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J DENT RES 2007 86: 451
DOI: 10.1177/154405910708600512

RESEARCH REPORTS
Biological

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J Dent Res 86(5):451-456, 2007

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ABSTRACT

Ozone has been proposed as an alternative oral antiseptic in dentistry, due to its antimicrobial power reported for gaseous and aqueous forms, the latter showing a high biocompatibility with mammalian cells. New therapeutic strategies for the treatment of periodontal disease and apical periodontitis should consider not only antibacterial effects, but also their influence on the host immune response. Therefore, our aim was to investigate the effect of aqueous ozone on the NF-κB system, a paradigm for inflammation-associated signaling/transcription. We showed that NF-κB activity in oral cells stimulated with TNF, and in periodontal ligament tissue from root surfaces of periodontally damaged teeth, was inhibited following incubation with ozonized medium. Under this treatment, IκBα proteolysis, cytokine expression, and κB-dependent transcription were prevented. Specific ozonized amino acids were shown to represent major inhibitory components of ozonized medium. In summary, our study establishes a condition under which aqueous ozone exerts inhibitory effects on the NF-κB system, suggesting that it has an anti-inflammatory capacity.

KEY WORDS: aqueous ozone, NF-κB, signaling, inflammation, oral.

Received March 19, 2006; Last revision December 2, 2006; Accepted January 1, 2007

A supplemental appendix to this article is published electronically only at http://www.dentalresearch.org.

INTRODUCTION

Currently, ozone is being discussed in dentistry as a possible alternative oral antiseptic agent. Its high antimicrobial power, including against oral pathogens, without resistance development has been reported not only for gaseous ozone (Paraskeva and Graham, 2002; Baysan and Lynch, 2004), but also for ozone in aqueous solution (Restaino *et al.*, 1995; Nagayoshi *et al.*, 2004a,b; Arita *et al.*, 2005). In the concentrations currently used in dentistry, ozone gas has been found to decrease the viability of oral cells significantly (Huth *et al.*, 2006). In comparison, aqueous ozone revealed a high level of biocompatibility to fibroblasts, cementoblasts, and epithelial cells (Filippi, 2001; Ebensberger *et al.*, 2002; Nagayoshi *et al.*, 2004b; Huth *et al.*, 2006), which suggests its use against oral infectious diseases, where it comes into contact with resident oral cells, *e.g.*, periodontal disease and apical periodontitis.

For the manifestation of these disease entities, bacterial colonization of the dento-gingival complex or the endodontic region, respectively, has been considered as the primary causative factor (Graves *et al.*, 2000; Nair, 2004). However, it is generally accepted that periodontal disease and apical periodontitis do not result from direct tissue destruction by pathogenic bacteria. Rather, the destructive potential of the host immune response leads mainly to signs and symptoms of these diseases (Honda *et al.*, 2006; Nair, 2004). Therefore, immune modulation effects of treatment strategies for these diseases may also be considered. Both disease entities are characterized by an inflammatory reaction involving different oral hard and soft tissues, *e.g.*, the gingiva, periodontal attachment fibers, or alveolar bone (Nair, 2004; Bartold and Narayanan, 2006). The inflammatory process is primarily induced by pathogen-associated molecular patterns (PAMPs), particularly by bacterial lipopolysaccharides (Madianos *et al.*, 2005). The subsequent activation of the inflammatory molecular cascade leads to the expression of several pro-inflammatory cytokines—*e.g.*, interleukin-1, interleukin-8, and tumor necrosis factor (TNF)—that ultimately mediate the destruction of the alveolar bone and periodontal connective tissue (Gamonal *et al.*, 2000; Graves *et al.*, 2000; Márton and Kiss, 2000).

The transcription factor NF-κB plays a pivotal role in inflammatory/immune processes and apoptosis (Bonizzi and Karin, 2004). NF-κB is also thought to be of paramount importance in the regulation of periodontal/periapical inflammatory reactions and the pathogenesis of periodontal disease and apical periodontitis (Nichols TC *et al.*, 2001; Sabeti *et al.*, 2005; Bartold and Narayanan, 2006). This transcription factor exists as a dimer which is trapped in the cytosol by inhibitory proteins, *e.g.*, IκBα (Bonizzi and Karin, 2004). The NF-κB system is activated by numerous agents, including cytokines (*e.g.*, TNF, interleukin-1) and microbial pathogens/products. The activation of NF-κB is mediated by the IκB kinase complex that phosphorylates IκB, which is subsequently degraded by the proteasome. The thus-freed NF-κB translocates to the nucleus, where it

binds to κB sequences in promoters/enhancers, thereby regulating the expression of various genes such as interleukin-1/-8 or TNF.

Ozone gas is known to activate NF-κB under certain conditions (Haddad *et al.*, 1996; Laskin *et al.*, 2002). However, it is not known if aqueous ozone also interferes with the NF-κB system. This is important, since an activation of NF-κB might adversely affect the therapeutic benefit of aqueous ozone when used against periodontal disease and apical periodontitis. Hence, the aim of the present study was to investigate the effect of aqueous ozone on NF-κB-associated signaling/transcription in oral cells.

MATERIALS & METHODS

Culture Conditions

Human oral epithelial cells (BHY, ACC404, DSMZ, Braunschweig, Germany), gingival fibroblasts (HGF-1, ATCC-CRL-2014, LGC-Promochem, Teddington, UK), and HeLa cells (DSMZ) were cultured under standard conditions: BHY and HGF-1 in DMEM (PromoCell, Heidelberg, Germany) and HeLa in RPMI-1640 medium (Biochrom, Berlin, Germany) (7% or 10% fetal calf serum, respectively), both containing 100 U/mL penicillin and 100 μg/mL streptomycin (Biochrom). The proteasome inhibitor PSI was from Calbiochem (Darmstadt, Germany). A potential toxicity of culture conditions was monitored by cell morphology/count, trypan-blue-exclusion, and the Luminescent-Cell-Viability-Assay (Promega, Mannheim, Germany). Furthermore, we acquired periodontal ligament tissue adherent to the middle third of the root surfaces of permanent teeth (Hou and Yaeger, 1993) following extraction due to periodontitis-associated destruction, with patients' informed written consent. Hematoxylin-eosin staining revealed the predominance of fibroblasts in this tissue (data not shown), as found earlier and reviewed recently (Nanci and Bosshardt, 2006). Samples were incubated in RPMI-1640 and, following the experimental procedure, were shock-frozen in liquid nitrogen and homogenized in a pre-chilled mortar before nuclear extracts were prepared (Appendix A).

Ozonation of Reagents

Aqueous ozone was applied to the cells in the form of ozonized medium without fetal calf serum (O_3 medium), or ozonized phosphate-buffered saline (O_3-phosphate-buffered saline) without/with 1 g/L amino acids or 2 g/L glucose. These solutions were treated with gaseous ozone (75 μg/mL, 15 min) in an ozone generator (Ozonosan-photonic, Dr. Hänsler, Iffezheim, Germany). This condition—which, when applied to water, would result in a final ozone concentration of 20 μg/mL (saturation point)—was defined as the 100% ozonation state. For dose-response experiments, the solutions were diluted accordingly. The ozone gas concentration was monitored by a photometer integrated into the ozone generator and confirmed by another ozone gas detector (GM-6000-NZL, Anseros, Tübingen, Germany). The ozone concentrations of the respective aqueous solutions were monitored photometrically (Palintest, Gateshead, England).

Electrophoretic Mobility Shift Assay

Nuclear extracts of cell lines or periodontal ligament tissue were prepared, and an electrophoretic mobility shift assay was performed (Page *et al.*, 1999; Appendix A). The prototypic immunoglobulin κ-chain oligonucleotide was used as a probe and labeled with the Klenow fragment of DNA polymerase I (Roche, Penzberg, Germany), together with [α-^{32}P]dCTP (PerkinElmer-LifeSciences, Brussels, Belgium). The Sp-1 consensus oligonucleotide (Promega) was labeled with [γ-^{32}P]ATP (PerkinElmer-LifeSciences) and T4-polynucleotide-kinase (Roche). Samples were run on non-denaturing 4% polyacrylamide gels and analyzed by autoradiography.

Western Blot Analysis

Cytosolic extracts were isolated, and electrophoresis was performed (Page *et al.*, 1999; Appendix A). After transfer, the membranes were incubated with IκBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or actin antibodies (Sigma-Aldrich, Deisenhofen, Germany), followed by secondary peroxidase-conjugated antibodies (Dianova, Hamburg, Germany). Antibody binding was visualized on x-ray film with Chemiluminescent-Reagent-Plus (PerkinElmer-LifeSciences).

Determination of Cytokines

Interleukin-8 and -1β concentrations in supernatants were measured by immunoassays (R&D-Systems, Wiesbaden, Germany; Bender-MedSystems, Vienna, Austria).

Luciferase Assay

For luciferase assays, HeLa cells were transiently transfected with a firefly-luciferase reporter plasmid (pGL2-3κB-Luc, 3-κB binding motifs), together with a constitutively active Renilla-luciferase plasmid, pRLtk (Promega) (Page *et al.*, 1999; Appendix A), according to a Superfect-based protocol (Qiagen, Hilden, Germany). Subsequent to stimulation, cells underwent lysis, and luciferase activity was determined with the Dual-Luciferase-Reporter-Assay (Promega). Results were expressed as relative luciferase activity (firefly-relative light-units divided by Renilla-relative light-units).

RESULTS

NF-κB Activation is Inhibited by O_3 Medium

First, we incubated oral epithelial cells (BHY), gingival fibroblasts (HGF-1), and HeLa epithelial cells in O_3 medium to investigate whether this was a condition that directly activated NF-κB. Dose-response and time-course experiments demonstrated that O_3 medium alone did not modulate the NF-κB system (data not shown). Next, we tested whether pre-incubation with O_3 medium affected the activation of NF-κB by other stimuli. Cells were pre-incubated with O_3 medium followed by exposure to TNF, and optimal cellular conditions were again evaluated by dose-response/time-course experiments (data not shown). In the absence of ozone, a marked activation of NF-κB by TNF was observed (Figs. 1A, 1B). Incubation with O_3 medium, however, inhibited this activation, whereas constitutive Sp-1 binding was unchanged. We excluded a potential toxicity of O_3 medium by monitoring cell morphology, trypan-blue exclusion, and ATP levels (Cell Viability Assay) (Appendix B). As a positive control, pre-incubation with the proteasome inhibitor PSI (10 μM, 1 hr) markedly prevented TNF-induced NF-κB activity (BHY, inhibition by 68 ± 7%; HeLa, inhibition by 75 ± 6%; n = 3). Furthermore, we investigated whether O_3 medium also modulated NF-κB activity in periodontal ligament tissue from the root surfaces of periodontally diseased teeth. These experiments revealed strong NF-κB binding activity when this

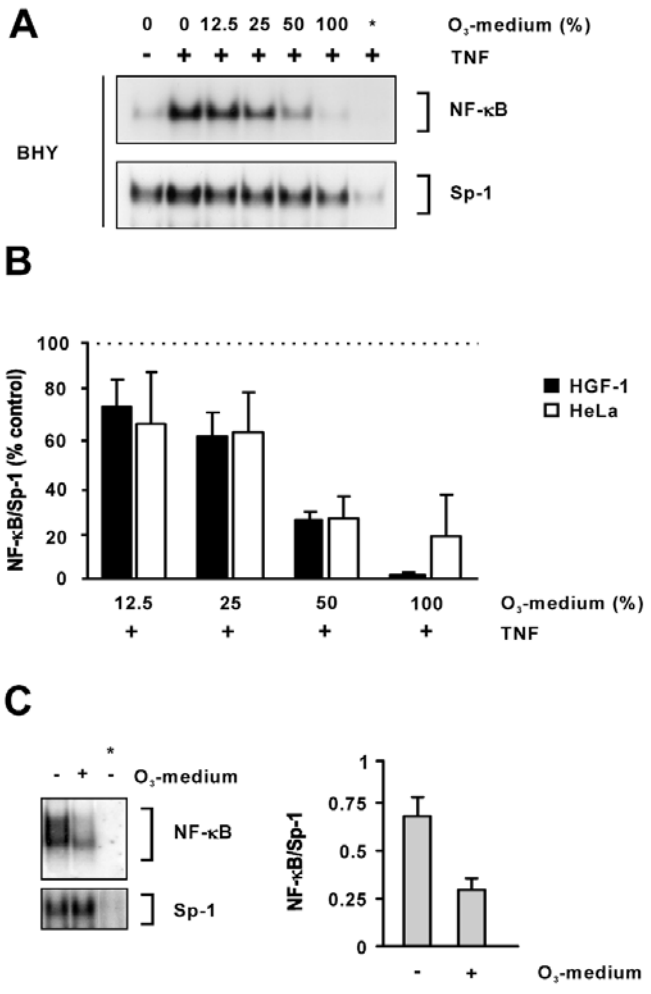

Figure 1. Activation of NF-κB was inhibited in the presence of O_3 medium. **(A)** BHY cells were pre-incubated with serum-free O_3 medium (15 min; the ozonation state is indicated in %) before TNF was added (20 ng/mL, 45 min), and an electrophoretic mobility shift assay was performed (NF-κB and Sp-1 binding). The asterisk marks a control reaction in which a 100-fold concentration of unlabeled consensus oligonucleotide was added. **(B)** Cells were treated with O_3 medium (HGF-1, 45 min; HeLa, 1 hr), followed by stimulation with TNF (HGF-1, 5 ng/mL for 45 min; HeLa, 1 ng/mL for 15 min). NF-κB activity normalized to Sp-1 is shown (densitometric analysis). NF-κB activity after TNF stimulation following pre-incubation with non-ozonized medium was defined as 100% (dashed line) (n = 3, mean ± SD). **(C)** Periodontal ligament tissue separated into equal-sized portions was incubated (1 hr, 37°C) with serum-free non-ozonized medium (indicated by "-") or O_3 medium (100% ozonation state, indicated by "+"), followed by two-hour incubation in fresh medium without ozone. The samples were shock-frozen and homogenized, and nuclear extracts were prepared. Left: Electrophoretic mobility shift assays were performed (NF-κB and Sp-1 binding). The asterisk marks a control reaction in which a 100-fold concentration of unlabeled consensus oligonucleotide was added. Right: Densitometrically measured NF-κB activity was normalized to Sp-1 (n = 3, mean ± SD).

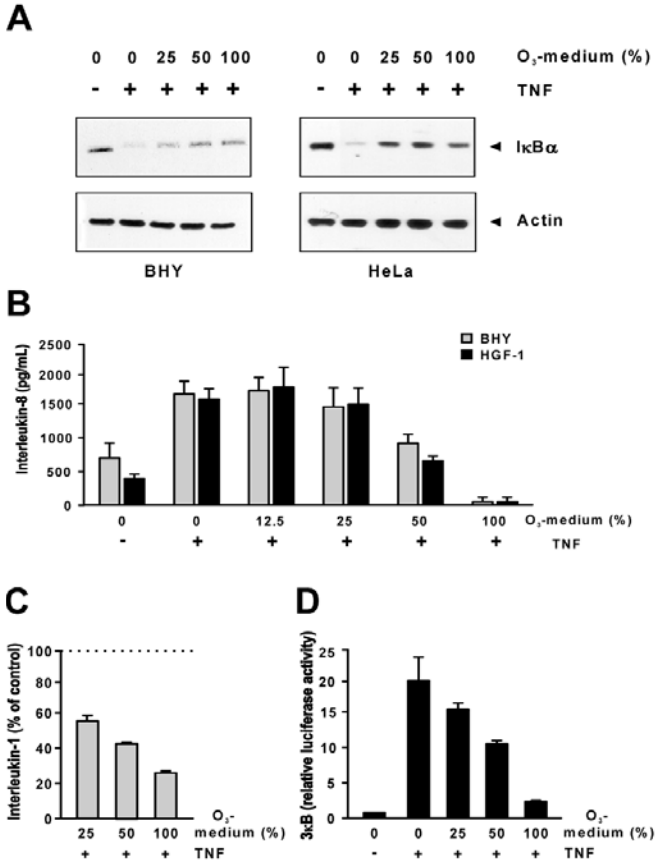

Figure 2. Effect of O_3 medium on IκBα proteolysis, NF-κB-dependent gene expression, and κB-dependent transcription. **(A)** IκBα proteolysis was prevented in O_3-medium-cultivated cells. BHY (left panel) and HeLa cells (right panel) were pre-incubated with serum-free O_3 medium (BHY, 15 min; HeLa, 1 hr) (ozonation state in %) before TNF was added (BHY, 20 ng/mL, 45 min; HeLa, 1 ng/mL, 15 min). IκBα and actin were determined by Western blot analysis. (B) and (C) NF-κB target gene expression was inhibited by pre-incubation with O_3 medium. **(B)** BHY and HGF-1 cells were pre-incubated with serum-free O_3 medium (BHY, 15 min; HGF-1, 45 min), followed by TNF (BHY, 20 ng/mL, 45 min; HGF-1, 5 ng/mL, 45 min). The supernatant was then replaced by regular medium, and interleukin-8 was measured after 5 hrs by an enzyme-linked immunosorbent assay (BHY, n = 2; HGF-1, n = 3; mean ± SD). **(C)** BHY cells were pre-treated with O_3 medium (15 min). The supernatant was replaced by regular medium, TNF (20 ng/mL, 45 min) was added, and interleukin-1β was measured after 12 hrs (immunoassay). The interleukin-1β level after TNF stimulation following pre-treatment with non-ozonized medium was defined as 100% (dashed line) (n = 3, mean ± SD). **(D)** κB-dependent transcription was inhibited by pre-incubation with O_3 medium. HeLa cells were transfected with pGL2-3κB-Luc and the Renilla plasmid. After 24 hrs, cells were treated with serum-free O_3 medium (1 hr), followed by TNF (1 ng/mL, 15 min). The supernatant was replaced by regular medium, and relative luciferase activity was measured after 5 hrs (n = 3, mean ± SD).

tissue was incubated in medium alone, which was markedly inhibited following treatment with ozonized medium (Fig. 1C).

IκBα Proteolysis is Inhibited in the Presence of O_3 Medium

Next, we investigated if the level of the NF-κB inhibitor IκBα was affected by O_3 medium. Stimulation of cells with TNF led to proteolysis of IκBα (Fig. 2A). However, in O_3-medium-pre-incubated cells, this TNF-induced IκBα degradation was inhibited, whereas constitutive actin levels were unchanged.

Target Gene Expression and κB-dependent Transcription Were Prevented

We studied functional consequences by measuring cytokine levels in culture supernatants. Consistent with the results above, pre-incubation with O_3 medium led to a dose-dependent

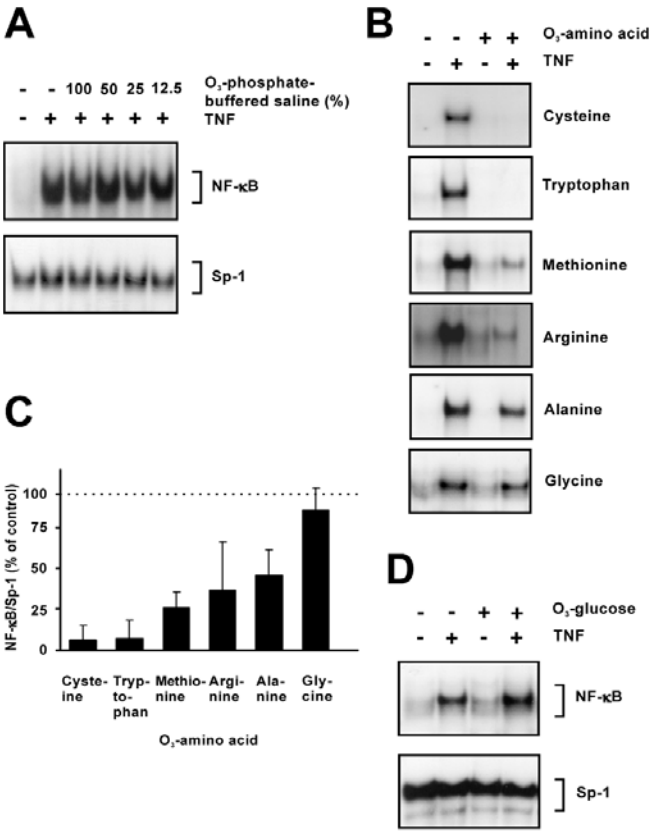

Figure 3. O_3 amino acids selectively inhibited the NF-κB system. **(A)** HeLa cells were incubated with O_3-phosphate-buffered saline (1 hr), followed by TNF stimulation (1 ng/mL, 15 min). The ozonation state is indicated in %. We performed an electrophoretic mobility shift assay to determine NF-κB activity as well as Sp-1 binding. **(B)** Cells were pre-incubated (1 hr) with non-ozonized or ozonized amino acids (medium concentration) dissolved in phosphate-buffered saline (100% ozonation state), followed by stimulation with TNF (1 ng/mL, 15 min). The activation of NF-κB was determined by an electrophoretic mobility shift assay. **(C)** Densitometric analysis of the NF-κB activity normalized to Sp-1 is shown (n = 3, mean ± SD). NF-κB activity of cells pre-incubated with non-ozonized amino acids in phosphate-buffered saline and stimulated with TNF was defined as 100% (dashed line). **(D)** HeLa cells were pre-incubated with non-ozonized or ozonized glucose (medium concentration), followed by TNF stimulation. The NF-κB activity compared with Sp-1 binding was determined by an electrophoretic mobility shift assay.

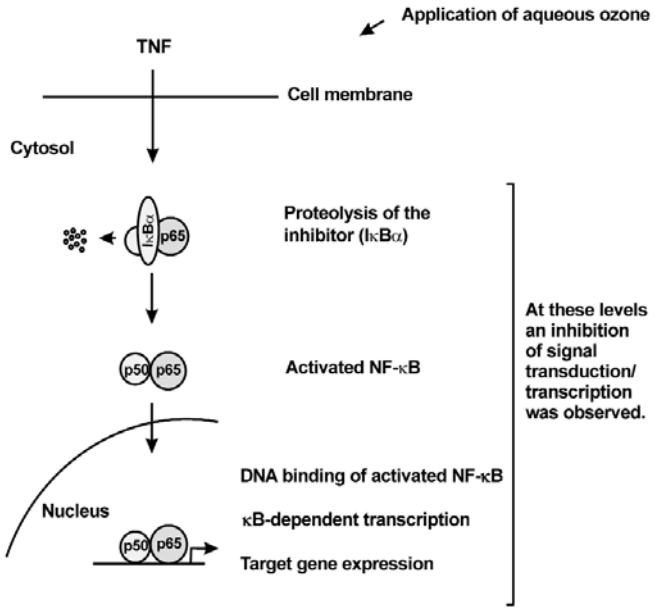

Figure 4. Inhibition of NF-κB by aqueous ozone. In this study, pre-incubation with aqueous ozone (O_3 medium) led, in TNF-stimulated cells, to an inhibition of IκBα proteolysis (Western blot analysis), NF-κB DNA binding (electrophoretic mobility shift assay), and κB-dependent transcription (luciferase assay), as well as to reduced expression of the target genes interleukin-8 and -1β (immunoassay). This indicates that aqueous ozone inhibited NF-κB signaling at the level and/or upstream of IκBα. In the Fig., the classic NF-κB dimer is depicted, consisting of the subunits p65 and p50. The activation pathway of NF-κB is described in more detail in the INTRODUCTION.

inhibition of TNF-induced production of interleukin-8 (Fig. 2B) and interleukin-1β (Fig. 2C). In addition, we transfected cells with a κB-dependent luciferase construct and treated them as described previously, to monitor a direct impact on κB-dependent transcription. TNF induced a marked increase in transcriptional activity, which again was strongly inhibited by O_3 medium (Fig. 2D).

O_3 Amino Acids Selectively Inhibited NF-κB

The inhibitory effect of O_3 medium could be due to ozone itself or to ozonized medium constituents. Therefore, cells were pre-incubated with O_3-phosphate-buffered saline without further ingredients. In contrast to the results obtained above, the NF-κB activation by TNF was not inhibited in all 3 cell lines by O_3-phosphate-buffered saline (Fig. 3A, data not shown). This indicated that the inhibitory effect of O_3 medium was not directly caused by ozone, but rather was mediated by the formation of medium-ozonation products. To identify these inhibitory ozonation products, we added various amino acids (medium concentration) of the different groups (Cataldo, 2003), as major medium components, to phosphate-buffered saline prior to ozonation. After pre-incubation of cells with ozonized and non-ozonized amino acids, TNF was added. Dependent on the different ozonized amino acids, NF-κB activity was variously affected: The sulfhydryl group containing cysteine and the aromatic tryptophan blocked the NF-κB signal almost completely (Figs. 3B, 3C); the sulfur-containing methionine resulted in clear inhibition, the basic arginine in a modest, and the non-polar alanine in a slight reduction of NF-κB activity, whereas the small, non-polar glycine or non-ozonized amino acids had no effect. Similarly, dose-dependent effects were observed in all 3 cell lines (data not shown). To determine whether the inhibition of TNF signaling by O_3 medium was also mediated by glucose, we added glucose (medium concentration) to phosphate-buffered saline, followed by ozonation, and treated the cells as described above. These experiments showed that the NF-κB activity was not inhibited by O_3-glucose (Fig. 3D). Taken together, these results signify that ozonized amino acids, to various extents, are an important component responsible for the inhibitory effect of O_3 medium.

DISCUSSION

The present study investigated the effect of aqueous ozone on the NF-κB system, which is involved in the pathogenesis of

periodontal disease and apical periodontitis (Nichols TC *et al.*, 2001; Sabeti *et al.*, 2005; Bartold and Narayanan, 2006). Analysis of our data revealed that activation of NF-κB induced by the potent cytokine TNF (Bonizzi and Karin, 2004) was dose-dependently inhibited in oral cells cultured in O_3 medium, while no toxic effects were observed. Supporting this finding, incubation with O_3 medium also inhibited NF-κB activity in periodontal ligament tissue of periodontally damaged teeth. This suggests that aqueous ozone under certain conditions displays anti-inflammatory effects.

Furthermore, our study demonstrated that TNF-stimulated proteolysis of the inhibitor IκBα (Bonizzi and Karin, 2004) was prevented in the presence of O_3 medium, which indicates that incubation with O_3 medium affects signaling at the level and/or upstream of IκB (Fig. 4). In addition, our experiments showed that κB-dependent transcription and the expression of the NF-κB target genes interleukin-8 and -1β (Pahl, 1999) were prevented by O_3 medium. Both cytokines have been correlated with the biological and clinical severity of periodontal disease (Gamonal *et al.*, 2000) and are secreted by various cells, *e.g.*, epithelial cells, fibroblasts, and monocytes/macrophages. Interleukin-8 expression shows a rapid onset in an early inflammatory stage and exerts chemotactic/activation effects on neutrophils (Okada and Murakami, 1998; Bartold and Narayanan, 2006). Interleukin-1β, when inadequately expressed, is known to mediate tissue-destructive effects, *e.g.*, bone resorption and loss of periodontal attachment (Gamonal *et al.*, 2000; Graves *et al.*, 2000).

Surprisingly, TNF-signaling was unaffected when O_3-phosphate-buffered saline was used instead of O_3 medium, which indicates that ozone itself is not able to inhibit NF-κB. Therefore, the question was which ozonation-product in the O_3 medium was responsible for this effect. To this end, we added various amino acids from the different groups to phosphate-buffered saline (medium concentration), and found that TNF-induced NF-κB activation was affected differently. The ozonized sulfhydryl group containing cysteine, as well as the aromatic tryptophan, were found to be most effective in inhibiting NF-κB, which agreed with earlier reports showing that the ozone attack is essentially directed toward the aromatic amino acids, and that there is a high oxidation reactivity of cysteine and methionine (Cataldo, 2003).

Earlier reports of the use of gaseous ozone throughout showed an activation of NF-κB (Haddad *et al.*, 1996; Laskin *et al.*, 2002). This discrepancy with our results may be explained by the fact that direct exposure of cells to gaseous ozone initiates an immediate oxidative stress by the increase of reactive oxygen species (Chen and Qu, 1997; Nichols BG *et al.*, 2001), a condition which directly activates NF-κB (Pahl, 1999). In contrast, the ozonation of amino acids in an aqueous milieu may lead to the production of modified amino acid molecules (Cataldo, 2003), which may interact with cellular signaling systems, thus preventing subsequent stimulation, as has been shown for lipid oxidation products such as 4-hydroxynonenal (Page *et al.*, 1999).

The clinical consequences of the inhibitory effects of aqueous ozone on NF-κB found herein remain to be elucidated. The attenuated activation of the host immune system may reduce periodontitis-associated tissue destruction (Bartold and Narayanan, 2006), which, as already mentioned, has been suggested to be caused by an inadequate and exaggerated inflammatory/immune response against a bacterial stimulus (Honda *et al.*, 2006). However, a prolonged inhibition of NF-κB should be avoided, to prevent a disequilibrium between the immune response and the bacterial challenge (Yamamoto and Gaynor, 2001).

In summary, our study establishes a condition under which the aqueous ozone exerts inhibitory effects on the NF-κB system, suggesting that it has anti-inflammatory and immune-modulatory capacities. Thereby, the data presented build an experimental basis for a comprehensive clinical study on the use of aqueous ozone in the oral cavity.

ACKNOWLEDGMENTS

We thank M. Krautkrämer and A. Ertl for excellent technical support. This work was financed by a grant from the Medical Faculty of the University of Munich (FöFoLe Reg.Nr. 401), the Stiftung für Pathobiochemie und Molekulare Diagnostik (DGKL), the Wilhelm-Sander-Stiftung, and the Department of Restorative Dentistry & Periodontology, University of Munich.

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