore, Klempt *et al*. (18) showed that C/EBPα (CCAAT enhancing binding protein), a transcription factor, was sufficient for the elevation of S100A9 expression in a monocytic cell line of HL-60 cells.

To elucidate the effects of stress on calprotectin expression, in the present study we investigated the actions of norepinephrine and cortisol, major stress modulators, on calprotectin expression in human monocytic cell line U-937 cells, and also examined the actions of norepinephrine and cortisol on C/EBPα DNA binding activity.

Material and methods

Cell cultures

U-937 cells (ATCC No. CRL-1593.2), a human monocytic cell line, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. U-937 cells were seeded in the culture medium at 1×10^7 cells/ml and incubated with 10^{-5} M norepinephrine (Sigma Chemical Co., St. Louis, MO, USA) or 3×10^{-6} M cortisol (Wako Pure Chemical Industries, Osaka, Japan) for 30 min (C/EBPα binding assay), 18 h (S100A8/A9 mRNA assay) or 36 h (calprotectin determination). In other experiments, cells were incubated with norepinephrine (10^{-5} M) in the presence or absence of 10^{-5} M propranolol (β1- and β2-adrenergic receptor antagonist, Sigma), 10^{-5} M atenolol (β1-adrenergic receptor antagonist, Sigma) or 10^{-5} M pentholamine (α-adrenergic receptor antagonist, Sigma) for 18 h.

Northern blotting

Total RNA was extracted from U-937 cells by the phenol–guanidinium thiocyanate–chloroform method using TRIZOL® Reagent (Invitrogen Life Technologies, Burlington, ON, Canada). Twenty micrograms of RNA was electrophoretically separated on 1% agarose gel–6% formaldehyde, and transferred to Hybond N^+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The cDNA probes for S100A8, S100A9 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were synthesized by polymerase chain reaction (PCR) amplification using the following primers: S100A8: 5′-GCTGGAGAAAGCCTTGAACTC-3′ and 5′-CCACGCCCATCTTTATCACCA-3′; S100A9: 5′-TCGCAGCTGGAACGCAACATA-3′ and 5′-AGCTCAGCTGCTTGTCTGCAT-3′; GAPDH: 5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′. These probes were labeled with α-[^{32}P]dCPT (Amersham Pharmacia Biotech) using a BcaBEST™ Labeling Kit (TaKaRa, Kusatu, Japan). Prehybridization and hybridization were performed at 42°C for 8 h and at 42°C for more than 12 h, respectively. The density of autoradiography bands was determined using a Fujix Bioimaging Analyzer (BAS 2000 II, Fuji Photo Film Co., Tokyo, Japan) and their levels were normalized to that of GAPDH mRNA.

Calprotectin determination

After the incubation of U-937 cells with norepinephrine or cortisol for 36 h, the cell and medium fractions were separated by centrifugation at 750 *g* for 5 min and mixed with a protease inhibitor cocktail. The amount of calprotectin in the cell and medium fractions was determined using Calprest® (CalproAS, Trieste, Italy), as described previously (5). Briefly, the samples were added to microtiter plates precoated with an anticalprotectin antibody and incubated for 45 min at room temperature. After washing the plate, an alkaline phosphatase conjugated antibody was added to the wells in the plate and it was incubated for 45 min at room temperature and washed. The plate was then incubated with substrate solution containing *p*-nitrophenol phosphate for 30 min at room temperature in the dark. The amount of calprotectin was calculated by comparison with a standard curve.

Electrophoretic mobility shift assays (EMSA)

Nuclear extract was isolated from U-937 cells using a NucBuster™ Protein Extraction Kit (Novagen, Darmstadt, Germany). Briefly, the incubated cells were collected by centrifugation (750 *g*, 5 min, 4°C) and the cell pellet was suspended in extraction reagent I

contained in the kit and incubated on ice for 5 min. After centrifugation (16,000 *g*, 5 min, 4°C), the precipitate was washed with phosphate-buffered saline and dissolved in extraction reagent II supplemented with 100 mM dithiothreitol and a protease inhibitor cocktail, incubated on ice for 5 min and centrifuged at 16,000 *g* for 5 min at 4°C. The supernatant was used as a nuclear extract. EMSA was performed using a DIG Gel Shift Kit (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, a double-stranded oligonucleotide containing a C/EBPα binding site (GGTTTGCTGCTTAAGATGCCTG) was used as a probe and labeled with digoxigenin (DIG). Ten micrograms of nuclear extract was incubated with DIG-labeled oligonucleotide (1 pmol) in the reaction mixture for 30 min at room temperature. For the competition assay, 100 pmol unlabeled oligonucleotide was added to the nuclear extract prior to the addition of a labeled probe. The C/EBPα binding complex was electrophoresed on 5% native polyacrylamide gel in 0.4 × Tris–borate–EDTA buffer (pH 8.0), transferred to a positively charged nylon membrane and reacted with an anti-DIG antibody conjugated with alkaline phosphatase and then with CSPD® (Tropix, Inc, Bedford, MA, USA), a chemiluminescent substrate for alkaline phosphatase.

Statistical analysis

Statistical analyses were performed by unpaired, two-tailed Student's *t*-test between each experimental group and the control. Differences of $p < 0.01$ were considered statistically significant.

Results

Norepinephrine stimulates S100A8 and S100A9 mRNA expression

Norepinephrine (10^{-5} M) clearly increased the expression of S100A8 and S100A9 mRNAs in U-937 cells to about six and four-fold those of non-treated cells (control), respectively (Fig. 1). However, cortisol (3×10^{-6} M) did not show significant effects on the expression of S100A8/S100A9 mRNAs, and cortisol at other concentrations ($3 \times 10^{-9} \sim 3 \times 10^{-5}$ M) had also no effect on their mRNA expression (data not shown). Norepinephrine is known to cause several physiological actions via binding to β-adrenergic receptors. Therefore, it was investigated whether or not norepinephrine stimulated the expression of S100A8/S100A9 mRNAs via β-adrenergic receptors, using several adrenergic receptor antagonists (Fig. 2). Propranolol, a β1- and β2-adrenergic receptor antagonist, strongly blocked the norepinephrine-stimulated expression of S100A8/S100A9 mRNAs, and atenolol, a selective β1-adrenergic receptor antagonist, partially inhibited those expressions. However, pentholamine, an α-adrenergic receptor antagonist, did not have any inhibitory effect. These results demonstrate that norepinephrine stimulates the expression of S100A8/S100A9 mRNAs via β-adrenergic receptors in U-937 cells.

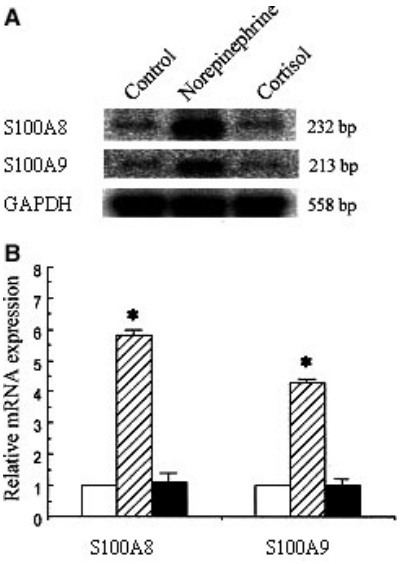

Fig. 1. Effects of norepinephrine and cortisol on the expression of S100A8/S100A9 mRNAs in U-937 cells. Cells were incubated with norepinephrine (10^{-5} M) or cortisol (3×10^{-6} M) for 18 h. The expression of S100A8/S100A9 mRNAs was analyzed by northern blotting (A). The mRNA levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and the data represent the mean of relative mRNA expression ± SD from three separate experiments (B). The open, hatched and closed columns represent control, norepinephrine and cortisol, respectively. Significantly different from control (*$p < 0.01$).

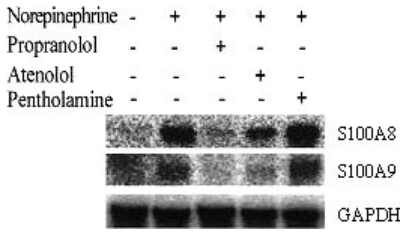

Fig. 2. Effects of propranolol, atenolol and pentholamine on norepinephrine-stimulated expression of S100A8/S100A9 mRNAs in U-937 cells. Cells were incubated with norepinephrine (10^{-5} M) in the presence or absence of propranolol (10^{-5} M), atenolol (10^{-5} M) or pentholamine (10^{-5} M) for 18 h. The expression of S100A8/S100A9 mRNAs was analyzed by northern blotting. Similar results were obtained from three experiments and typical data are shown.

Norepinephrine stimulates calprotectin production

The amount of calprotectin in the cell and medium fractions was determined in order to investigate the effect of norepinephrine on calprotectin production. Norepinephrine (10^{-5} M) increased the amount of calprotectin in the cell fraction to about 3.6-fold that of the control level, but cortisol did not show any significant effect (Fig. 3). The amount of calprotectin in the medium fraction of all samples was less than the detectable limit of the kit used. These results suggest that norepinephrine induces the production of calprotectin following the expression of S100A8/S100A9 mRNAs in U-937 cells.

Norepinephrine increases C/EBPα DNA binding activity

To investigate the mechanism of norepinephrine's action on calprotectin expression, the effect of this stress modulator on C/EBPα DNA binding activity was investigated (Fig. 4). The C/EBPα DNA binding complex was detected in samples of non-treated (control), norepinephrine- and cortisol-treated cells. The activity in norepinephrine-treated cells was stronger than that of control cells, whereas the

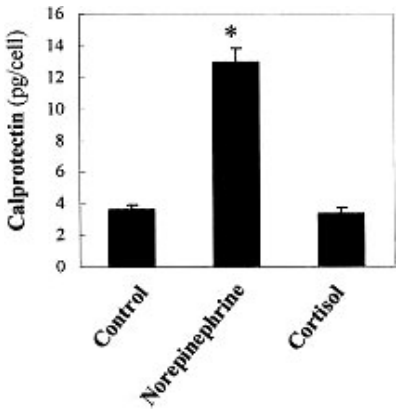

Fig. 3. Effects of norepinephrine and cortisol on calprotectin production in U-937 cells. Cells were incubated with norepinephrine (10^{-5} M) or cortisol (3×10^{-6} M) for 36 h. The amount of calprotectin in cells was determined by enzyme-linked immunosorbent assay. Values are expressed as means ± SD from six samples. Significantly different from control (* $p < 0.01$).

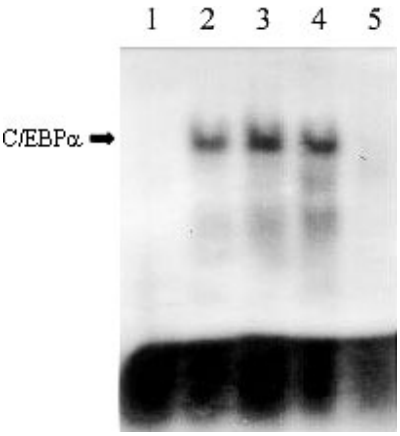

Fig. 4. Effects of norepinephrine and cortisol on C/EBPα DNA binding activity in U-937 cells. Cells were incubated with norepinephrine (10^{-5} M) or cortisol (3×10^{-6} M) for 30 min and nuclear extracts were isolated from the treated cells. C/EBP α DNA binding activity was investigated by electrophoretic mobility shift assays. Lane 1: nuclear extract-free; 2: control; 3: norepinephrine; 4: cortisol; 5: norepinephrine and an excess of unlabeled oligonucleotide for the competition assay. Similar results were obtained from three experiments and typical data are shown.

activity in cortisol-treated cells was similar to that of control cells. The C/EBPα binding complex was not observed in the competitive binding assay using excess unlabeled oligonucleotide. EMSA was performed in three separate experiments and similar results were obtained.

Discussion

Calprotectin is an antimicrobial peptide contained in leukocytes such as defensin, lactoferrin and lysozyme, and is detected in gingival tissue with inflammation and gingival crevicular fluid (1, 4–7, 19). We previously found that inflammatory factors such as *Porphyromonas gingivalis* lipopolysaccharide, tumor necrosis factor-α and interleukin-1β stimulated calprotectin release and its production in human peripheral blood monocytes (5, 10). In the present study, calprotectin expression in monocytes was stimulated by norepinephrine, a stress modulator. A signal of emotional or psychological stress is transmitted to the CNS and activates the autonomic nervous system, inducing norepinephrine release (14–16). Norepinephrine promotes the differentiation of T-cells and antibody production in B-cells, and stimulates the expression of interleukin-10, an immunomodulated cytokine, in monocytic cells (20, 21), suggesting that norepinephrine activates immune responses. Acute stress enhances skin immunity by stress-induced leukocyte trafficking and cytokine expression via norepinephrine (22), and also enhances innate immunity, but suppresses acquired immunity (23). There are few reports related to norepinephrine action in periodontal diseases. Roberts *et al.* (24) reported that norepinephrine promoted the growth of periodontopathic bacteria, including *Actinomyces naeslundii* and *Eikenella corrodens*, but inhibited that of *P. gingivalis* and *Bacteroides forsythus*, and suggested that norepinephrine showed a contrary action in the pathogenesis of periodontal diseases. We speculate that stress-induced norepinephrine may initiate a protective response in periodontal tissues via calprotectin because calprotectin induces the recruitment of leukocytes to inflammatory sites, shows antimicrobial action against *P. gingivalis*, and plays a significant role in innate immunity (1, 2, 13).

Norepinephrine causes stimulative and suppressive actions via binding with α- and β-adrenergic receptors in organs innervated by noradrenergic nerve fibers. Norepinephrine-induced S100A8/S100A9 expressions were inhibited by β-adrenergic receptor antagonists (propranolol and atenolol) in U-937 cells (Fig. 2), and isoproterenol, a β-adrenergic agonist, also increased the expression of S100A8/S100A9 mRNAs to a similar level as norepinephrine (data not shown). U-937 cells possess β-adrenergic receptors (25). These results indicate that norepinephrine up-regulates S100A8/S100A9 expression via β-adrenergic receptors in U-937 cells.

Glucocorticoid (cortisol) is also known as a major stress modulator in addition to catecholamines (14–16). This steroid inhibits the number or accumulation of neutrophils, monocytes/macrophages and lymphocytes *in vivo*, and their functions including chemotaxis, secretion, antigen presentation and the production of several cytokines (15), indicating that glucocorticoid suppresses immune responses. In the present study, cortisol ($3 \times 10^{-9} \sim 3 \times 10^{-5}$ M) did not show any significant effect on calprotectin expression in monocytic cells, whereas Gebhardt *et al.* (26) reported that dexamethasone, a synthetic glucocorticoid, inhibited phorbol ester-induced S100A8/S100A9 expression in murine skin. We do not yet know the effect of this steroid on calprotectin expression because U-937 cells were not stimulated by any factors before the treatment with cortisol in the present experiments.

C/EBPα is a member of the basic leucine zipper class of transcription factors and is implicated in the differentiation of hepatocytes, adipocytes and myelomonocytic cells (27, 28). C/EBPα binding sites are located on the promoter region of the human S100A9 gene and C/EBPα is a significant transcription factor for S100A9 expression in monocytic cells (29). Norepinephrine elevated C/EBPα DNA binding activity compared to control and cortisol treatment in U-937 cells (Fig. 4), and this effect of norepinephrine may be related to the finding

that it increased the expression of S100A8/S100A9 mRNAs and calprotectin. Norepinephrine stimulates C/EBPα expression via β-adrenergic receptors in mature brown adipocytes (30). In myeloid cells, the expression of lactoferrin, antimicrobial protein, is transcriptionally regulated by C/EBPα and C/EBPε (31). Our results and some other reports suggest that norepinephrine increases the production of antimicrobial proteins such as calprotectin and lactoferrin via the stimulation of C/EBPα DNA binding activity.

We have reported the relationship between calprotectin and periodontal inflammation (5–8), and in the present study we first demonstrated that norepinephrine, a stress modulator, stimulated calprotectin expression in monocytic cells. Calprotectin expression may be regulated by stress, in addition to virulence factors such as periodontopathic bacteria and cytokines.

Acknowledgements

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