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N-Acetylcysteine Prevents LPS-Induced Pro-inflammatory Cytokines and MMP2 Production in Gingival Fibroblasts

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Periodontitis is an inflammatory process that ultimately results in tooth loss. Although the primary etiologic agent for periodontitis is bacteria, the majority of periodontal tissue destruction is thought to be caused by an inappropriate host response. Reactive oxygen species (ROS) have been known to be involved in periodontal tissue destruction. We treated human gingival fibroblasts with lipopolysaccharide (LPS) obtained from *E. coli* and the periodontopathogens *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis,* and examined their inflammatory responses in the presence and absence of the antioxidant N-acetylcysteine (NAC). LPS enhanced ROS production, as well as, expression of pro-inflammatory cytokines such as interleukin-1β, interleukin-6, interleukin-8 and tumor necrosis factor-α, and the production and activation of MMP2. NAC suppressed all LPS-induced inflammatory responses examined, suggesting that LPS-induced ROS may play a major regulatory role in these responses in gingival fibroblasts. In addition, NAC prevented LPS-induced activation of p38 MAPK and JNK but not phosphorylation and subsequent degradation of IkB. These results indicate that NAC exerts anti-inflammatory effects in LPS-stimulated gingival fibroblasts, functioning at least in part via down-regulation of JNK and p38 MAPK activation. Furthermore, this work suggests that antioxidants may be useful in adjunctive therapies that complement conventional periodontal treatments.

Key words: Gingival fibroblasts, LPS, MMP2, N-Acetylcysteine, Pro-inflammatory cytokines

INTRODUCTION

Periodontitis is an inflammatory process initiated by plaque biofilm. It leads to degradation of connective tissue and alveolar bone, and ultimately results in tooth loss. Among the many Gram-negative bacteria harbored in periodontal pockets, *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* have been implicated as etiological agents of juvenile and adult periodontitis (Haffajee and Socransky, 2005). These bacteria generate products such as lipopolysaccharide (LPS), that participate in inflammation and tissue destruction. LPS is considered to be the major virulence factor of Gram-negative bacteria and it activates immunomodulatory cells by binding to specific Toll-like receptors (TLR), which results in induction of inflammatory response (Heumann and Roger, 2002). Although bacteria are the primary etiological agents for periodontitis, most periodontal tissue destruction is thought to be caused by an inappropriate host response to those bacteria and their products (Graves and Cochran, 2003; Haffajee and Socransky, 2005). Gingival fibroblasts (GFs) are the major constituent of gingival connective tissue and in periodontitis lesions they interact directly with bacteria and bacterial products such as LPS (McCulloch and Bordin, 1991). It has been suggested that GFs play a critical role in the periodontal response to LPS, in part through production of pro-inflammatory cytokines and matrix metalloproteases (MMPs;

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Okada and Murakami, 1998; Bodet *et al.*, 2007).

Reactive oxygen species (ROS) have been implicated in the pathogenesis of a variety of diseases, including periodontitis (Chapple and Matthews, 2007). Several studies have demonstrated that neutrophils from patients with periodontitis exhibit a higher ROS production capacity than those from healthy controls (Kimura *et al.*, 1993; Gustafsson and Asman, 1996). In addition, the total antioxidant capacity is lower in plasma and gingival crevicular fluid samples from periodontitis patients (Chapple *et al.*, 2002; Brock *et al.*, 2004). These reports suggest that antioxidants may be useful in adjunctive therapies that complement conventional periodontal treatments. Supporting evidence has been obtained in a rodent model of periodontitis, whereby Di Paola *et al.* (2005) demonstrated that tempol, a free radical scavenger, could exert a significant decrease in inflammation parameters.

Previous studies have demonstrated that ROS production is induced by LPS binding TLR4 and that LPS-induction of ROS is associated with nuclear factor-κB (NF-κB) activation and pro-inflammatory cytokine production in phagocytes (Asehnoune *et al.*, 2004; Ryan *et al.*, 2004). However, the pathways leading to these responses differ between myeloid and nonmyeloid cells (Andreakos *et al.*, 2004). In GFs, the roles played by ROS in modulating LPS-induced responses and intracellular signaling events remains unclear. We found that in LPS-stimulated GFs, ROS were involved in enhancing production of pro-inflammatory cytokines and MMP2. In addition, we determined that LPS-induced p38 mitogen activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) activation were antioxidant dependent, whereas LPS-induced phosphorylation and degradation of inhibitor of NF-κB (IκB) were not.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS) was purchased from Bio-Whittker (Walkersville, MD) and Dulbecco's modified Eagles medium (DMEM) from Hyclone (Logan, UT). The other tissue culture reagents and 10% gelatin zymogram gels were obtained from Invitrogen Life Technologies (Carlsbad, CA). easy-BLUE™ for total RNA extraction and *Star*Taq™ polymerase for polymerase chain reaction (PCR) were obtained from iNtRON Biotechnology (Sungnam, Korea). AccuPower RT-PreMix for first-strand cDNA synthesis was from Bioneer (Daejeon, Korea). PCR primers were synthesized by TaKaRa Korea (Seoul, Korea). Supex for detecting chemiluminescence was from Dyne-Bio (Sungnam, Korea). Anti-IκBα, anti-phospho-IκBα, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-JNK, anti-phospho-JNK, anti-extracellular signal-regulated kinase (ERK) and anti-phospho-ERK antibodies were from Cell Signaling Technology (Beverly, MA). ImmunoPure goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated and bovine anti-goat IgG HRP-conjugated antibodies were purchased from Santa Cruz (Santa Cruz, CA). BIOXYTECH GSH/GSSG-412™ kit was from *Oxis*Research (Portland, OR). CD14, LPS-binding protein (LBP) and Quantikine Immunoassay kits for detection of interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α), were obtained from R&D systems (Minneapolis, MN). N-acetylcysteine (NAC), 2'7'-dichlorofluorescin diacetate (DCFH-DA) and *E. coli* LPS were obtained from Sigma-Aldrich (St. Louis, MO). LPS from periodontopathogens was isolated from whole cells of *A. actinomycetemcomitans* (ATCC 33384) and *P. gingivalis* (ATCC 33277) using an LPS Extraction Kit (iNtRon, Seoul, Korea).

Primary culture of GFs

Gingival tissues were obtained from patients undergoing extraction of the first premolar during orthodontic treatment at Seoul National University Dental Hospital. Gingival tissue attached to the extracted tooth was rinsed three times with DMEM containing antibiotics, and then minced and seeded in tissue culture dish containing DMEM supplemented with 20% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. This culture medium was changed every 2 d until the cells reached confluency, after which cells were subcultured and incubated in DMEM supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Fibroblasts used in these experiments were between the 4th and 7th passage.

Measurement of intracellular ROS

Intracellular ROS were measured using DCFH-DA, which diffuses into cells where it is hydrolyzed to DCFH, and then converted by ROS-mediated oxidation to the highly-fluorescent derivative *2',7'*-dichlorofluorescein (DCF; Bass *et al.*, 1983). GFs (5×10^4/well) were seeded into 96-well culture plates, incubated overnight and serum-starved for 18 h. Cells were then pre-incubated for 30 min in serum-free DMEM containing 50 μM DCFH-DA, transferred to fresh serum-free DMEM and incubated for the times indicated in the presence of the reagents indicated. DCF fluorescence was measured using a FLUOstar OPTIMA (BMG Lab-technologies, Offenburg, Germany).

Measurement of intracellular glutathione

GFs (5×10^5) were seeded into 60 mm culture dishes and incubated for 2 d in DMEM supplemented with 10% FBS. They were then cultured for 2 h in the presence of the reagents indicated and harvested. Total glutathione and the ratio of its reduced to oxidized forms were determined using a BIOXYTECH GSH/GSSG-412™ kit. The

ratio of GSH/GSSG was calculated using the following equation: Ratio = (total glutathione – 2GSSG) / GSSG.

Cell proliferation assay

GFs (1×10^4) were seeded into 96-well culture plates and incubated for 24 h. The cells were then incubated for 2 d in DMEM supplemented with 10% FBS and NAC, at concentrations indicated. Finally, cells were trypsinized and the numbers of viable cells counted using trypan blue staining.

Reverse transcription-polymerase chain reaction (RT-PCR)

Expression levels of pro-inflammatory cytokine genes were examined by semi-quantitative RT-PCR. GFs (5×10^5) were seeded into 60 mm tissue culture dishes and incubated overnight. The cells were then pretreated with or without 20 mM NAC for 30 min, after which 100 ng mL^{-1} LPS, 200 ng mL^{-1} CD14 and 50 ng mL^{-1} LBP were added to the culture. After 4 h incubation, total cellular RNA was extracted using easy-BLUE™ reagent. Complementary DNA (cDNA) was synthesized from 1 μg total RNA using the AccuPower RT PreMix and PCR was performed using *Star*Taq™ polymerase on 10% of the cDNA. The following primers were used for PCR: IL-1β-forward (f) 5'-GACACATGGGATAACGAGGC-3', IL-1β-reverse (r) 5'-ACGCAGGACAGGTACAGATT-3'; IL-6-f 5'-ATGAACTCCTTCTCCACAAG-3', IL-6-r 5'-GTGCCTGCAGCTTCGTCAGCA-3'; IL-8-f 5'-ATGACTTCCAAGCTGGCCGTGGCT-3', IL-8-r 5'-TCTCAGCCCTCTTCAAAAACTTCTC-3'; TNF-α-f 5'-TCAGATCATCTTCTCGAACC-3', TNF-α-r 5'-CAGATAGATGGGCTCATACC-3'; β-actin-f 5'-CGGGAAATCGTGCGTGACAT-3' and b-actin-r 5'-GAACTTTGGGGGATGCTCGC-3'. PCR products were separated by electrophoresis on 1.2% agarose gels, stained with ethidium bromide and visualized under UV light.

Enzyme-linked immunosorbent assay (ELISA)

GFs (2.5×10^5) were seeded into 35 mm tissue culture dishes, incubated overnight and serum-starved for 18 h. Cells were transferred to fresh serum-free DMEM and incubated for 48 h in the presence of the reagents indicated. Cell supernatants were obtained and cytokine levels (IL-1β, IL-6, IL-8 and TNF-α) determined using the Quantikine Immunoassay kit.

Gelatin zymography

Levels of secreted pro- and active MMP2 were evaluated using gelatin zymography. GFs (1×10^5) were seeded into 4-well culture plates, incubated overnight and serum-starved for 18 h. Cells were transferred to fresh serum-free DMEM, supplemented with 0.1% bovine serum albumin (BSA), 200 ng mL^{-1} CD14 and 50 ng mL^{-1} LBP and incubated for times indicated in the presence of the reagents indicated. At the end of culture, aliquots of conditioned media were separated by electrophoresis in 10% gelatin zymogram gels, after which the gels were washed twice in renaturing buffer (2.5% Triton X-100, 50 mM Tris-Cl [pH 7.5]) at room temperature for 30 min, followed by developing buffer (150 mM NaCl, 10 mM $CaCl_2$, 50 mM Tris-Cl [pH 7.5]) at 37°C for 16 - 18 h. Gels were stained with 0.1% Coomassie brilliant blue in 10% methanol and 10% acetic acid. After destaining, pro- and active MMP2 were detected as clear bands (72 kDa and 62 kDa, respectively).

Immunoblot analysis

GFs (5×10^5) were seeded into 60 mm tissue culture dishes, incubated overnight and serum-starved for 18 h. Cells were then transferred to fresh serum-free DMEM and incubated for times indicated in the presence of the reagents indicated. The cells were washed with PBS and scraped into lysis buffer (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM PMSF, 1 mg mL^{-1} aprotinin, 1 mM leupetin, and 1 mM pepstatin) and sonicated briefly. Protein concentrations were determined using the BCA protein assay. Samples containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis, after which the separated proteins were transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with primary antibody followed by HRP-conjugated secondary antibody. Luminescence was detected using Supex reagent in a LAS1000 (Fuji PhotoFilm, Tokyo, Japan).

Statistical analysis

All results were expressed as means ± S.E. Statistical differences were analyzed using the unpaired Student's *t* test. p values < 0.05 were considered to indicate a statistically significant difference.

RESULTS

NAC suppresses LPS-induced ROS production

To confirm the ability of LPS to increase intracellular ROS production in GF cells, we observed ROS levels using DCF-DA. Under serum-starved conditions, GFs produced ROS spontaneously (Fig. 1). To increase the responsiveness of GFs to LPS, we added 200 ng mL^{-1} CD14 and 50 ng mL^{-1} LBP to the medium. ROS production increased significantly in the presence of LPS obtained from *P. gingivalis*, *A. actinomycetemcomitans* and *E. coli*. As expected, NAC suppressed both spontaneous

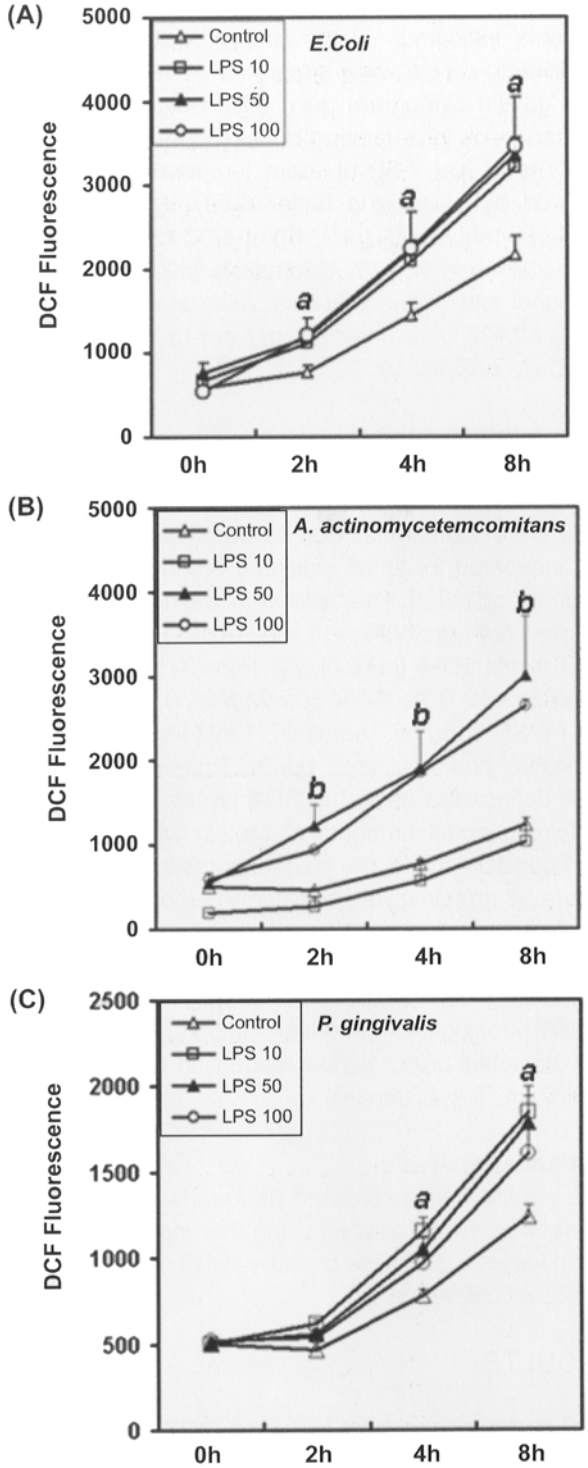

Fig. 1. LPS increases intracellular ROS production in GFs. Cells were serum-starved, and then incubated in the presence of LPS at the concentrations indicated (ng mL^{-1}) in serum-free DMEM supplemented with CD14 (200 ng mL^{-1}) and LBP (50 ng mL^{-1}). Intracellular ROS levels were measured by detecting ROS-mediated conversion of DCFH-DA to DCF. Data represent means ± S.E. (N = 6). [a] All values from LPS-treated groups were significantly different from the controls ($p < 0.05$). [b] Groups treated with 50 and 100 ng mL^{-1} LPS exhibited ROS values that were significantly different from the control ($p < 0.05$).

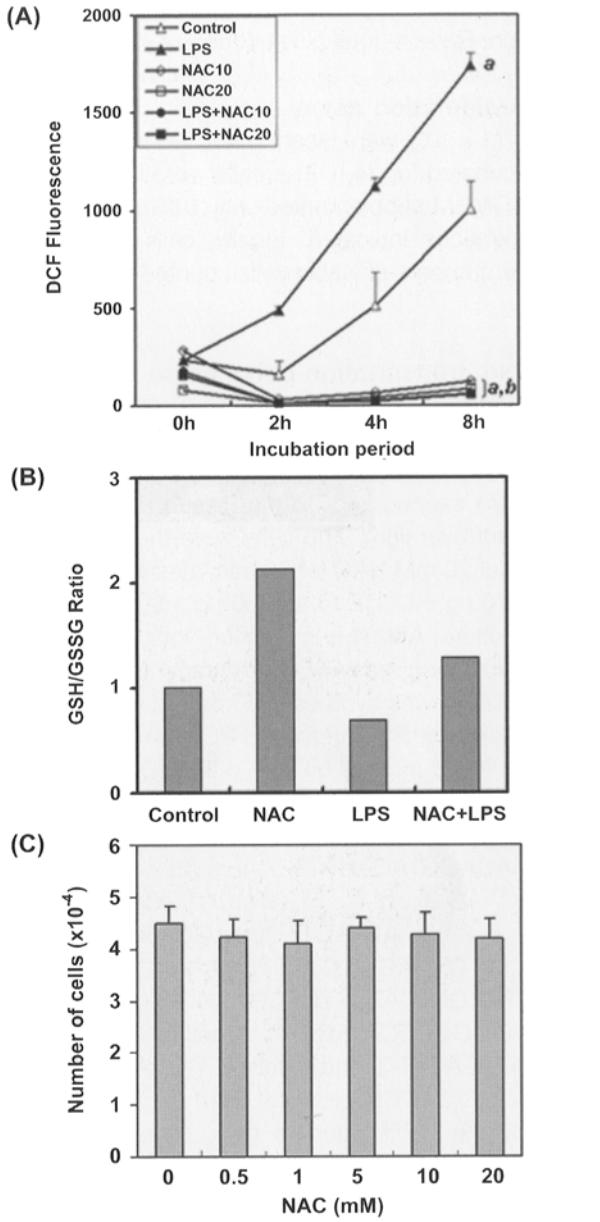

Fig. 2. NAC prevents LPS-induced ROS production and decreases the ratio of reduced to oxidized glutathione. (A) Cells were pretreated with or without NAC (mM) for 30 min and then stimulated with LPS (100 ng mL^{-1}) in serum-free DMEM supplemented with CD14 (200 ng mL^{-1}) and LBP (50 ng mL^{-1}). Intracellular ROS levels were measured and data represent means ± S.E. (N = 6). [a] Significant difference from the control after 2, 4 and 8 h incubation ($p < 0.05$). [b] Significant difference from samples treated with LPS alone after 2, 4 and 8 h incubation ($p < 0.05$). (B) Cells were incubated in the presence of the reagents indicated for 2 h and the amount of total and oxidized glutathione was determined using a BIOXYTECH GSH/GSSG-412 kit. The ratio of GSH/GSSG was calculated as follows: Ratio = (total glutathione – 2GSSG) / GSSG. Reagent concentrations: 20 mM NAC, 100 ng/mL LPS. (C) NAC did not affect the proliferation or viability of GFs. Cells were incubated for 2 d in the presence of NAC at the concentrations indicated, after which the number of viable cells was counted. Data represent means ± S.E. (N = 4).

and LPS-mediated ROS production significantly (Fig. 2A). Since NAC is a precursor of glutathione (Winyard and Blake, 1997), we examined the effects of NAC and LPS on intracellular glutathione levels. Although the total glutathione content of GFs was not affected significantly by 2 h of either treatment (data not shown), the ratio of the reduced to oxidized forms of glutathione increased and decreased in response to NAC and LPS, respectively (Fig. 2B). As expected, NAC pretreatment prevented LPS-mediated oxidation of glutathione. At the concentrations used in this study, NAC did not affect cell viability or proliferation of GFs (Fig. 2C).

NAC down-regulates LPS-induced Pro-inflammatory cytokine production

We used RT-PCR to examine expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α, in order to evaluate the effect of NAC on GFs stimulated

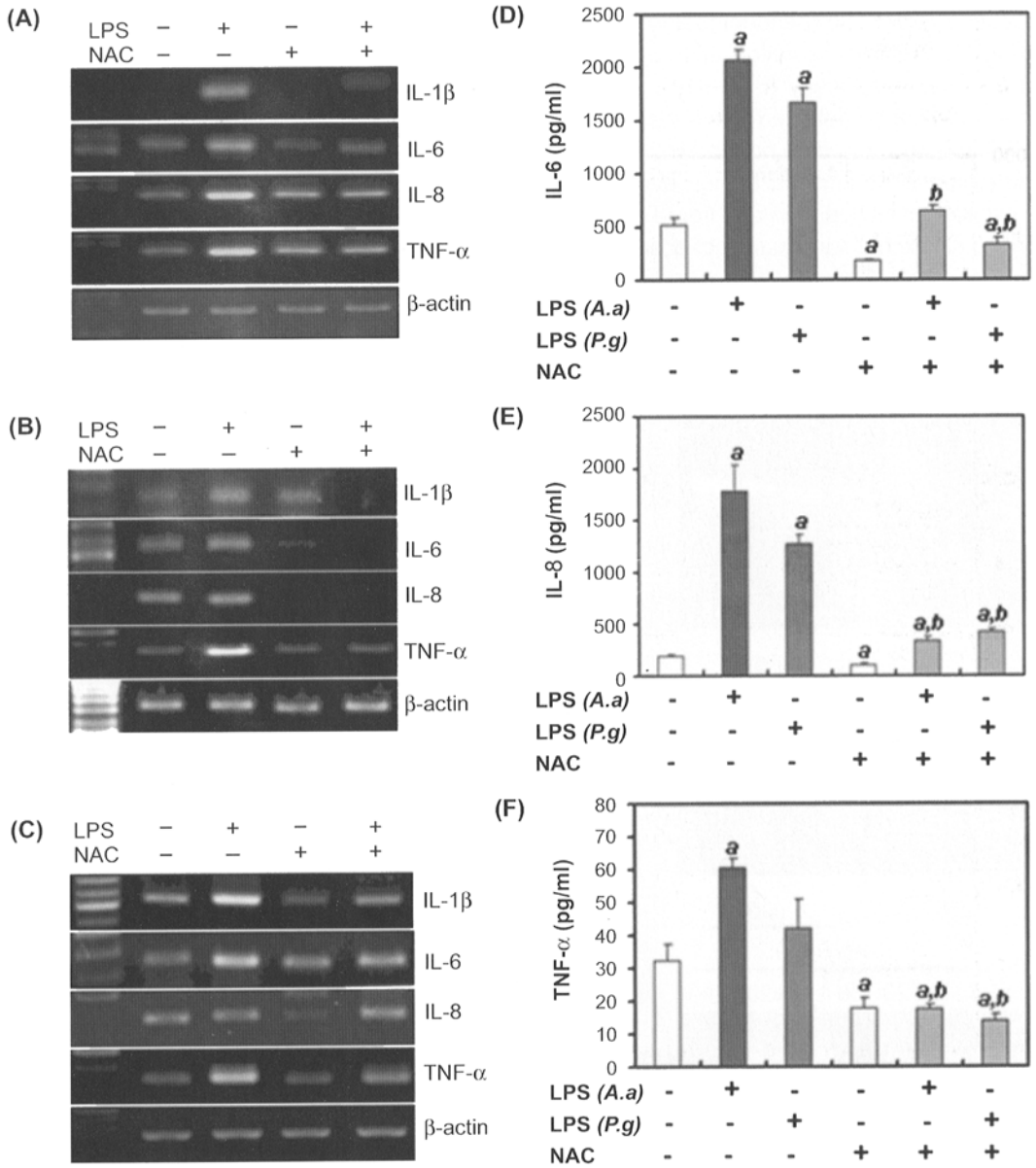

Fig. 3. NAC suppresses LPS-induced pro-inflammatory cytokines expression. (A-C) Cells were pretreated with or without 20 mM NAC for 30 min, and then incubated for 4 h in the presence or absence of LPS (100 ng mL^{-1}) from *E. coli* (A), *A. actinomycetemcomitans* (B) or *P. gingivalis* (C). Gene expression levels were examined using semiquantitative RT-PCR. (D-F) Cells were serum-starved, and then incubated for 48 h in the presence of the reagents indicated. The levels of IL-6 (D), IL-8 (E) and TNF-α (F) in conditioned media were determined using the Quantikine Immunoassay kit. Data represent means ± S.E. (N = 6). [a] Significant difference from the control ($p < 0.05$). [b] Significant difference from samples treated with LPS alone ($p < 0.05$). Reagent concentrations: 20 mM NAC, 100 ng mL^{-1} LPS.

with LPS. Previously we demonstrated that LPS-induced pro-inflammatory cytokine mRNA expression peaks 4 h after LPS treatment of GFs (Park *et al.*, 2004). We therefore chose that time point for observation of NAC effects. Stimulation of GFs with *E. coli* LPS (100 ng mL^{-1}) augmented mRNA expression of all the cytokines examined, whereas NAC (20 mM) treatment prevented their induction (Fig. 3A). Similarly, LPS (100 ng mL^{-1}) from the periodontopathogens induced mRNA expression of pro-inflammatory cytokines, though to a lesser extent than was observed for *E. coli* LPS (Fig. 3B, C). Cytokine stimulation by LPS from the peridontopathogens was confirmed using ELISA, and secretion of IL-6, IL-8 and TNF-α could be observed clearly (Fig. 3D-F). However, IL-1β secretion could not be detected within the sensitivity range used (3.9-250 pg mL^{-1}; data not shown). LPS from *A. actinomycetemcomitans* induced higher levels of pro-inflammatory cytokine secretion than that from *P. gingivalis* and NAC effected significant suppression of both the basal and LPS-induced secretion of the cytokines (Fig. 3D-F).

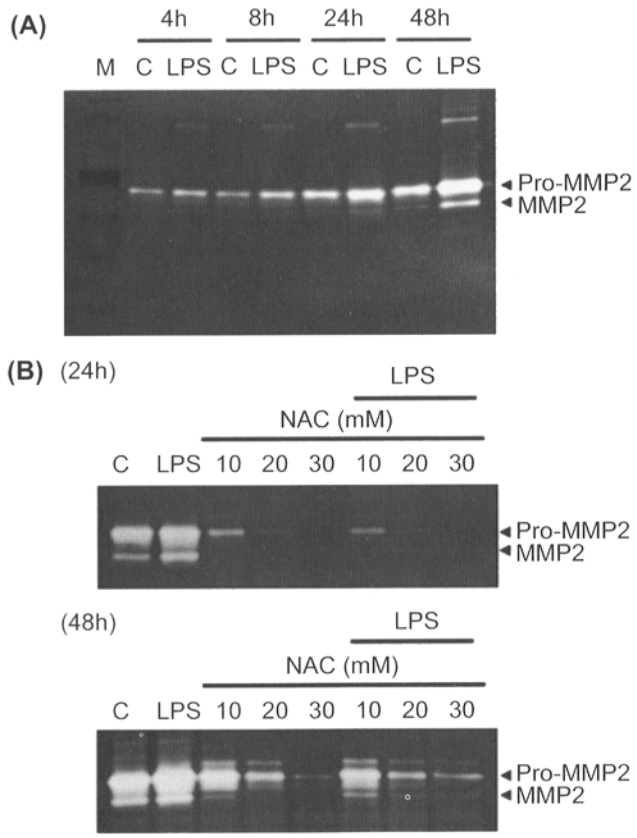

Fig. 4. NAC suppresses LPS-induced production and activation of pro-MMP2. (A) GFs were serum-starved, and then incubated in DMEM supplemented with 0.1% BSA, 200 ng mL^{-1} CD14 and 50 ng mL^{-1} LBP. The levels of pro- and active MMP2 in the conditioned media were determined using gelatin zymography. (B) GFs were incubated for 24 or 48 h (*upper* and *lower panels*, respectively) in the presence of the reagents indicated, followed by gelatin zymography. Reagent concentration: 100 ng mL^{-1} LPS.

NAC inhibits production of pro- and active MMP2

Since GFs responded similarly to LPS from the periodontopathogens and *E. coli*, we used *E. coli* LPS for examination of MMP2 production. In the absence of LPS, GFs produce basal levels of pro- and active-MMP2, whereas following treatment (100 ng mL^{-1} *E. coli* LPS), secretion of pro-MMP2 increased and its conversion to active MMP2 occurred in a time-dependent manner (Fig. 4A). NAC caused significant down-regulation of MMP2 production and activation in the presence and absence of LPS induction (Fig. 4B). Furthermore, this down-regulation occurred in a concentration-dependent manner.

NAC blocks LPS-mediated activation of p38 MAPK and JNK

Since LPS is known to activate several signaling molecules including MAPKs and NF-κB pathways (Means *et al.*, 2000), we examined which pathways were affected by NAC treatment. *E. coli* LPS strongly induced phosphorylation of p38 MAPK, JNK and IkB, while it stimulated ERK phosphorylation slightly (Fig. 5). NAC almost completely blocked LPS-induced activation of p38 MAPK and JNK, but did not prevent LPS-induced phosphorylation of IkB and ERK.

DISCUSSION

In this study, we investigated the effects of NAC on

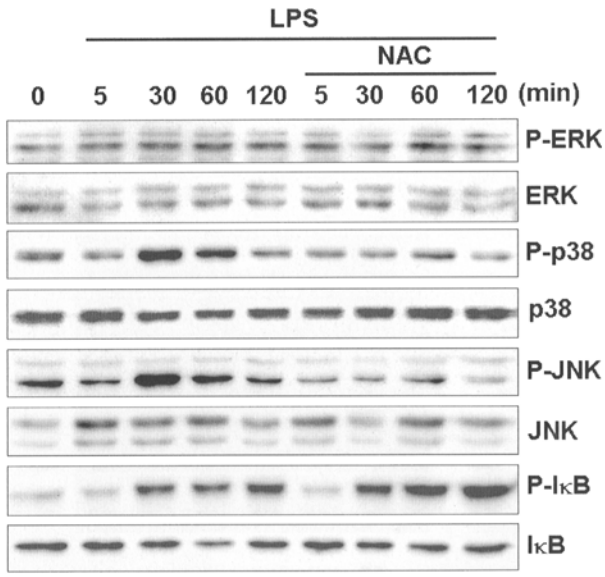

Fig. 5. NAC prevents LPS-induced activation of p38 MAPK and JNK, but not of NF-κB. GFs were serum-starved, pretreated with or without 20 mM NAC for 30 min, and then incubated in the presence of the reagents indicated. Western blot analysis was performed on cell lysates. Reagent concentrations: 100 ng mL^{-1} LPS.

LPS-induced responses in GFs. The results demonstrated that LPS enhanced production of ROS, pro-inflammatory cytokines and MMP2, whereas NAC suppressed these effects significantly, at least in part via down-regulation of JNK and p38 MAPK activation.

The host uses an array of antioxidant enzymes and ROS scavenger compounds to protect its tissues from the harmful effects of oxidative stress (Victor *et al.*, 2003). Discovery of millimolar levels of glutathione in gingival crevicular fluid led to the hypothesis that this ubiquitous thiol may represent a fundamental innate defense strategy in periodontal tissues (Chapple and Matthews, 2007). Glutathione is synthesized intracellularly, where it plays a major role in maintaining the redox equilibrium. Cysteine is the rate-limiting substrate in glutathione production and it is the cysteinyl moiety of this compound that provides the reactive thiol. NAC is a precursor for glutathione and interest in this compound has increased recently in light of its ability to inhibit synthesis of pro-inflammatory molecules, as well as to enhance the immune functions of phagocytes and lymphocytes (Winyard and Blake, 1997; De la Fuente and Victor, 2000; Victor and De la Fuente, 2002). Furthermore, NAC has been shown to suppress osteoclast differentiation, which leads to inhibition of bone resorption (Hall *et al.*, 1995; Lee *et al.*, 2005). Thus, as an antioxidant and immunomodulatory molecule, NAC may be useful for the control of periodontitis.

Periodontopathogens cause the strong release of inflammatory mediators and the etiological correlation between periodontitis and high levels of pro-inflammatory cytokines in the periodontal tissues and gingival crevicular fluid has been reported extensively (Birkedal-Hansen, 1993a). Although antioxidants can prevent expression of LPS-induced inflammatory mediators in phagocytic cells (Hsu and Wen, 2002; Kim *et al.*, 2004; Ryan *et al.*, 2004), other investigators have demonstrated that NAC up-regulates these mediators in human chondrocytes (Mathy-Hartert *et al.*, 2002, 2003). This discrepancy may be due in part to differences between human myeloid and non-myeloid cells, which use different LPS-response pathways for inflammatory cytokine production (Andreakos *et al.*, 2004). Therefore, we examined the effect of NAC on LPS-induced production of pro-inflammatory cytokines in GFs. As observed previously in phagocytic cells, NAC prevented LPS-stimulation of inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) in GFs, which suggests that production of LPS-induced cytokines requires increased ROS generation.

In addition to these pro-inflammatory cytokines, MMPs are believed to play an important role in periodontal tissue degradation (Birkedal-Hansen, 1993b). MMP2 is secreted primarily from fibroblasts such as GFs (Makela *et al.*, 1994). MMP2 degrades denatured type I collagen and the type IV collagen found in the basement membrane (Nagase and Woessner, 1999). In addition, it degrades native type I collagen and is considered to be a major enzyme in turnover of soft connective tissue (Creemers *et al.*, 1998). The increased levels of active MMP2 found in the gingival samples from periodontitis patients suggest that this enzyme play a role in the degradation of the periodontium (Korostoff *et al.*, 2000). As reported previously, LPS stimulates MMP2 activation in GFs, as well as increased secretion of pro-MMP2 (Tiranathanagul *et al.*, 2004; Bodet *et al.*, 2007). We observed that in LPS-stimulated GFs, NAC suppressed both the secretion and activation of pro-MMP2. This result is in agreement with previous reports indicating that MMP2 production can be activated by ROS in vascular smooth muscle cells and hepatic stellate cells (Rajagopalan *et al.*, 1996; Galli *et al.*, 2005). These results suggest that in GFs, pro-MMP2 expression and activation depend upon intracellular ROS generation.

LPS activates several signaling molecules within the MAPK and NF-κB pathways (Means *et al.*, 2000). There are three major MAPKs in mammalian cells (ERK, JNK and p38 MAPK; Seger and Krebs, 1995) and these contribute to activation of activator protein-1 (AP-1), which is a redox-sensitive transcription factor (Karin, 1992; Minden and Karin, 1997). Previous studies have demonstrated that ROS are associated commonly with activation of both the redox-sensitive transcription factors AP-1 and NF-κB (Chapple and Matthews, 2007). Furthermore, the signal transduction pathways that mediate the transcriptional regulation of many pro-inflammatory cytokines and MMPs require activation of MAPKs, AP-1 and NF-κB (Mastronarde *et al.*, 1998; Chakraborti *et al.*, 2003; Morse *et al.*, 2003; Bodet *et al.*, 2007). However, the relative importance of ROS in activation of the individual MAPKs and NF-κB, as well as the roles played by these signaling molecules in LPS-induced production of pro-inflammatory cytokines and MMP2, differs according to cell type and experimental conditions (Hsu and Wen, 2002; Cho *et al.*, 2003; Asehnoune *et al.*, 2004; Kim *et al.*, 2004; Strassheim *et al.*, 2004; Sanchez-Tillo *et al.*, 2007). Therefore, we examined the effect of NAC on LPS-induced activation of MAPKs and NF-κB in GFs. We observed that LPS-induced activation of p38 MAPK and JNK1 was prevented by NAC treatment. NAC has been shown to up-regulate LPS-induced JNK activity in murine macrophages (Hsu and Wen, 2002), and disruption of the JNK1 locus enhances LPS-induced release of TNF-α and IL-6 in microglial cells (Hidding *et al.*, 2002). However, our results are similar to previous reports which found that LPS-activated p38 MAPK regulates production of IL-1β, IL-6 and TNF-α in murine macrophages (Beyaert *et al.*, 1996; Brook *et al.*, 2000; Hsu and Wen, 2002). Furthermore, Sanchez-Tillo *et al.* (2007) demonstrated that LPS phosphorylated only the JNK1 isoform; in mouse macrophages,

this isoform is required for production of IL-1β, IL-6 and TNF-α. These results suggest that in GFs, p38 MAPK and JNK1 are important for LPS-induced ROS-mediated production of pro-inflammatory cytokines and MMP2.

NF-κB is a crucial regulator of inflammation responses (Janssen-Heininger *et al.*, 2000) and under normal cellular conditions it is maintained in an inactive state via association with IκB. Following stimulation, IκB kinases phosphorylate IκB, which leads to the ubiquitination and subsequent degradation of the inhibitor. The free NF-κB protein complex then translocates to the nucleus, where it binds to target DNA sequences. Several lines of evidence indicate that activation of NF-κB can be controlled by ROS such as superoxide and hydrogen peroxide (Janssen-Heininger *et al.*, 2000; Asehnoune *et al.*, 2004) and that antioxidants block cytokine transcription by preventing NF-κB migration to the nucleus (Schubert *et al.*, 2002; Cho *et al.*, 2003; Ryan *et al.*, 2004). However, other evidence indicates that ROS are not involved in NF-κB activation, suggesting that the effects of ROS on NF-κB activation may be both cell- and stimulus-dependent (Schreck *et al.*, 1992; Anderson *et al.*, 1994; Brennan and O'Neill, 1995). Some authors have demonstrated that LPS-dependent activation of NF-κB is unrelated to increased ROS production (Haddad, 2001; Woo *et al.*, 2004) and in keeping with these findings we observed that NAC did not alter the phosphorylation or subsequent degradation of IκB in GFs. These results suggest that in GFs, LPS-induced NF-κB activation is a ROS-independent event, and that although important, its activation alone is insufficient for transcriptional activation of pro-inflammatory cytokines and MMP2.

Although our data have shown that NAC reduced intracellular ROS levels and increased the reduced form of glutathione in GFs, it is not clear whether its inhibitory effects on LPS-induced inflammatory responses are solely dependent on its antioxidant activity. Because there are the possibility of direct modification of certain signaling molecules by NAC (Zafarullah *et al.*, 2003), it will be necessary to determine whether the same results are obtained by approaches such as overexpression of antioxidant enzymes.

This study demonstrated that in LPS-stimulated GFs, NAC is able to inhibit ROS production and suppresses expression of pro-inflammatory cytokines and MMP-2 activity. These finding suggest that antioxidants may be useful for adjunctive therapies to complement conventional periodontal treatments.

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REFERENCES

Anderson, M. T., Staal, F. J., Gitler, C., Herzenberg, L. A., and Herzenberg, L. A., Separation of oxidant-initiated and redox-regulated steps in the NF-kappa B signal transduction pathway. *Proc. Natl. Acad. Sci. U.S.A.*, 91, 11527-11531 (1994).

Andreakos, E., Sacre, S. M., Smith, C., Lundberg, A., Kiriakidis, S., Stonehouse, T., Monaco, C., Feldmann, M., and Foxwell, B. M., Distinct pathways of LPS-induced NF-kappa B activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. *Blood*, 103, 2229-2237 (2004).

Asehnoune, K., Strassheim, D., Mitra, S., Kim, J. Y., and Abraham, E., Involvement of reactive oxygen species in Toll-like receptor 4-dependent activation of NF-kappa B. *J. Immunol.*, 172, 2522-2529 (2004).

Bass, D. A., Parce, J. W., Dechatelet, L. R., Szejda, P., Seeds, M. C., and Thomas, M., Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J. Immunol.*, 130, 1910-1917 (1983).

Beyaert, R., Cuenda, A., Vanden Berghe, W., Plaisance, S., Lee, J. C., Haegeman, G., Cohen, P., and Fiers, W., The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. *EMBO J.*, 15, 1914-1923 (1996).

Birkedal-Hansen, H., Role of cytokines and inflammatory mediators in tissue destruction. *J. Periodontal Res.*, 28, 500-510 (1993a).

Birkedal-Hansen, H., Role of matrix metalloproteinases in human periodontal diseases. *J. Periodontol.*, 64, 474-484 (1993b).

Bodet, C., Andrian, E., Tanabe, S. I., and Grenier, D., Actinobacillus actinomycetemcomitans lipopolysaccharide regulates matrix metalloproteinase, tissue inhibitors of matrix metalloproteinase, and plasminogen activator production by human gingival fibroblasts: A potential role in connective tissue destruction. *J. Cell. Physiol.*, 212, 189-194 (2007).

Brennan, P. and O'Neill, L. A., Effects of oxidants and antioxidants on nuclear factor kappa B activation in three different cell lines: evidence against a universal hypothesis involving oxygen radicals. *Biochim. Biophys. Acta*, 1260, 167-175 (1995).

Brock, G. R., Butterworth, C. J., Matthews, J. B., and Chapple, I. L. C., Local and systemic total antioxidant capacity in periodontitis and health. *J. Clin. Periodontol.*, 31, 515-521 (2004).

Brook, M., Sully, G., Clark, A. R., and Saklatvala, J., Regulation of tumour necrosis factor alpha mRNA stability by the mitogen-activated protein kinase p38 signalling cascade.

FEBS Lett., 483, 57-61 (2000).

Chakraborti, S., Mandal, M., Das, S., Mandal, A., and Chakraborti, T., Regulation of matrix metalloproteinases: an overview. *Mol. Cell. Biochem.*, 253, 269-285 (2003).

Chapple, I. L. and Matthews, J. B., The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol 2000.*, 43, 160-232 (2007).

Chapple, I. L. C., Brock, G., Eftimiade, C., and Matthews, J. B., Glutathione in gingival crevicular fluid and its relation to local antioxidant capacity in periodontal health and disease. *Mol. Pathol.*, 55, 367-373 (2002).

Cho, S. Y., Park, S. J., Kwon, M. J., Jeong, T. S., Bok, S. H., Choi, W. Y., Jeong, W. I., Ryu, S. Y., Do, S. H., Lee, C. S., Song, J. C., and Jeong, K. S., Quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-kappaB pathway in lipopolysaccharide-stimulated macrophage. *Mol. Cell. Biochem.*, 243, 153-160 (2003).

Creemers, L. B., Jansen, I. D., Docherty, A. J., Reynolds, J. J., Beertsen, W., and Everts, V., Gelatinase A (MMP-2) and cysteine proteinases are essential for the degradation of collagen in soft connective tissue. *Matrix Biol.*, 17, 35-46 (1998).

De la Fuente, M. and Victor, V. M., Anti-oxidants as modulators of immune function. *Immunol. Cell Biol.*, 78, 49-54 (2000).

Di Paola, R., Mazzon, E., Zito, D., Maiere, D., Britti, D., Genovese, T., and Cuzzocrea, S., Effects of Tempol, a membrane-permeable radical scavenger, in a rodent model periodontitis. *J. Clin. Periodontol.*, 32, 1062-1068 (2005).

Galli, A., Svegliati-Baroni, G., Ceni, E., Milani, S., Ridolfi, F., Salzano, R., Tarocchi, M., Grappone, C., Pellegrini, G., Benedetti, A., Surrenti, C., and Casini, A., Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism. *Hepatology*, 41, 1074-1084 (2005).

Graves, D. T. and Cochran, D., The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J. Periodontol.*, 74, 391-401 (2003).

Gustafsson, A. and Asman, B., Increased release of free oxygen radicals from peripheral neutrophils in adult periodontitis after Fc delta-receptor stimulation. *J. Clin. Periodontol.*, 23, 38-44 (1996).

Haddad, J. J., L-Buthionine-(S,R)-sulfoximine, an irreversible inhibitor of gamma-glutamylcysteine synthetase, augments LPS-mediated pro-inflammatory cytokine biosynthesis: evidence for the implication of an IkappaB-alpha/NF-kappaB insensitive pathway. *Eur. Cytokine Netw.*, 12, 614-624 (2001).

Haffajee, A. D. and Socransky, S. S., Microbiology of periodontal diseases: introduction. *Periodontol. 2000*, 38, 9-12 (2005).

Hall, T. J., Schaeublin, M., Jeker, H., Fuller, K., and Chambers, T. J., The role of reactive oxygen intermediates in osteoclastic bone resorption. *Biochem. Biophys. Res. Commun.*, 207, 280-287 (1995).

Heumann, D. and Roger, T., Initial responses to endotoxins and Gram-negative bacteria. *Clin. Chim. Acta*, 323, 59-72 (2002).

Hidding, U., Mielke, K., Waetzig, V., Brecht, S., Hanisch, U., Behrens, A., Wagner, E., and Herdegen, T., The c-Jun N-terminal kinases in cerebral microglia: immunological functions in the brain. *Biochem. Pharmacol.*, 64, 781-788 (2002).

Hsu, H. Y. and Wen, M. H., Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J. Biol. Chem.*, 277, 22131-22139 (2002).

Janssen-Heininger, Y. M., Poynter, M. E., and Baeuerle, P. A., Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB. *Free Radic. Biol. Med.*, 28, 1317-1327 (2000).

Karin, M., Signal transduction from cell surface to nucleus in development and disease. *FASEB J.*, 6, 2581-2590 (1992).

Kim, S. H., Kim, J., and Sharma, R. P., Inhibition of p38 and ERK MAP kinases blocks endotoxin-induced nitric oxide production and differentially modulates cytokine expression. *Pharmacol. Res.*, 49, 433-439 (2004).

Kimura, S., Yonemura, T., and Kaya, H., Increased oxidative product formation by peripheral blood polymorphonuclear leukocytes in human periodontal diseases. *J. Periodontal Res.*, 28, 197-203 (1993).

Korostoff, J. M., Wang, J. F., Sarment, D. P., Stewart, J. C., Feldman, R. S., and Billings, P. C., Analysis of in situ protease activity in chronic adult periodontitis patients: expression of activated MMP-2 and a 40 kDa serine protease. *J. Periodontol.*, 71, 353-360 (2000).

Lee, N. K., Choi, Y. G., Baik, J. Y., Han, S. Y., Jeong, D. W., Bae, Y. S., Kim, N., and Lee, S. Y., A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood*, 106, 852-859 (2005).

Mastronarde, J. G., Monick, M. M., Mukaida, N., Matsushima, K., and Hunninghake, G. W., Activator protein-1 is the preferred transcription factor for cooperative interaction with nuclear factor-kappaB in respiratory syncytial virus-induced interleukin-8 gene expression in airway epithelium. *J. Infect. Dis.*, 177, 1275-1281 (1998).

Mathy-Hartert, M., Deby-Dupont, G. P., Reginster, J. Y., Ayache, N., Pujol, J. P., and Henrotin, Y. E., Regulation by reactive oxygen species of interleukin-1beta, nitric oxide and prostaglandin E(2) production by human chondrocytes. *Osteoarthritis Cartilage*, 10, 547-555 (2002).

Mathy-Hartert, M., Martin, G., Devel, P., Deby-Dupont, G., Pujol, J. P., Reginster, J. Y., and Henrotin, Y., Reactive oxygen species downregulate the expression of pro-inflammatory genes by human chondrocytes. *Inflamm. Res.*, 52, 111-118 (2003).

McCulloch, C. A. and Bordin, S., Role of fibroblast subpopulations in periodontal physiology and pathology. *J. Periodontal Res.*, 26, 144-154 (1991).

Means, T. K., Golenbock, D. T., and Fenton, M. J., Structure and function of Toll-like receptor proteins. *Life. Sci.*, 68, 241-258 (2000).

Minden, A. and Karin, M., Regulation and function of the JNK subgroup of MAP kinases. *Biochim. Biophys. Acta*, 1333, F85-F104 (1997).

Morse, D., Pischke, S. E., Zhou, Z., Davis, R. J., Flavell, R. A., Loop, T., Otterbein, S. L., Otterbein, L. E., and Choi, A. M., Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. *J. Biol. Chem.*, 278, 36993-36998 (2003).

Nagase, H. and Woessner, J. F. Jr., Matrix metalloproteinases. *J. Biol. Chem.*, 274, 21491-21494 (1999).

Okada, H. and Murakami, S., Cytokine expression in periodontal health and disease. *Crit. Rev. Oral Biol. Med.*, 9, 248-266 (1998).

Park, S. Y., Kim, Y. H., Jun, J. H., Kim, G. S., Min, B. M., and Baek, J. H., P. gingivalis and A. actinomycetemcomitans LPS differentially regulate the expression of inflammatory cytokines and chemokines in gingival fibroblast and periodontal ligament fibroblast. *Int. J. Oral Biol.*, 29, 7-16 (2004).

Rajagopalan, S., Meng, X. P., Ramasamy, S., Harrison, D. G., and Galis, Z. S., Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J. Clin. Invest.*, 98, 2572-2579 (1996).

Ryan, K. A., Smith, M. F. Jr., Sanders, M. K., and Ernst, P. B., Reactive oxygen and nitrogen species differentially regulate Toll-like receptor 4-mediated activation of NF-kappa B and interleukin-8 expression. *Infect. Immun.*, 72, 2123-2130 (2004).

Sanchez-Tillo, E., Comalada, M., Xaus, J., Farrera, C., Valledor, A. F., Caelles, C., Lloberas, J., and Celada, A., JNK1 is required for the induction of MKP1 expression in macrophages during proliferation and LPS-dependent activation. *J. Biol. Chem.*, 282, 12566-12573 (2007).

Schreck, R., Albermann, K., and Baeuerle, P. A., Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic. Res. Commun.*, 17, 221-237 (1992).

Schubert, S. Y., Neeman, I., and Resnick, N., A novel mechanism for the inhibition of NF-kappaB activation in vascular endothelial cells by natural antioxidants. *FASEB J.*, 16, 1931-1933 (2002).

Seger, R. and Krebs, E. G., The MAPK signaling cascade. *FASEB J.*, 9, 726-735 (1995).

Strassheim, D., Asehnoune, K., Park, J. S., Kim, J. Y., He, Q., Richter, D., Mitra, S., Arcaroli, J., Kuhn, K., and Abraham, E., Modulation of bone marrow-derived neutrophil signaling by H_2O_2: disparate effects on kinases, NF-kappaB, and cytokine expression. *Am. J. Physiol. Cell Physiol.*, 286, C683- C692 (2004).

Tiranathanagul, S., Yongchaitrakul, T., Pattamapun, K., and Pavasant, P., Actinobacillus actinomycetemcomitans lipopolysaccharide activates matrix metalloproteinase-2 and increases receptor activator of nuclear factor-kappaB ligand expression in human periodontal ligament cells. *J. Periodontol.*, 75, 1647-1654 (2004).

Victor, V. M. and De la Fuente, M., N-acetylcysteine improves in vitro the function of macrophages from mice with endotoxin-induced oxidative stress. *Free Radic. Res.*, 36, 33-45 (2002).

Victor, V. M., Rocha, M., and De la Fuente, M., N-acetylcysteine protects mice from lethal endotoxemia by regulating the redox state of immune cells. *Free Radic. Res.*, 37, 919-929 (2003).

Winyard, P. G. and Blake, D. R., Antioxidants, redox-regulated transcription factors, and inflammation. *Adv. Pharmacol.*, 38, 403-421 (1997).

Woo, C. H., Lim, J. H., and Kim, J. H., Lipopolysaccharide induces matrix metalloproteinase-9 expression via a mitochondrial reactive oxygen species-p38 kinase-activator protein-1 pathway in Raw 264.7 cells. *J. Immunol.*, 173, 6973-6980 (2004).

Zafarullah, M., Li, W. Q., Sylvester, J., and Ahmad, M., Molecular mechanisms of N-acetylcysteine actions. *Cell. Mol. Life Sci.*, 60, 6-20 (2003).