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Triclosan inhibits tumor necrosis factor-α-stimulated urokinase production in human gingival fibroblasts

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Background and Objectives: Destruction of the supporting periodontal tissues is mediated by the action of several proteolytic enzymes. Urokinase is a serine protease that plays a key role in connective tissue destruction through conversion of plasminogen into plasmin. The present study was conducted to evaluate the effect of triclosan on the production and activity of urokinase in cultured gingival fibroblasts.

Material and Methods: Urokinase production was studied in primary cultures of human gingival fibroblasts stimulated with tumor necrosis factor-alfa. Urokinase activity and production were evaluated using casein zymography and western blotting, respecively. Urokinase mRNA expression was evaluated using the reverse transcription–polymerase chain reaction. Triclosan was used to interfere with this stimulatory effect. The roles of different cell-signaling cascades involved in urokinase production were assessed through western blotting and immunofluorescence using several cell-signaling inhibitors.

Results: Tumor necrosis factor-alfa was found to be a strong stimulus for urokinase production and triclosan was able to inhibit this response at the protein and mRNA levels. Triclosan was also able to inhibit conversion of plasminogen into plasmin. Tumor necrosis factor-alfa-stimulated urokinase production was shown to be dependent on the nuclear factor-κB and c-Jun N-terminal kinase signaling pathways. Triclosan inhibited c-Jun N-terminal kinase phosphorylation and c-Jun production.

Conclusions: Within the limits of this study, these results show that triclosan may inhibit urokinase production and plasminogen activation in gingival fibroblasts through modulation of the c-Jun N-terminal kinase signaling pathway.

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Triclosan is a widely used lipid-soluble antibacterial agent that has been incorporated into dentifrices and mouthrinses (1). Initially it was described that this pharmacological agent had a direct effect on plaque reduction and that this property was associated with a significant reduction in gingival inflammation (2–7). However, more recent studies have shown that triclosan may also exert an anti-inflammatory effect. Local application of triclosan has been reported to inhibit inflammation on oral mucosa and to reduce chemically induced inflammatory reactions in human skin (8,9). Triclosan may also inhibit prostanoid formation in human gingival fibroblasts stimulated with tumor necrosis factor-alfa or interleukin-1 beta (10,11). This effect has been explained by the ability of triclosan to reduce the mRNA and protein expression of microsomal prostaglandin E

synthase-1 in gingival fibroblasts (12). Other studies have reported that triclosan may also promote the healing of periodontal lesions after treatment (13,14).

Clinical studies have reported that triclosan may retard the progression of periodontal disease when used in a dentifrice (15–17). This therapeutic property has been explained by the ability of triclosan to induce qualitative and quantitative changes in the subgingival microflora of patients with periodontitis (18). Besides the role of bacteria in periodontal disease pathogenesis, a key step in progressive periodontitis is the degradation of connective tissue molecules involved in periodontal attachment (19). Fibroblasts are the predominant cell type in gingival connective tissues and are actively involved in connective tissue formation and destruction in both health and disease (20). The extracellular matrix is actively degraded by several groups of enzymes, including the serine protease urokinase-type plasminogen activator (21). The urokinase-type plasminogen activator is able to convert plasminogen into plasmin, another serine protease responsible for the degradation of fibrin and for the conversion of latent matrix metalloproteinases into their active forms (21,22). Production of the urokinase-type plasminogen activator is subject to regulation and its expression may be induced by growth factors and cytokines (23). Tumor necrosis factor-alpha is one of the key cytokines implicated in inflammatory responses and it has been found at increased quantities in inflamed periodontal tissues (24–26). Therefore, production of urokinase-type plasminogen activator upon stimulation with tumor necrosis factor-alfa may be envisaged as an important mechanism involved in connective-tissue destruction.

In the present study we analyzed the ability of triclosan to regulate the production of urokinase-type plasminogen activator in tumor necrosis factor-alfa-stimulated gingival fibroblasts. Moreover, we analyzed some of the cell-signalling pathways involved in the production of urokinase-type plasminogen activator that may be affected by triclosan.

Material and methods

Cell culture

Primary cultures of human gingival fibroblasts were established using the explant method (27). Tissue explants were obtained from the retromolar tissue of three female patients and three male patients undergoing extraction of third molars at a private dental practice in Santiago, Chile. Informed consent was obtained from all patients before biopsy was performed. The protocols utilized in this study were approved by the Ethics Committee of the Faculty of Dentistry of the University of Chile. No previous history of inflammation of the retromolar tissue was reported. No relevant pre-existing medical or drug histories were cited during the last 6 mo. Cells were cultured in alpha-minimal essential medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco BRL), 100 μg/mL of penicillin (Sigma, St Louis, MO, USA), 100 μg/mL of streptomycin (Sigma) and 50 μg/mL of gentamycin (Sigma), at 37°C in a 5% CO_2 atmosphere. All experiments were performed using cells between the fourth and tenth passages.

Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using Trizol (Gibco BRL) from appropriately stimulated cells, and genomic DNA was eliminated using RQ1-RNase-free DNase (Promega, Madison, WI, USA). cDNA was synthesized for 1 h (42°C) with Moloney murine leukemia virus reverse transcriptase (Promega) using oligo dT (Gibco BRL), and RT-PCR analyses were carried out as described previously (28). Primer sequences were as follows: urokinase-type plasminogen activator forward 5′-GCA GGA ACC CAG ACA ACC G-3′/urokinase-type plasminogen activator reverse 5′-GAC CCA GGT AGA CGA TGT AG-3′ (yielding an amplified PCR product of 357 bp). Glyceraldehyde-3-phosphate dehydrogenase forward 5′-ACC ACA GTC CAT GCC ATC AC-3′/glyceraldehyde-3-phosphate dehydrogenase reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′ (yielding an amplified PCR product of 452 bp). In all cases the annealing temperature was 58°C. Polymerase chain reaction products were subjected to electrophoresis on a 1.5% agarose (Gibco BRL) gel, that contained 0.5 μg/mL ethidium bromide (Sigma) for DNA observation.

Casein zymography and radial diffusion assay

The urokinase-type plasminogen activator-secreted activity of cell cultures was determined by caseinolytic zymography (29). Briefly, aliquots of conditioned serum-free media, normalized for the protein content measured in the cell lysate, were subjected to electrophoresis through 10% sodium dodecyl sulfate–polyacrylamide (BioRad, Hercules, CA, USA) gels under nonreducing conditions. Sodium dodecyl sulfate (Sigma) was removed by extensive washing in 2.5% Triton X-100 (Sigma). Thereafter, gels were placed over a 1% agarose gel containing 0.5% casein (Sigma) and 1 μg/mL of plasminogen (Sigma) and then incubated at 37°C for 24 h. Plasmin-dependent proteolysis was visible as a clear area in a white–blue field. Quantification of these bands was performed by densitometric analysis. Radial diffusion assays, to study plasminogen activation, were carried out in 1% agarose gels containing 0.5% casein and 2 mg/mL of plasminogen. In brief, aliquots of culture medium normalized for the number of cells were applied to holes previously punched in the gels and incubated at 37°C for 16 h. Gels lacking plasminogen were used as controls. The diameters of the radial zones of caseinolysis were measured using densitometric analysis (29).

Immunofluorescence

Cells were plated on glass coverslips. After appropriate stimulation, cells

were washed once with phosphate-buffered saline and fixed with 4% paraformaldehyde (Sigma) for 10 min, permeabilized with 0.25% Triton X-100 for 5 min and incubated with phosphate-buffered saline containing 4% bovine serum albumin (Rockland, Gilbertsville, PA, USA) for 30 min at room temperature (20 °C). Primary antibodies diluted in phosphate-buffered saline containing 1% bovine serum albumin were used at a dilution of 1:100 for anti-p65 (SantaCruz Biotechnology, Santa Cruz, CA, USA) and incubated for 30 min at room temperature (20 °C). Afterwards, antigen–antibody complexes were washed and incubated with fluorescein isothiocyanate-conjugated secondary antibodies (Rockland). Fluorescence images were collected on an immunofluorescence microscope (Carl Zeiss, Dresden, Germany) and photographed using a digital camera (Carl Zeiss).

Detection of mitogen-activated protein kinase activation through western blotting

Gingival fibroblasts were stimulated with tumor necrosis factor-alfa (R&D Systems, Minneapolis, MN, USA) for different periods of time in serum-free medium. In selected experiments, triclosan (2,4,4-trichloro-2-hydroxy diphenyl ether) (Calbiochem, San Diego, CA, USA) was added 60 min before stimulation with tumor necrosis factor-alfa. Then, cells were lysed with a buffer containing 1% Nonidet P-40 (Calbiochem), 0.5% sodium deoxycolate (Calbiochem), 0.2% sodium dodecyl sulfate (Calbiochem), 150 mM NaCl (Calbiochem) and 50 mM Tris–HCl (Calbiochem), pH 7.4, in the presence of 2 mM phenylmethylsulfonyl fluoride (Calbiochem), 2 mg/mL of pepstatin (Calbiochem), 2 μg/mL of leupeptin (Calbiochem) and 1 mM sodium orthovanadate (Calbiochem) at 4°C. The cell lysate was centrifuged (19,000 *g*, 10 min, 4°C). Proteins were resolved by electrophoresis through a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences, Boston, MA, USA). Membranes were exposed to primary antibodies against c-Jun (Calbiochem), extracellular signal-regulated kinase (ERK) (Upstate Biotechnology, Lake Placid, NY, USA), phosphorylated ERK (Upstate Biotechnology), phosphorylated c-Jun N-terminal kinase (Upstate Biotechnology), c-Jun N-terminal kinase (Santa Cruz Biotechnology) and beta-actin (Sigma), then to secondary antibodies coupled to horseradish peroxidase and finally developed using an enhanced chemiluminescence kit (PerkinElmer Life Sciences).

Signal transduction experiments

To identify the cell-signaling pathways involved in the production of urokinase-type plasminogen activator, cells were incubated in the presence of SP600125 (Biolmol, Plymouth Meeting, PA, USA), PD98059 (Calbiochem), SN50 (Calbiochem) or SN50M (Calbiochem), and then stimulated with tumor necrosis factor-alfa for the periods ot time indicated in the 'Results' section.

Detection of urokinase-type plasminogen activator production through western blotting

To evaluate the production of urokinase-type plasminogen activator at the protein level, conditioned media (2 mL per treatment) from cell cultures were collected and concentrated to 200 μL by centrifugation (1500 *g*, 20 min, 4°C) in an ultracentrifuge tube (Millipore, Bedford, MA, USA). Concentrated conditioned media were resolved by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel under reducing conditions and transferred to polyvinylidene difluoride transfer membrane (PerkinElmer Life Sciences). Membranes were exposed to primary antibodies against urokinase-type plasminogen activator (American Diagnostica, Temecula, CA, USA), to secondary antibodies coupled to horseradish peroxidase and finally developed using an enhanced chemiluminescence kit (PerkinElmer Life Sciences).

Statistics

Statistical significance for each data set was tested using the Student's *t*-test, with the significance level set at $p < 0.05$.

Results

Triclosan inhibits tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator production and plasmin generation in gingival fibroblasts

Serum-starved human gingival fibroblasts were stimulated with a range of concentrations of tumor necrosis factor-alfa for 48 h and the conditioned media were analyzed using casein zymography. As shown in Fig. 1A, tumor necrosis factor-alfa stimulated a dose-dependent increase in urokinase-type plasminogen activator activity. Given that the greatest increase in urokinase-type plasminogen activator activity was detected at 20 ng/mL of tumor necrosis factor-alfa, this concentration of cytokine was selected to assess the effect of different concentrations of triclosan on urokinase-type plasminogen activator activity. To achieve this, serum-starved gingival fibroblasts were first exposed to a range of triclosan concentrations (0.25–1.0 μg/mL) for 1 h and then stimulated with 20 ng/mL of tumor necrosis factor-alfa. Analysis of the conditioned media using casein zymography demonstrated that tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator activity was effectively inhibited by triclosan in a dose-dependent manner (Fig. 1B). Quantification of urokinase-type plasminogen activator bands obtained from three independent experiments demonstrated that tumor necrosis factor-alfa stimulated a statistically significant increase in urokinase-type plasminogen activator activity ($p < 0.01$) and that 1.0 μg/mL of triclosan also exerted a significant reduction in the tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator activity ($p < 0.01$) (Fig. 1C).

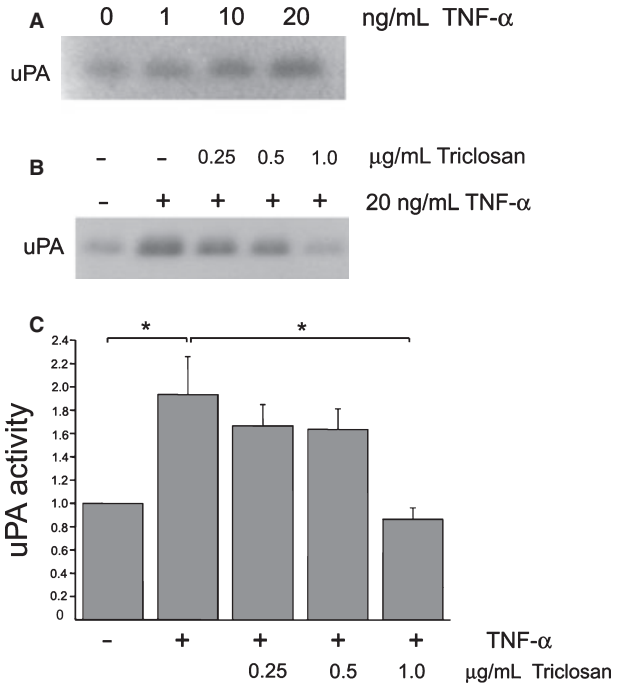

Fig. 1. Triclosan inhibits tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator activity in human gingival fibroblasts. Serum-starved human gingival fibroblast cultures were stimulated with a range of tumor necrosis factor-alfa concentrations (1–20 ng/mL). In a parallel set of experiments, cells were exposed to triclosan (0.25–1 μg/mL) 1 h prior to stimulation with tumor necrosis factor-alfa. After 48 h, urokinase-type plasminogen activator activity present in the conditioned media was analyzed using casein zymography, as described in the Material and methods (A and B). A quantitative analysis of urokinase-type plasminogen activator bands was performed. These data, obtained from three independent experiments, were expressed as the average and standard error. The asterisks (*) indicate statistically significant differences (C). TNF-α, tumor necrosis factor-alfa; uPA, urokinase-type plasminogen activator.

Because urokinase-type plasminogen activator has an important role in the conversion of the zymogen plasminogen into plasmin, we investigated whether triclosan might modulate plasmin generation. Serum-starved gingival fibroblasts were exposed to triclosan for 1 h (1.0 μg/mL) and then stimulated with 20 ng/mL of tumor necrosis factor-alfa. After 48 h the conditioned media derived from control cells, from tumor necrosis factor-alfa-stimulated cells and from tumor necrosis factor-alfa + triclosan-stimulated cells were analyzed using the radial diffusion assay. This experimental approach evaluates the balance between urokinase-type plasminogen activator and its inhibitor, plasminogen activator inhibitor-1, in terms of the conversion of plasminogen into plasmin (29). As shown in Fig. 2A, tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator activity was able to stimulate the conversion of plasminogen into plasmin, and triclosan effectively inhibited this response. A quantitative analysis of three independent experiments showed that both of these responses reached statistical significance ($p < 0.05$ and $p < 0.01$ respectively) (Fig. 2B).

To evaluate the effect of triclosan at the transcriptional level, serum-starved gingival fibroblasts were exposed to triclosan for 1 h (1.0 μg/mL) and then stimulated with 20 ng/mL of tumor necrosis factor-alfa. After 8 h the urokinase-type plasminogen activator mRNA level was assessed using RT-PCR. Figure 2C shows a representative result, derived from three independent experiments, which revealed that tumor necrosis factor-alfa was able to stimulate urokinase-type plasminogen activator mRNA expression. In the presence of triclosan, urokinase-type plasminogen activator mRNA expression was effectively inhibited. To assess whether this effect of tumor necrosis factor-alfa and triclosan on urokinase-type plasminogen activator activity is also observed at the protein level, serum-starved gingival fibroblasts were exposed to 1.0 μg/mL of triclosan for 1 h and then stimulated with 20 ng/mL of tumor necrosis factor-alfa. After 48 h the conditioned media were concentrated, and urokinase-type plasminogen activator activity and protein levels were analyzed using casein zymography and western blotting, respectively. As shown in Fig. 2D, casein zymography and western blotting demonstrated a high level of correlation and confirmed the above-described results concerning the effect of tumor necrosis factor-alfa and triclosan on urokinase-type plasminogen activator production. Quantitative analysis of three independent experiments showed that the effect of tumor necrosis factor-alfa and triclosan on urokinase-type plasminogen activator production reached statistically significant levels ($p < 0.01$). Although urokinase-type plasminogen activator production and activity seemed to be slightly stimulated by triclosan alone (Fig. 2D), quantification of these responses did not show a statistically significant effect (Fig. 2E).

The nuclear factor-κB pathway is involved in tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator production but is not affected by triclosan

Previous studies have demonstrated that the nuclear factor-κB pathway has a role in the regulation of urokinase-type plasminogen activator production in distinct cell types (30). To study its involvement in urokinase-type plasminogen activator production, serum-starved gingival fibroblasts were exposed to SN50, a cell-permeable peptide carrying the nuclear localization sequence of p50 nuclear factor-κB and thereby able to interrupt its signaling response (31). As a control, a nonfunctional peptide mutant was used (SN50M). After exposure of cells

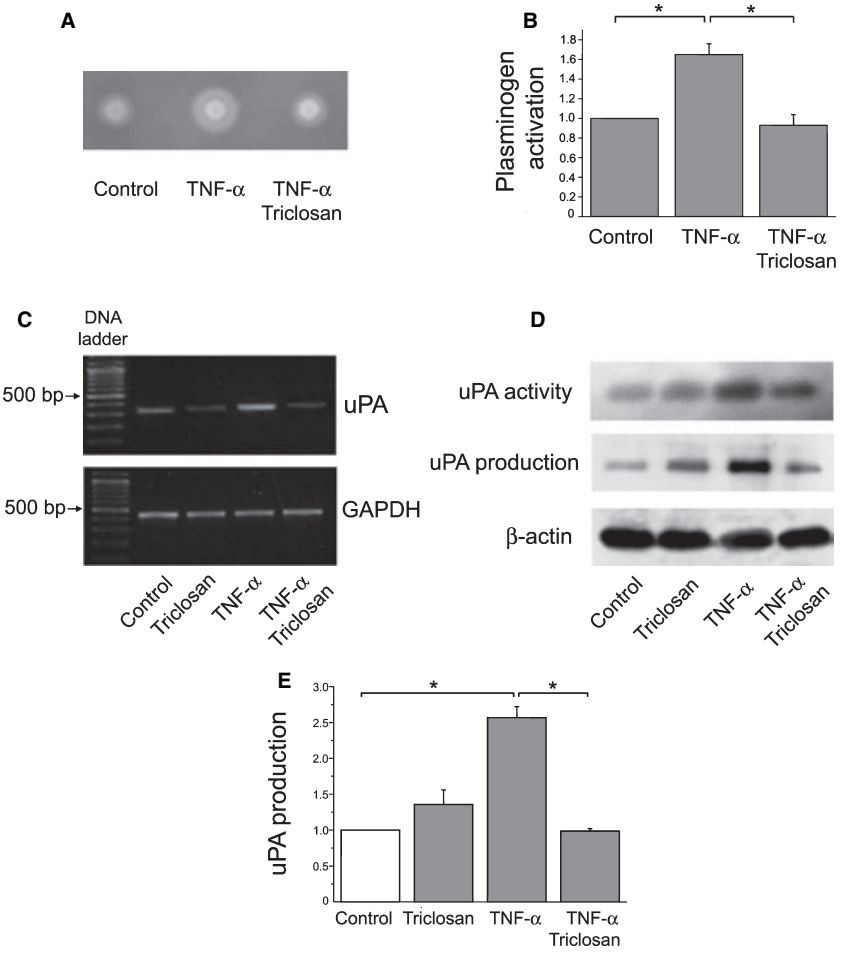

Fig. 2. Triclosan inhibits tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator production and plasmin activation in human gingival fibroblasts. Serum-starved human gingival fibroblast cultures were exposed to triclosan (1 μg/mL) for 1 h prior to stimulation with tumor necrosis factor-alfa (20 ng/mL). After 48 h, the ability of the conditioned media to stimulate the conversion of plasminogen into plasmin was assessed using the radial diffusion assay, as previously described (A). A quantitative analysis of the plasmin-derived proteolytic activity was performed. These data were expressed as the average and standard error. Asterisks (*) indicate statistically significant differences (B). Serum-starved human gingival fibroblast cultures were exposed to triclosan (1 μg/mL) for 1 h and were then stimulated for 8 h with tumor necrosis factor-alfa (20 ng/mL). Thereafter, the cells were lysed and RNA was extracted as described in the Material and methods. Urokinase-type plasminogen activator and glyceraldehyde-3-phosphate dehydrogenase mRNA expression levels were analyzed using the semiquantitative reverse transcription–polymerase chain reaction. Polymerase chain reaction products were visualized after electrophoresis through agarose (C). Serum-starved human gingival fibroblast cultures were exposed to triclosan (1 μg/mL) for 1 h and then stimulated for 48 h with tumor necrosis factor-alfa (20 ng/mL). Urokinase-type plasminogen activator activity and protein production were evaluated through casein zymography and western blotting, respectively. Beta-actin was used as a loading control (D). A quantitative analysis of urokinase-type plasminogen activator bands derived from western blots was performed. These data, obtained from three independent experiments, were expressed as the average and standard error. Asterisks (*) indicate statistically significant differences (E). β-actin, beta-actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tumor necrosis factor-alfa; uPA, urokinase-type plasminogen activator.

to these peptides, the cells were stimulated with tumor necrosis factor-alfa for 48 h and urokinase-type plasminogen activator activity was evaluated through casein zymography of the conditioned media. As shown in Fig. 3A, SN50 effectively inhibited the tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator activity in gingival fibroblasts. In the presence of the control peptide (SN50M), tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator activity was not altered. Statistical analysis of three independent experiments showed that SN50, but not SN50M, inhibited tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator activity at statistically significant levels ($p < 0.05$, Fig. 3B). In order to verify the activation of the nuclear factor-κB pathway in gingival fibroblasts, cells were stimulated with tumor necrosis factor-alfa and translocation of the p65 subunit was verified through immunofluorescence. As shown in Fig. 3C, unstimulated cells did not demonstrate nuclear staining for the p65 subunit. After 15 and 30 min of stimulation with tumor necrosis factor-alfa, a large proportion of cells showed strong nuclear immunofluorescent staining for p65, suggesting that this pathway in fact activated this cytokine. The proportion of cells with nuclear p65 staining was quantified in three independent experiments and is shown in Fig. 3D. Nuclear p65 translocation reached a similar level after 15–30 min of stimulation. In order to identify whether triclosan might affect the translocation of the p65 subunit, cells were previously exposed to 1 μg/mL of triclosan for 1 h and then stimulated with 20 ng/mL of tumor necrosis factor-alfa. This experiment did not show any change in the proportion of p65-stained cells (data not shown), suggesting that triclosan may not affect the activation of the nuclear factor-κB pathway.

Tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator production is dependent on the activity of the c-Jun N-terminal kinase signaling pathway

To identify the role of the ERK and c-Jun N-terminal kinase pathways in tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator production, serum-starved gingival

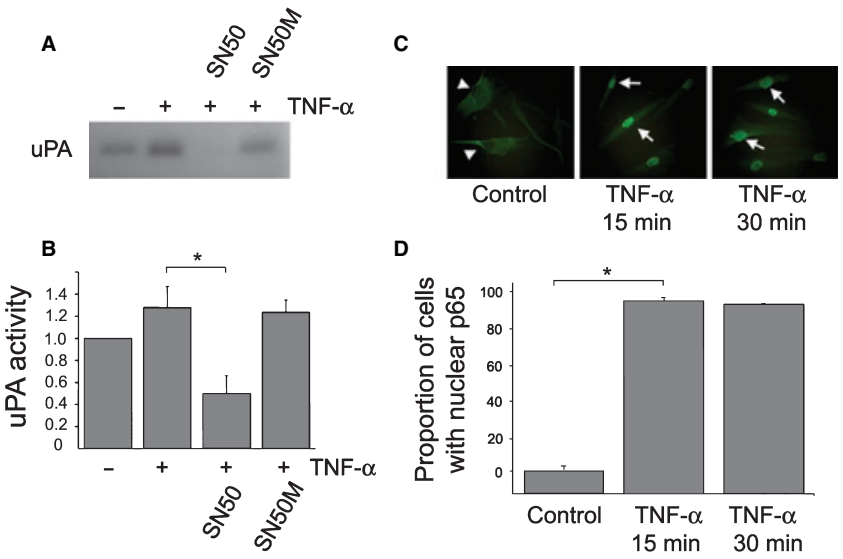

Fig. 3. Nuclear factor-κB is implicated in tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator production but is not affected by triclosan. Serum-starved human gingival fibroblast cultures were exposed to SN50 or SN50M (50 μg/mL) for 1 h and then stimulated with tumor necrosis factor-alfa (20 ng/mL). After 48 h, urokinase-type plasminogen activator activity present in the conditioned media was analyzed using casein zymography, as described in the Material and methods (A). A quantitative analysis of urokinase-type plasminogen activator bands was performed. These data, obtained from three independent experiments, were expressed as the average and standard error. Statistical analysis was performed using the Student's *t*-test. The asterisk (*) indicates a statistically significant difference (B). Serum-starved human gingival fibroblasts were stimulated with 20 ng/mL of tumor necrosis factor-alfa, and translocation of the p65 subunit was analyzed through immunofluorescence, as described in the Material and methods (C). A quantitative analysis of the number of cells positive for nuclear p65 staining was performed. These data, obtained from three independent experiments, were expressed as the average and standard error. Statistical analysis was performed using the Student's *t*-test. The asterisk (*) indicates statistically significant differences (D). TNF-α, tumor necrosis factor-alfa; uPA, urokinase-type plasminogen activator.

fibroblasts were exposed to a range of concentrations of the selective inhibitors for mitogen activated protein kinase kinase 1 (MEK-1) (PD98059; 5–25 μM) and c-Jun N-terminal kinase (SP600125; 1–5 μM) (Fig. 4). After 30 min of pre-incubation with the inhibitors, gingival fibroblasts were stimulated with 20 ng/mL of tumor necrosis factor-alfa for 48 h and the conditioned media were analyzed using casein zymography. In the absence of stimulation with tumor necrosis factor-alfa, treatment with 5 μM SP600125 or 25 μM PD98059 induced a limited reduction in urokinase-type plasminogen activator activity that did not reach statistical significance. Although PD98059 moderately inhibited tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator activity, none of these drug concentrations reached statistical significance. On the contrary, in the case of the c-Jun N-terminal kinase inhibitor SP600125, all three concentrations assayed (1–5 μM) demonstrated a statistically significant reduction in the tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator activity ($p < 0.05$).

To verify the effect of SP600125 on tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator production at the protein level, serum-starved gingival fibroblasts were exposed to 5 μM SP600125 and then stimulated with 20 ng/mL of tumor necrosis factor-alfa. After 48 h the conditioned media were concentrated and urokinase-type plasminogen activator protein levels were analyzed using western blotting. As shown in Fig. 4B, western blotting demonstrated that SP600125 effectively abrogated the tumor necrosis factor-alfa stimulus on urokinase-type plasminogen activator production. Quantitative analysis of three independent experiments showed that the effect of SP600125 on urokinase-type plasminogen activator production reached statistical significance ($p < 0.01$) (Fig. 4C).

Triclosan inhibits the signalling activity of the c-Jun N-terminal kinase pathway in gingival fibroblasts

To test whether the experimental conditions that promote urokinase-type plasminogen activator expression correlate with the activation of ERK and c-Jun N-terminal kinase pathways by tumor necrosis factor-alfa, serum-starved gingival fibroblasts were stimulated with 20 ng/mL of tumor necrosis factor-alfa and the activation of these routes was evaluated through western blotting of the cell lysates at different time-points. As revealed in Fig. 5A, stimulation of cells with tumor necrosis factor-alfa did not induce a significant increase in the proportion of phosphorylated ERK 1/2. Conversely, tumor necrosis factor-alfa was able to stimulate the phosphorylation of c-Jun N-terminal kinase 2 following a bimodal pattern. A consistent increase in the phosphorylation of c-Jun N-terminal kinase 2 was observed at 5 min of stimulation, followed by a reduction in the phosphorylattion of c-Jun N-terminal kinase 2 between 15 and 30 min and a subsequent, and more sustained, increase after 60 and 180 min of tumor necrosis factor-alfa stimulation. This pattern of c-Jun N-terminal kinase activation was observed in at least four independent experiments and was found to be very consistent (data not shown).

To identify whether triclosan was able to modify the activation/phosphorylation of c-Jun N-terminal kinase 2 in gingival fibroblasts, serum-starved cells were exposed to 1 μg/mL of triclosan for 1 h and then stimulated at different time-points with 20 ng/mL of tumor necrosis factor-alfa. As demonstrated in Fig. 5A, a decreased phosphorylation of c-Jun N-terminal kinase

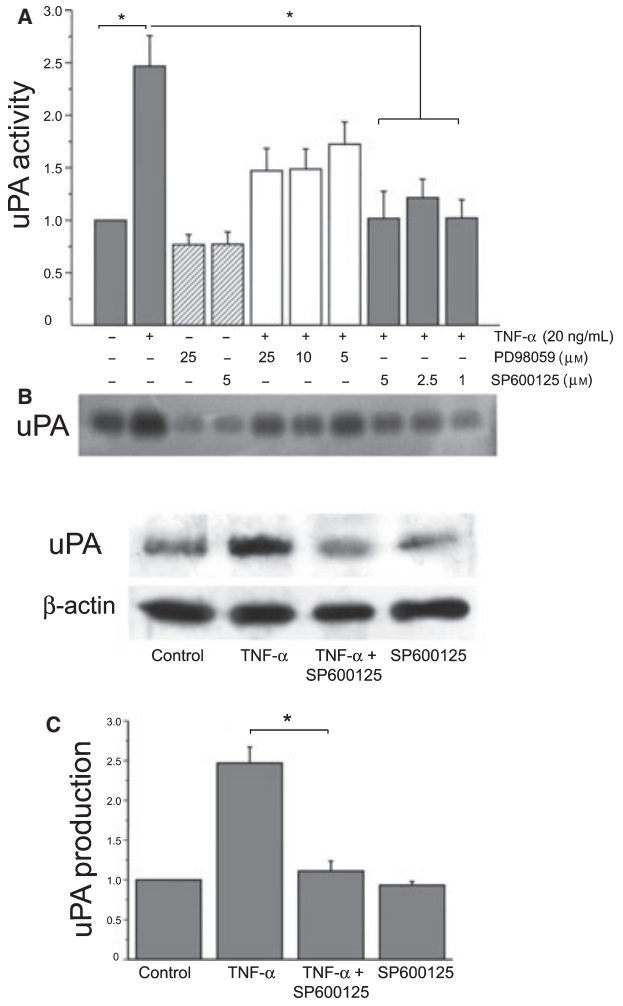

Fig. 4. c-Jun N-terminal kinase inhibition interferes with the production of tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator. Serum-starved gingival fibroblasts were incubated in the presence of SP600125 (1–5 μM) or PD98059 (5–25 μM) for 1 h and subsequently stimulated with 20 ng/mL of tumor necrosis factor-alfa. The conditioned medium derived from each experimental condition was analyzed using casein zymography, as described in the Material and methods. Caseinolytic bands were digitalized, quantified and are expressed in the graph as the average and standard error. Data were obtained from three independent experiments. Asterisks (*) indicate statistically significant differences (A). Serum-starved gingival fibroblast cultures were incubated in the presence of SP600125 (5 μM) for 1 h and subsequently stimulated with 20 ng/mL of tumor necrosis factor-alfa. The conditioned medium derived from each experimental condition was concentrated and analyzed using western blotting, as described in the Material and methods. Beta-actin was used as a loading control (B). A quantitative analysis of urokinase-type plasminogen activator bands derived from western blotting was performed. These data, obtained from three independent experiments, were expressed as the average and standard error. The asterisk (*) indicates a statistically significant difference (C). β-actin, beta-actin; TNF-α, tumor necrosis factor-alfa; uPA, urokinase-type plasminogen activator.

2 was observed at all experimental time-points evaluated. These data strongly suggested that the c-Jun N-terminal kinase pathway was involved in the production of urokinase-type plasminogen activator stimulated by tumor necrosis factor-alfa (Fig. 4) and that triclosan may affect the activation of this signalling pathway.

To assess, quantitatively, the effect of triclosan on the c-Jun N-terminal kinase 2 pathway, serum-starved gingival fibroblasts were exposed to triclosan and then stimulated with tumor necrosis factor-alfa. After 60 min, cells were lysed and phosphorylation of c-Jun N-terminal kinase 2 was estimated through western blotting. Figure 5B shows that triclosan consistently inhibited the activation of c-Jun N-terminal kinase 2. Analysis of the results of five independent experiments, performed using cell cultures derived from different patients, demonstrated that this inhibition was statistically significant (Fig. 5C).

Considering the inhibitory effect of triclosan on the activation/phosphorylation of c-Jun N-terminal kinase 2, we further evaluated the effect of this drug on c-Jun protein expression as a functional assay of c-Jun N-terminal kinase. It has been demonstrated that active c-Jun induces the expression of a number of proteins, including itself. Therefore, the rate of normal c-Jun expression represents a satisfactory functional assay for c-Jun N-terminal kinase activity (32). Serum-starved gingival fibroblasts were exposed to 1 μg/mL of triclosan for 1 h and then stimulated with 20 ng/mL of tumor necrosis factor-alfa. After 12 h, cells were lysed and c-Jun production was evaluated through western blotting. As observed in Fig. 6A, tumor necrosis factor-alfa was able to stimulate c-Jun production and triclosan effectively inhibited this effect. Analysis of three independent experiments demonstrated that this inhibition was statistically significant (Fig. 6B). In the absence of tumor necrosis factor-alfa, triclosan was not able to induce a significant change in c-Jun production.

Discussion

The present study shows that triclosan, a pharmacological agent used in dentifrices and mouthrinses, may have a novel effect on specific cellular events associated with gingival-derived extracellular matrix degradation. Our results obtained in gingival fibroblasts demonstrated that tumor necrosis factor-alfa, a cytokine whose activity has been associated with inflammatory events, stimulates the production of urokinase-type plasminogen activator. In the presence of triclosan, tumor

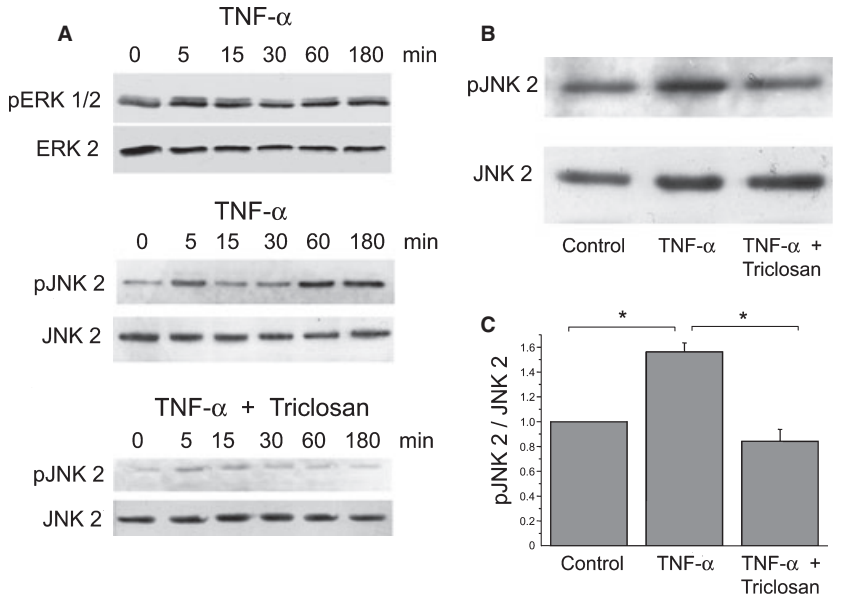

Fig. 5. Triclosan alters tumor necrosis factor-alfa-stimulated c-Jun N-terminal kinase phosphorylation. Serum-starved gingival fibroblasts were exposed or not exposed to triclosan (1 μg/mL) and were then stimulated with 20 ng/mL of tumor necrosis factor-alfa. Phosphorylated forms of both extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase 2 were evaluated at different time-points in the cell lysate using western blotting, as described in the Material and methods. As loading controls, the presence of total ERK and c-Jun N-terminal kinase 2 were probed for in the cell lysate (A). Serum-starved gingival fibroblasts were exposed or not exposed to triclosan (1 μg/mL) and were then stimulated with 20 ng/mL of tumor necrosis factor-alfa. After 1 h of stimulation, phosphorylated forms of c-Jun N-terminal kinase 2 were detected in the cell lysate using western blotting. As a loading control, the presence of total c-Jun N-terminal kinase 2 was probed for in the cell lysate (B). After image analysis, phosphorylated c-Jun N-terminal kinase 2 bands, normalized against total c-Jun N-terminal kinase 2 bands, were expressed as the average and standard error. Data were derived from three independent experiments. Asterisks (*) indicate statistically significant differences (C). JNK 2, c-Jun N-terminal kinase 2; pERK, phosphorylated extracellular signal-regulated kinase; pJNK 2, phosphorylated c-Jun N-terminal kinase 2; TNF-α, tumor necrosis factor-alfa.

necrosis factor-alfa-stimulated urokinase-type plasminogen activator production and plasmin generation were effectively inhibited. Moreover, our results showed that triclosan is able to control urokinase-type plasminogen activator production by inhibiting the activation of c-Jun N-terminal kinase 2 and preventing the production of the downstream transcription factor, c-Jun.

Significant amounts of tumor necrosis factor-alfa have been detected in inflamed gingival tissues and gingival crevicular fluid (25,26,33). Therefore, it is highly probable that increased levels of this cytokine, as those detected by previous studies in periodontal lesions, may stimulate gingival fibroblasts to produce urokinase-type plasminogen activator. Our study is the first to show that primary cultures of human gingival fibroblasts respond to tumor necrosis factor-alfa with an increase in urokinase-type plasminogen activator production and a consequent conversion of plasminogen into plasmin. Tumor necrosis factor-alfa-stimulated urokinase-type plasminogen activator production was blocked by inhibitors for nuclear factor-κB (SN50) and c-Jun N-terminal kinase (SP600125). PD98059, a MEK-1 inhibitor, did not produce a significant blockade of urokinase-type plasminogen activator activity (Fig. 4A). In accordance with these results, we also found that tumor necrosis factor-alfa was able to stimulate nuclear factor-κB and c-Jun N-terminal kinase activation; nonetheless, its effect on ERK phosphorylation was weak. In addition, it has been described that tumor necrosis factor-alfa is a stronger activator of c-Jun N-terminal kinase when compared with ERK (34). Previous studies have also indicated that tumor necrosis factor-alfa may activate nuclear factor-κB and c-Jun N-terminal kinase pathways in several cell types, including gingival fibroblasts, and these cascades have also been involved in inflammatory diseases (35–37).

Considering these results, our efforts were focused on analysing the role of triclosan as a regulator of the nuclear factor-κB and c-Jun N-terminal kinase pathways, signaling routes that are engaged in an active functional cross-talk (38). Our results showed that triclosan was unable to alter the nuclear factor-κB-dependent translocation of the p65 subunit into the nucleus, a key event in the activation of nuclear factor-κB-regulated genes (39). This result is consistent with the result of a previous study which showed that triclosan was unable to modify nuclear factor-κB activation in human gingival fibroblasts at similar concentrations (12). However, previous studies have shown that the combination of triclosan and cetylpirydinium chloride is able to inactivate nuclear factor-κB phosphorylation (40). Although the molecular basis that may explain this difference is unknown, further studies should define the experimental conditions which allow nuclear factor-κB inhibition in triclosan-treated cells.

A significant observation of our study was that triclosan inhibited c-Jun N-terminal kinase 2 phosphorylation and the production of c-Jun transcription factor. The c-Jun N-terminal kinase pathway has been involved in the production of urokinase-type plasminogen activator stimulated by different growth factors, cytokines and stress-inducing agents such as ultraviolet light (28). Because the expression of c-Jun transcription factor is highly dependent on the activity of the c-Jun N-terminal kinase pathway, it may represent a relevant end-point of the activity of this pathway and a promis-

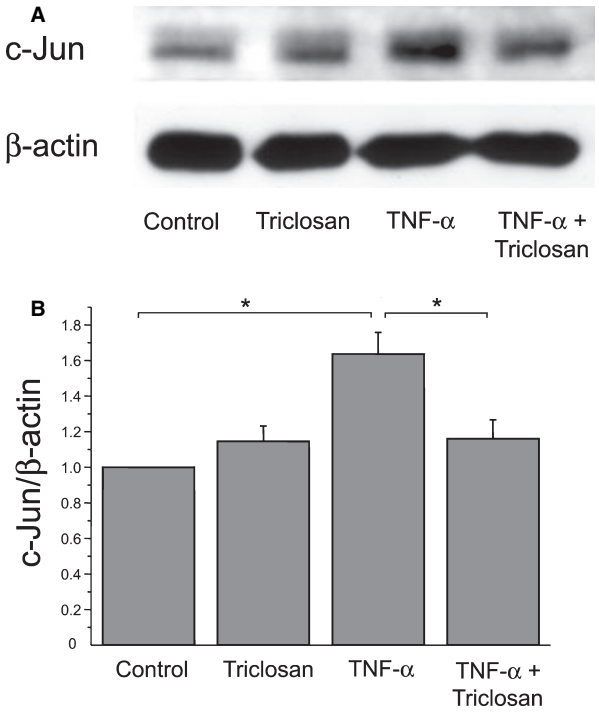

Fig. 6. Triclosan alters tumor necrosis factor-alfa-stimulated c-Jun production. Serum-starved gingival fibroblasts were exposed or not exposed to triclosan (1 μg/mL) and were then stimulated with 20 ng/mL of tumor necrosis factor-alfa. The amount of c-Jun produced was evaluated in the cell lysate after 12 h of stimulation. Beta-actin was used as a loading control (A). A quantitative analysis was performed of c-Jun bands derived from western blots. These data, obtained from three independent experiments, were expressed as the average and standard error. Asterisk (*) indicates statistically significant differences (B). β-actin, beta-actin; TNF-α, tumor necrosis factor-alfa.

sory target for therapeutic interventions (41). Our study is the first to report that the c-Jun N-terminal kinase signaling cascade may be inhibited by triclosan. Further studies are needed to identify other c-Jun N-terminal kinase-regulated genes that may be controlled by this pharmacological agent.

Progression of periodontal disease has been explained by several factors, including variations in the level of inflammatory cytokines, altered ratios between proteolytic enzymes and their inhibitors, and changes in the subgingival microflora, among others (42). Long-term use of a dentifrice containing triclosan may reduce loss of attachment in adolescents and slows the progression of periodontal disease in adults (15,17,18). These results have been explained by the observation that individuals using the triclosan-containing toothpaste demonstrated reductions in the numbers of periodontal pathogens at subgingival sites (16). Because the progression of periodontal disease has also been associated with an increased extracellular matrix turnover, the results of the present study may offer an additional explanation for the effect of triclosan on periodontal tissue destruction (42). Although the present results were obtained in an *in vitro* cell-culture system using primary cultures of human gingival fibroblasts, they suggest that triclosan may regulate urokinase-type plasminogen activator production and activity in gingival connective tissues. It is important to note that, besides fibroblasts, other cell types, such as macrophages, are also involved in urokinase-type plasminogen activator and tumor necrosis factor-alfa production in inflamed connective tissues (43). Therefore, future studies should be performed to analyze the effect of triclosan also on other significant cell phenotypes.

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