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Triclosan reduces microsomal prostaglandin E synthase-1 expression in human gingival fibroblasts

M. Mustafa[1], B. Wondimu[1], T. Yucel-Lindberg[1], A. T. Kats-Hallström[1], A. S. Jonsson[2], T.Modéer[1]

[1]Department of Pediatric Dentistry, Institute of Odontology; [2]Department of Medicine, Karolinska Institutet, Huddinge, Sweden

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Abstract

Objective: The effect of triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether) on the expression of cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) and on the translocation of the nuclear factor-κB (NF-κB) in relation to prostaglandin E_2 (PGE_2) production was investigated in human gingival fibroblasts challenged with tumor necrosis factor α (TNFα).

Methods: Fibroblasts were established from gingival biopsies obtained from six children. COX-2 mRNA and protein expression was quantified using mRNA quantitation and enzyme immunometric assay kits. mPGES-1 mRNA was analysed by RT-PCR, mPGES-1 protein and NF-κB translocation by immunoblotting. PGE_2 was determined by radioimmunoassay.

Results: The cytokine TNFα enhanced the expression of mRNA as well as the protein levels of both COX-2 and mPGES-1 and subsequently the production of PGE_2 in gingival fibroblasts. Treatment of gingival fibroblasts with triclosan (1 μg/ml) significantly reduced the stimulatory effect of TNFα (10 ng/ml) on the expression of mPGES-1 at both the mRNA and the protein level by an average of 21% and 43%, respectively, and subsequently the production of PGE_2 ($p < 0.01$). Triclosan did not, however, affect the translocation of NF-κB or the expression of COX-2 in TNFα-stimulated cells.

Conclusion: The results show that triclosan reduces the augmented biosynthesis of PGE_2 by inhibiting the mRNA and the protein expression of mPGES-1 in gingival fibroblasts. This finding may partly explain the anti-inflammatory effect of the agent previously reported in clinical studies.

Key words: gingival fibroblasts; membrane-bound prostaglandin E synthase-1; prostaglandin E_2; triclosan

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Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether) is a lipid-soluble antibacterial agent that is included in dentifrices and mouthrinses (Saxton 1989). The agent has been shown to exhibit a broad spectrum of antimicrobial activity against oral bacteria (Ritchie & Jones 1988, Ellingsen & Rölla 1994). Several clinical trials have reported that triclosan exhibits an inhibitory effect on gingival inflammation in addition to what can be accounted for the plaque reduction (Stephen et al. 1990, Lindhe et al. 1993). Furthermore, long-term use of a triclosan/copolymer dentifrice was shown to reduce attachment loss in adolescents (Ellwood et al. 1998) and slows the progression of periodontal disease in adults (Cullinan et al. 2003). Moreover, local application of triclosan has been reported to have an anti-inflammatory effect on oral mucosa (Skaare et al. 1996) and to reduce inflammatory reactions in human skin induced by sodium lauryl sulfate in experimental studies (Barkvoll & Rölla 1994). Triclosan has been shown to inhibit prostanoid formation in human gingival fibroblasts challenged with tumor necrosis factor α (TNFα) or interleukin-1β (IL-1β). The anti-inflammatory effect of triclosan is hypothesized to be partly related to its inhibitory effect on prostaglandin E_2 (PGE_2) biosynthesis (Gaffar et al. 1995, Modéer et al. 1996). The mechanism by which triclosan affects the biosynthesis of PGE_2 is, however, unclear.

The production of PGE_2 is regulated by key enzymes including phospholipase A_2, which release arachidonic acid (AA) from membrane phospholipids, and the two isozymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), which convert AA to prostaglandins (Dewitt 1991). Recently, however, the terminal enzyme prostaglandin E synthase (PGES), which converts PGH_2 into PGE_2, has been identified (Jakobsson et al. 1999). At present, three isoforms of PGES exist: the constitutive PGES which is expressed in the cytosol (cPGES), a membrane-bound PGES that is constitutively expressed (mPGES-2), and an inducible PGES located in the microsomal compartment (mPGES-1) (Thorén & Jakobsson 2000, Murakami et al. 2003).

It has been reported that the expression of mPGES-1 in human orbital fibroblasts and rheumatoid synovial cells, in contrast to cPGES and mPGES-2, is upregulated in response to pro-inflammatory cytokines such as TNFα or IL-1β (Stichtenoth et al. 2001, Han et al. 2002). Recently, we reported that gingival fibroblasts express mPGES-1 mRNA constitutively and this expression was enhanced in response to IL-β and TNFα (Yucel-Lindberg et al. 2004). In light of these findings and because of lack of information concerning the mechanisms involved in the inhibitory effect of triclosan on PGE_2 biosynthesis, the present study was undertaken. The aim was to investigate the effect of triclosan on the expression of COX-2 and mPGES-1 and on the translocation of nuclear factor-κB (NF-κB) in relation to PGE_2 production in human gingival fibroblasts.

Material and Methods

Fibroblast culture

Cultures of fibroblast cells were established from gingival biopsies obtained from six children (N-14, N-25, N-29, N-33, N-34, N-35) with no clinical sign of periodontal disease. The plan to use gingival fibroblasts for triclosan experiments was approved by the Ethical Committee of Karolinska Institutet, Huddinge University Hospital. Minced pieces of gingival tissue were explanted to 25 cm^2 Falcon tissue culture flasks containing 5 ml Eagle's basal medium (BME). The fibroblasts were obtained by trypsinization of the primary outgrowth of cells as previously described (Modéer et al. 1982). The cells used for the experiments proliferated in logarithmic phase between the eighth and 15th passage.

Quantification of COX-2 mRNA

Fibroblasts, harvested from the culture flasks, were counted in a Neubauer hemacytometer and seeded (1.5×10^4 cells/cm^2) in Petri dishes (100 mm) in a medium containing 5% fetal calf serum (FCS) at 37°C for 48 h. Then, the cells were treated in a serum-free medium with TNFα(6 h) in the presence or absence of triclosan or dexamethasone (Dex). After 6 h incubation at 37°C, the medium was withdrawn, acidified to pH 3.5, and stored at -70°C for PGE_2 analysis. The fibroblasts were washed three times in ice-cold phosphate buffered saline (PBS), frozen immediately in liquid nitrogen, and stored at -80°C for subsequent isolation of RNA. Total RNA was prepared by phenol–chloroform extraction and ethanol precipitation as described previously (Durnam & Palmiter 1983). The pellets were resuspended in 100 μl distilled water and total RNA was quantified by spectrophotometer at 260 nm. COX-2 mRNA levels were determined using a colorimetric mRNA quantitation kit (R&D, Minneapolis, MN, USA).

Quantification of COX-2 protein

Fibroblasts (1.5×10^4 cells/cm^2) were seeded in Petri dishes (60 mm) in a medium containing 5% FCS at 37°C for 48 h. After that the cells were treated in a serum-free medium with TNFα (24 h) in the presence or absence of triclosan or Dex. Thereafter, the medium was withdrawn, acidified to pH 3.5, and stored at -70°C for PGE_2 analysis. The fibroblasts were washed three times in ice-cold PBS, frozen immediately in liquid nitrogen, and then stored at -80°C for subsequent analysis of COX-2. The cells were scraped in TNE lysis buffer (10 mM Tris, pH 8, 0.15 M NaCl, 1% NP-40, 1 mM EDTA). The supernatant was collected after 5 min centrifugation at $12{,}000 \times g$ and assayed immediately using an enzyme immunometric assay kit (TiterZyme EIA, Assay Design Inc., Ann Arbor, MI, USA).

Analysis of mPGES-1 mRNA by reverse transcription polymerase chain reaction (RT-PCR)

Fibroblasts were seeded, treated, lysed and the total RNA was prepared as described under quantification of COX-2 mRNA. Total RNA (1.0 μg) was reverse transcribed using Superscript II in a total volume of 20 μl to obtain first-strand complementary DNA (cDNA). The obtained cDNA, 2.0 μl, was used for PCR amplification using AmpliTaq Gold DNA polymerase. The specific primers for mPGES-1 were sense 5′-CCAAGTGAGGCTGCGGAAGAA and mPGES-1 antisense 3′-GCTTCCCA-GAGGATCT GCAGA. The specificity of the RT-PCR products was confirmed by sequencing (CyberGene) using genebank database (Yucel-Lindberg et al. 2004). The GAPDH primers 5′-AAAG GGTCATCATCTCTGCC-3′ and 5′-TG ACAAAGTGGTCGTTGAGG-3′ were supplied by R&D Systems, and the reactions were performed according to the manufacturer&aposs recommendation. A 10-μl sample of each PCR reaction product was run on 3% NuSieve agarose gel, together with 1 kb DNA-ladder standard, and visualized with ethidium bromide. The PCR products were quantified with Kodak Electrophoresis Documentation and Analysis System (EDAS 290, Eastman Kodak Company, Rochester, NY, USA), using ROI Analysis to measure band intensities.

Immunoblot analysis of mPGES-1

Fibroblasts (1.5×10^4 cells/cm^2) were seeded in Petri dishes (60 mm) in a medium containing 5% FCS at 37°C for 48 h. After that the cells were treated in a serum-free medium with TNFα (24 h) in the presence or absence of triclosan or Dex. Thereafter, the medium was withdrawn, acidified to pH 3.5, and stored at -70°C for PGE_2 analysis. After that, the cells were washed three times in PBS and harvested in 0.4 ml cold buffer (10 mM, HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml of aprotinin, leupeptin, pepastatin). The cell lysate was centrifuged at $13{,}000 \times g$ for 10 min and the protein content in the lysates was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amount of protein (20 μg of total protein/sample) was fractionated on sodium dodecyl sulfate (SDS)-Page-separating gels and transferred electrophoretically to a nitrocellulose membrane (Bio-Rad). The membranes were then blocked in 5% dry milk and incubated with a primary polyclonal antibody against

mPGES-1 (diluted 1:1000) and secondary horseradish peroxidase-conjugated, swine anti-rabbit immunoglobulins (diluted 1:5000) for 1 h. The mPGES-1 protein was detected using an enhanced chemiluminescence system (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK) with autoradiographic film. The bands were quantified with Electrophoresis Documentation and Analysis System (EDAS) 290 using ROI Analysis to measure the band intensities.

Immunoblot analysis of NF-κB translocation

Fibroblasts were seeded, treated, and lysed as described under immunoblot analysis of mPGES-1. The cell lysate was separated into nuclear and cytoplasmic extracts. The nuclei were isolated by centrifugation at 13,000 × *g*, the supernatant was withdrawn, and the nuclear pellet was sonicated in 50 μl ice-cold buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA 1 mM DTT, 1 mM PMSF). Immunoblot analysis was performed using a primary antibody against NF-κB (polyclonal p65 diluted 1:3000) and a secondary antibody (horseradish peroxidase-conjugated, swine anti-rabbit immunoglobulins diluted 1:5000).

PGE_2 production

The amount of PGE_2 in the medium was determined by radioimmunoassay (RIA) using a commercially available kit.

Chemicals

BME, Hepes buffer, FCS, penicillin, and streptomycin were obtained from Life Technologies (Paisley, UK). Quantitation kits for COX-2 mRNA were purchased from R&D and enzyme immunometric assay kits for COX-2 protein from Assay Designs Inc., Ann Arbor, MI, USA. The protease inhibitors aprotinin, leupeptin, and pepastatin and human recombinant TNFα (specific activity $>2 \times 10^6$ U/mg) were supplied by Amersham Pharmacia Biotech (Little Chalfont, UK) The ^{125}I-PGE_2 RIA kit was obtained from NEN Life Science Products (Boston, MA, USA) Dex and pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The agent triclosan (2,4,4′-trichloro-2′-hydroxyldiphenyl ether) was a gift from Denofa OG Lilleborg Fabriker (Oslo, Norway). Polyclonal antibody against NF-κB (p65) was obtained from Oncogene Research Products (Cambridge, UK). Polyclonal antibody against mPGES-1 was supplied by Cayman Chemical (Ann Arbor, MI, USA) and peroxidase-conjugated swine anti-rabbit IgG by Dako (Golstrup, Denmark).

Statistics

Student's *t*-test (two-tailed) was used in the statistical analysis.

Results

In agreement with the previous findings (Modéer et al. 1992, Brunius et al. 1993), we here report that PGE_2 formation was enhanced in 24 h cultures of human gingival fibroblasts challenged with TNFα (10 ng/ml) or with exogenous AA (10 μM) (Fig. 1A, B). When triclosan (1 μg/ml) was added simultaneously with TNFα, the agent reduced ($p<0.01$) the stimulatory effect of TNFα (24 h) on PGE_2 production (Fig. 1A). Triclosan also reduced ($p<0.01$) the stimulatory effect of exogenous AA on PGE_2 production in a dose-dependent manner (Fig. 1B).

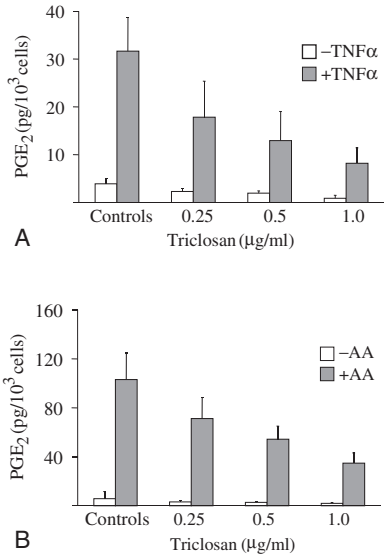

Fig. 1. Effect of triclosan in the presence or absence of (A) tumor necrosis factor α (TNFα) (10 ng/ml) or (B) exogenous arachidonic acid (AA, 10 μM) on prostaglandin E_2 (PGE_2) production in 24 h cultures of gingival fibroblasts. Mean ± SD of three cell lines (N-14, N-25, N-33).

In the next series of experiments, we investigated the effect of triclosan on the translocation of NF-κB and on the mRNA and protein expression of COX-2 in gingival fibroblasts challenged with TNFα (10 ng/ml). Our results showed that triclosan (1 μg/ml) did not affect the translocation of NF-κB (1 h) (Fig. 2A, B), the expression of COX-2 mRNA (6 h), or the protein level of COX-2 (24 h) in the controls or in the TNFα-stimulated cells (Fig. 3A, B). On the contrary, PDTC (100 μM) and Dex (1 μM), used as positive controls, reduced the TNFα-induced translocation of NF-κB ($p<0.05$) (Fig. 2A, B) and the mRNA ($p<0.01$), and protein expression ($p<0.01$) of COX-2 (Fig. 3A, B), respectively, in gingival fibroblasts.

In another series of experiments, we studied the effect of triclosan on the mRNA expression of mPGES-1, a terminal enzyme of the COX-2-mediated PGE_2 biosynthesis pathway. In agreement with the previous findings (Yucel-Lindberg et al. 2004), we here report that gingival fibroblasts constitutively express mPGES-1 mRNA (Fig. 4A, B). Furthermore, TNFα (10 ng/ml) enhanced the expression of mPGES-1 mRNA (6 h) and protein levels (24 h) of mPGES-1 in cultures of gingival fibro-

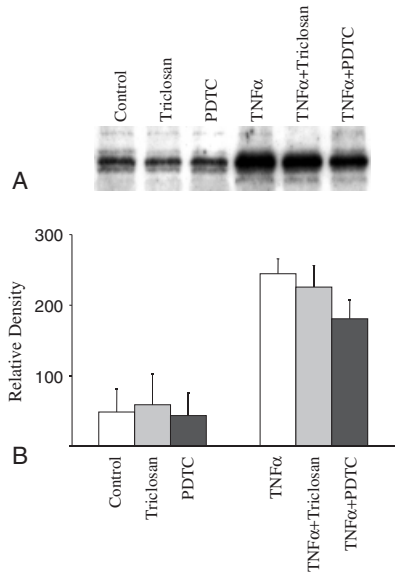

Fig. 2. Effect of triclosan (1 μg/ml) in the presence or absence of tumor necrosis factor (TNFα) (10 ng/ml) on nuclear factor-κB (NF-κB) translocation in 1 h cultures of gingival fibroblasts. NF-κB antagonist pyrrolidine dithiocarbamate (PDTC, 100 μM) was used as a positive control. (A) Western Blot analysis (N-35). (B) Relative density (mean ± SD) of three cell lines, (N-14, N-29, N-35).

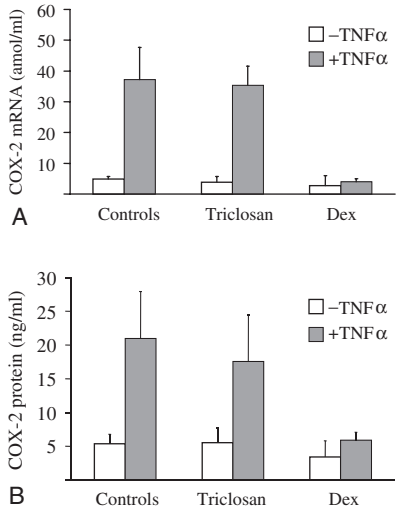

Fig. 3. Effect of triclosan (1 μg/ml) on (A) cyclooxygenase-2 (COX-2) mRNA (6 h) and (B) protein expression (24 h) induced by tumor necrosis factor α (TNFα) (10 ng/ml) in cultures of the gingival fibroblasts. Dexamethasone (Dex, 1 μM) was used as a positive control. Mean ± SD of three cell lines (N-14, N-33, N-34).

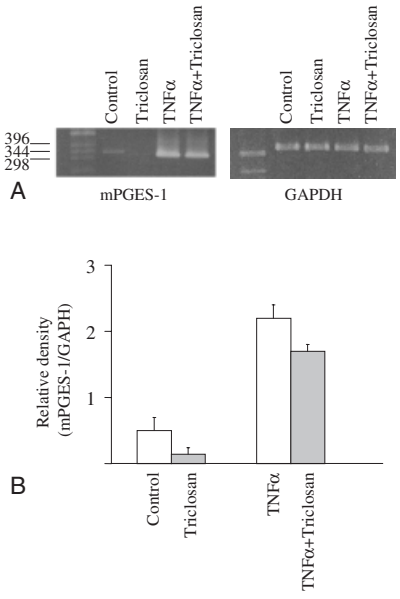

Fig. 4. Effect of triclosan (1 μg/ml) on microsomal prostaglandin E synthase-1 (mPGES-1) mRNA (6 h) induced by tumor necrosis factor α (TNFα) (10 ng/ml) in cultures of gingival fibroblasts. (A) Reverse transcription polymerase chain reaction (RT-PCR) analysis (N-34). (B) Relative density (mean ± SD) of three cell lines (N-25, N-33, N-34).

blasts (Figs 4A and 5A). Treatment of the cells (6 h) with triclosan (1 μg/ml) reduced the expression of mPGES-1 mRNA, both in the controls ($p<0.01$)

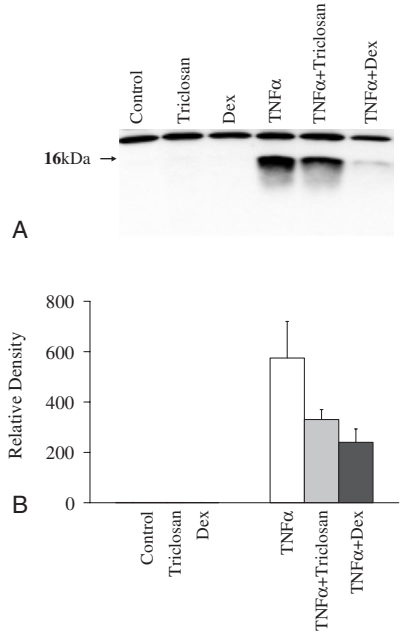

Fig. 5. Effect of triclosan (1 μg/ml) on microsomal prostaglandin E synthase-1 (mPGES-1) protein expression (24 h) induced by tumor necrosis factor α (TNFα) (10 ng/ml) in cultures of gingival fibroblasts (N-34). Dexamethasone (Dex) (1 μM) was used as a positive control. (A) Western blot analysis (N-34). (B) Relative density (mean ± SD) of three cell lines (N-25, N-29, N-34).

and in TNFα-stimulated cells ($p<0.05$) (Fig. 4A, B). Semi-quantification of PCR products showed that triclosan reduced the expression of mPGES-1 mRNA by 66% as an average (range 50–70) in the control cells and by 21% (range, 20–22) in TNFα-stimulated cells (N-34, N-25, N-33) (Fig. 4B). In addition, semi-quantification of western blot analysis showed that triclosan also reduced ($p<0.01$) the protein expression of mPGES-1 in 24 h cultures of gingival fibroblasts (N-34, N-29, N-25) challenged with TNFα by 43% (range, 36–51) (Fig. 5B). In the present study, mPGES-1 protein was undetectable in the control cells (Fig. 5A, B). Dex (1 μM), used as a positive control, reduced the TNFα-induced expression of mPGES-1 mRNA (data not shown) and the protein level of mPGES-1 (Fig. 5A, B).

Discussion

The effect of triclosan on the expression of COX-2 and mPGES-1 as well as on the translocation of NF-κB, in relation to PGE_2 production, was investigated in human gingival fibroblasts challenged with TNFα. The novel finding in the present study is that triclosan inhibits the production of PGE_2 by reducing the mRNA and the protein expression of mPGES-1 in gingival fibroblasts.

Several clinical trials have demonstrated that triclosan exhibits an inhibitory effect on gingival inflammation in addition to its antimicrobial effect (Lindhe et al. 1993, Suresh et al. 2001, Cullinan et al. 2003). Previous experimental studies have also demonstrated that triclosan has an inhibitory effect on prostanoid formation, and suggested that the anti-inflammatory effect of triclosan may be related to the inhibitory effect of the agent on PGE_2 formation (Gaffar et al. 1995, Modéer et al. 1996). PGE_2 is an important mediator of inflammation, and the level of PGE_2 in the gingival crevicular fluid is correlated with the degree of gingival inflammation and periodontitis (Offenbacher et al. 1993, Preshaw & Heasman 2002).

The biosynthesis of PGE_2 has been shown to involve the translocation of NF-κB (Crofford et al. 1997) and the expression of several enzymes including COX-2 (Dewitt 1991). In agreement with the previous findings (Nakao et al. 2002), we here report that TNFα induces NF-κB translocation, enhances COX-2 expression, and subsequently increases the production of PGE_2 in gingival fibroblasts. Our findings showed that treatment of the cells with triclosan reduces the production of PGE_2 without affecting the translocation of NF-κB or the expression of COX-2 in gingival fibroblasts. These findings indicate that the inhibitory effect of triclosan on the formation of PGE_2 is probably not related to COX-2 on the transcription or the translation level. However, we cannot rule out the possibility that triclosan may affect the activity of COX-2 and thereby reduce PGE_2 biosynthesis, since we did not succeed in determining the activity of COX-2 in gingival fibroblasts in the present study. Notably, triclosan has been reported to reduce the activity of COX-2 isolated from sheep placenta (Gaffar et al. 1995). However, the inhibitory effect of triclosan on the production of PGE_2 may involve other enzymes downstream of COX-2 in the PGE_2 biosynthetic pathway as well.

The recent identification of several isoforms of the terminal enzyme PGES (Jakobsson et al. 1999) has led to the proposal that these enzymes may play a regulatory role in the formation of PGE_2. The inducible isoform mPGES-

1, which converts the COX-2-derived PGH_2 into PGE_2, has been shown to be upregulated by pro-inflammatory cytokines such as IL-β and TNFα (Thoren & Jakobsson 2002, Han et al. 2002). Recently, Han et al. (2002) suggested that a functional coupling exist between COX-2 and mPGES-1 since the known selective COX-2 inhibitor SC58125 dramatically downregulates the expression of mPGES-1 in human orbital fibroblasts. Notably, triclosan in the present study reduced the upregulation of mPGES-1 without affecting COX-2 expression in gingival fibroblasts. This finding suggests that the agent triclosan, by reducing mPGES-1 expression, inhibits PGE_2 production in gingival fibroblasts. Further investigations on dose-response effect of triclosan, however, are needed to elucidate this issue.

Recent studies conducted in transfected cells have indicated that functional coupling may occur between COX-1 and mPGES-1 in cells treated with high concentration (10 μM) of exogenous AA (Murakami et al. 2000, 2003). This finding is compatible with our view that triclosan reduces the expression of mPGES-1 since the agent also reduced the PGE_2 biosynthesis stimulated by high concentrations of exogenous AA. Whether or not triclosan affects the signal pathways of the functional coupling of COX-1/mPGES-1 has to be further studied.

It was recently suggested that mPGES-1 plays a significant role in joint inflammation in rat adjuvant-induced arthritis (Claveau et al. 2003). Based on the fact that PGE_2 is strongly associated with the progression of periodontal disease (Champagne et al. 2003), it is reasonable to assume that mPGES-1 may be of importance in the pathogenesis of periodontal diseases. In this context, the finding that triclosan downregulates the production of PGE_2 by decreasing the expression of mPGES-1 may partly be of clinical relevance for the anti-inflammatory effect of the agent previously demonstrated in clinical studies (Lindhe et al 1993, Cullinan et al. 2003).

In conclusion, the agent triclosan reduces the augmented biosynthesis of PGE_2 by inhibiting the mRNA and protein expression of mPGES-1 in gingival fibroblasts challenged with TNFα. These findings may partly explain the previously reported anti-inflammatory effect of the agent on gingival inflammation.

Acknowledgments

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Address:
Thomas Modéer
Department of Pediatric Dentistry
Institute of Odontology
Karolinska Institutet
Box 4064, SE-141 04 Huddinge
Sweden
Fax: +46-8-7743395
E-mail: thomas.modeer@ofa.ki.se