Benzydamine reduces prostaglandin production in human gingival fibroblasts challenged with interleukin-1β or tumor necrosis factor α

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Benzydamine [1-benzyl-3-(3-dimethylamino)propoxy-1H-indazole] is a drug with analgesic, anesthetic, antimicrobial and anti-inflammatory activity. The purpose of the present study was to investigate the effect of benzydamine on prostaglandin production in human gingival fibroblasts. Benzydamine significantly reduced the basal production of both prostaglandin E_2 (PGE_2) and 6-keto-$PGF_{1\alpha}$, the stable breakdown product of prostaglandin I_2(PGI_2), in unstimulated human gingival fibroblasts. When the cells were treated simultaneously with benzydamine and the cytokines IL-1β or TNFα, the agent benzydamine reduced ($P < 0.05$) the stimulatory effect of IL-1β and TNFα respectively, on PGE_2 and PGI_2 production in human gingival fibroblasts. Furthermore, benzydamine reduced ($P < 0.05$) both the basal level and the cytokine-induced ^{3}H-arachidonic acid release ^{3}H-(AA) in gingival fibroblasts. The addition of exogenous arachidonic acid to the cells resulted in enhanced PGE_2 production, which was reduced ($P < 0.05$) in the presence of benzydamine. The study indicates that benzydamine reduces the prostaglandin synthesis in gingival fibroblasts, partly at the level of phospholipase A_2, by diminishing the liberation of arachidonic acid (AA) from phospholipids, and partly at the level of cyclooxygenase. The inhibitory effect of benzydamine on prostaglandin production may explain the anti-inflammatory effect of the drug in the management of patients with oral inflammatory conditions. □ *Interleukin-1β; prostaglandin E_2; prostaglandin I_2; tumor necrosis factor α*

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Benzydamine [1-benzyl-3-(3-dimethylamino)propoxy-1H-indazole] was introduced in 1960 and is available under a number of trade names, including Andolex®. The drug exhibits analgesic, anti-inflammatory and antimicrobial activity (1–6). The agent has been used in the treatment of oral inflammatory conditions, including pharyngitis, radiation mucositis, and aphthous stomatitis (6). In addition, it has been demonstrated that benzydamine treatment reduces the plaque accumulation, gingival inflammation, as well as pain reduction in periodontal postsurgical patients (7).

The anti-inflammatory mechanism of benzydamine is not fully understood, although stabilization of cellular membranes and reduction of prostaglandin biosynthesis (8) have been suggested as an action of the agent. Furthermore, it has also been demonstrated that the agent benzydamine reduces the production of pro-inflammatory cytokines in human mononuclear cells challenged with lipopolysaccharides (9, 10).

The proinflammatory mediators interleukin-1 (IL-1α and IL-1β) and tumor necrosis factor α (TNFα) are a family of closely related cytokines produced in inflammatory lesions mainly by macrophages (11–13). The cytokines IL-1 and TNFα stimulate prostaglandin biosynthesis in various types of cells, including gingival fibroblasts (14, 15).

Prostaglandins are important mediators of inflammation and the synthesis is mediated by the release of arachidonic acid (AA) from phospholipids by a family of enzymes of phospholipase A_2 (PLA_2) and converted to prostaglandins by two different isoenzymes of cyclooxygenase (COX-1 and COX-2) (16). To our knowledge, no information is available concerning the effect of benzydamine on prostaglandin production in cells derived from oral tissue. The present investigation was therefore undertaken to study whether benzydamine reduces the prostaglandin production in gingival fibroblasts challenged with the inflammatory mediators IL-1β or TNFα.

Materials and methods

Cultures of fibroblast cells were established from gingival biopsies obtained from five patients, 7–17 years of age (N-17, N-25, N-27, N-28, N-34) with clinical healthy gingiva. The plan to take biopsies was approved by the Ethics Committee of the Karolinska Institute. Minced pieces of the gingival biopsy tissue were explanted to 25 cm^2 Falcon tissue culture flasks containing 5 mL of Eagle's basal medium (BME). The fibroblasts were obtained by trypsinization of the primary outgrowth of cells as previously described (17). The cells were grown in BME supplemented with sodium-glutamine (4 mmol/L), fetal calf serum, FCS (5%), penicillin (50 IU/mL) and streptomycin (50 μg/mL). The fibroblasts were incubated

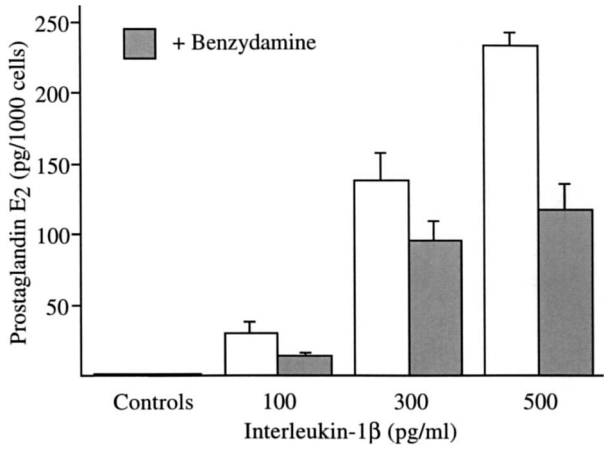

Fig. 1. Effect of IL-1β at different concentrations, in the absence or presence of benzydamine (1.0 μM) on PGE_2 production in human gingival fibroblasts (N − 17). Cell density was 1.4×10^4 cells/cm^2. Mean value of triplicates ± SD. PGE_2 levels significantly different ($P < 0.05$) in cells treated with the combination of IL-1β and benzydamine compared to IL-1β treated cells.

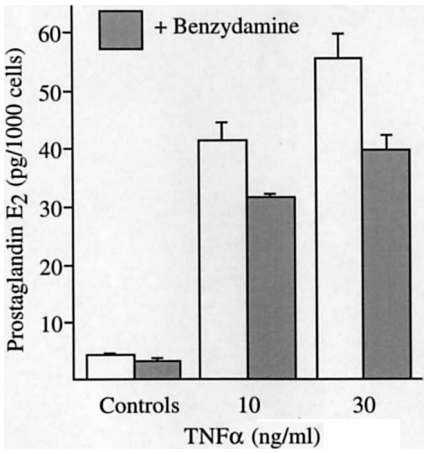

Fig. 3. Effect of TNFα at different concentrations, in the absence or presence of benzydamine (1.0 μM) on PGE_2 production in human gingival fibroblasts (N − 34). Cell density was 1.4×10^4 cells/cm^2. Mean value of triplicates ± SD. PGE_2 levels significantly different ($P < 0.05$) in cells treated with the combination of TNFα and benzydamine compared to TNFα treated cells.

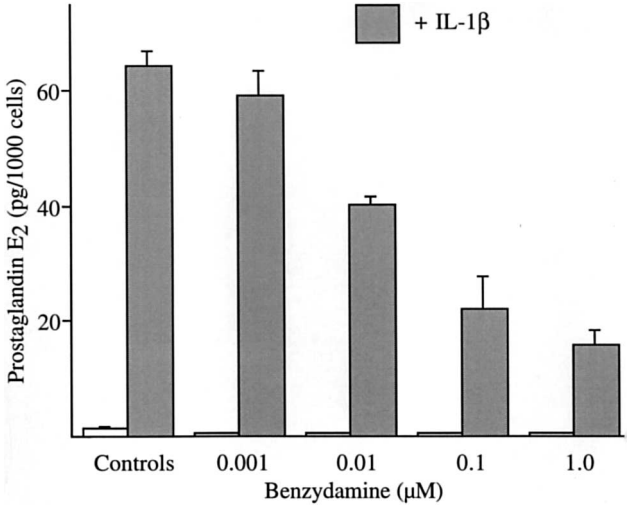

Fig. 2. Effect of benzydamine, at different concentrations, in the absence or presence of IL-1β (300 pg/ml) on PGE_2 production in human gingival fibroblasts (N − 27). Cell density was 1.4×10^4 cells/cm^2. Mean value of triplicates ± SD. PGE_2 levels significantly different ($P < 0.05$) in cells treated with benzydamine (≥0.01 μM).

at 37°C in a humidified incubator gassed with 5% CO_2 in air, and routinely passaged using 0.025% trypsin in phosphate-buffered saline (PBS) containing 0.02% EDTA. The cells used for the experiments proliferated in logarithmic phase between the 6th and 14th passages.

Chemicals

Eagle's BME, sodium-glutamine, Hepes buffer, fetal calf serum, and penicillin-streptomycin were obtained from Life Technologies Inc., Paisley, Scotland. Unlabeled arachidonic acid and lidocaine were purchased from the Sigma Chemical Co. (St. Louis, USA). Human recombinant interleukin-1β (specific activity $>5 \times 10^8$ units/mg) and tumor necrosis factor α (specific activity $>2 \times 10^6$ units/mg) were obtained from Amersham (Buckinghamshire, England). Radioimmunoassay kits for PGE_2, 6-keto-$PGF_{1\alpha}$, as well as [^{3}H] arachidonic acid (specific activity 79.9 Ci/mmol) were procured from Du Pont/New England Nuclear Chemicals (Austria). Benzydamine hydrochloride was donated by 3M, Health Care Ltd (Loughborough, England).

Analysis of prostaglandin production

Fibroblasts (4×10^4cells/dish) were seeded in Nunc multiwell dishes (24-well plate) in the BME in the presence of 5% FCS and grown for 24 h at 37°C. The cell layers were then rinsed three times in serum-free BME followed by the addition of 0.3 ml serum-free BME containing IL-1β, TNFα, or AA in the presence or absence of benzydamine at the concentrations indicated in the legends to the figures and tables. After 24 h the medium was withdrawn, acidified to pH 3.5, frozen, and stored at −70°C until used for determination of PGE_2 and 6-keto-$PGF_{1\alpha}$ levels. The amount of PGE_2 and 6-keto-$PGF_{1\alpha}$ in the medium was determined using commercially available radioimmunoassay kits. The PGE_2 antisera had 30% cross-reactivity with PGE_1.

Analysis of [^{3}H]-AA release

Gingival fibroblasts (4×10^4 cells/dish) were seeded in Nunc multiwell dishes (24-well plate) in BME containing 5% FCS and grown for 24 h at 37°C. The cell layers were

Table 1. Effect of benzydamine (1.0 μM) on 6-keto-$PGF_{1\alpha}$ production induced by IL-1β or TNFα in 24 h cultures of gingival fibroblasts (N – 25). Cell density was 1.4×10^4 cells/cm^2. Mean value of triplicates ± SD

	6-Keto-$PGF_{1\alpha}$ (pg/10^5 cells)	
	– Benzydamine	+ Benzydamine
Control	124 ± 21	63 ± 16[a]
IL-1β (100 pg/mL)	1310 ± 21	780 ± 53[a]
TNFα (10 ng/mL)	261 ± 42	177 ± 19[a]

[a] Significantly different from cells not treated with benzydamine ($P < 0.05$).

then rinsed three times in serum-free medium and incubated in 0.3 ml serum-free medium per dish containing 1.0 μCi/ml [^{3}H]-AA. After 24 h, the medium was withdrawn and the cells washed three times in serum-free medium. Subsequently, 0.3 ml of media with IL-1β or TNFα in the presence or absence of benzydamine was added and the cells were further incubated for 24 h at 37°C. Samples of the media were then withdrawn and analyzed to determine the amount of [^{3}H] released, using a scintillation counter. The amount of [^{3}H] found in the supernatant represents free [^{3}H]-AA as well as [^{3}H]-labeled metabolites.

[^{3}H]-thymidine incorporation (cell proliferation)

Gingival fibroblasts (1.0×10^4 – 1.5×10^4 cells/dish) were allowed to settle in Nunc multiwell dishes (96-well plate) in BME in the presence of 5% FCS for 4 h. The adherent cells were washed three times in serum-free BME and left untreated for 20 h at 37°C. Thereafter, the effect of IL-1β, TNFα or AA in the presence or absence of benzydamine was tested on the incorporation of thymidine (1.0 μCi/ well) into DNA during a 24 h pulse. The incorporation of [^{3}H]-thymidine was measured using a liquid scintillation counter.

Statistics

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

Results

Concurring with earlier findings (15), IL-1β dose-dependently stimulated PGE_2 production in human gingival fibroblasts. When the cells were treated simultaneously with benzydamine (1.0 μM) and IL-1β at different concentrations the drug significantly reduced the stimulatory effect of IL-1β on PGE_2 production (Fig. 1). The inhibitory effect of benzydamine on PGE_2 production in gingival fibroblasts in the presence of IL-1β was dependent on the concentration of the drug, as shown in Fig. 2. In addition, benzydamine (1.0 μM) also significantly reduced the stimulatory effect of TNFα on the production of PGE_2 in gingival fibroblasts (Fig. 3). Furthermore, the production of 6-keto-$PGF_{1\alpha}$ (the stable breakdown product of PGI_2) in gingival fibroblasts challenged with IL-1β or TNFα was significantly reduced by benzydamine (Table 1). The agent benzydamin also reduced the basal production of both PGE_2 and 6-keto-$PGF_{1\alpha}$ in unstimulated control fibroblasts (Fig. 2, Table 1).

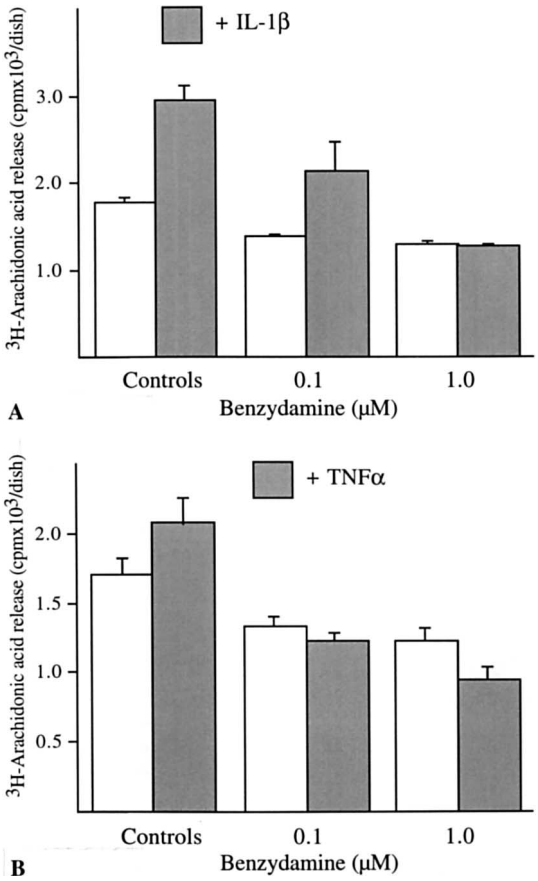

Fig. 4. Effect of benzydamine, at different concentrations, in the absence or presence of IL-1β (300 pg/ml) (A) and TNFα (20 ng/ml) (B), on the release of [^{3}H]-AA and [^{3}H]-labeled metabolites from human gingival fibroblasts (N – 34) prelabeled with [^{3}H]-AA. Cell density was 1.4×10^4 cells/cm^2. Mean value of triplicates ± SD. The ^{3}H-AA release significantly different ($P < 0.01$) in cells treated with benzydamine compared to untreated cells. The ^{3}H-AA release significantly different in cells treated with the combination of IL-1β and benzydamine ($P < 0.05$) as well as TNFα together with benzydamine ($P < 0.01$) compared to IL-1β and TNFα treated cells, respectively.

The effect of benzydamine was also studied on the release of arachidonic acid (^{3}H-AA) from prelabeled gingival fibroblasts in the absence or presence of IL-1β or TNFα (Fig. 4A, B). The agent benzydamine (0.1 and 1.0 μM) significantly reduced the basal level of ^{3}H-AA release in unstimulated gingival fibroblasts (Fig. 4). When benzydamine was added simultaneously with IL-1β

Table 2. Effect of arachidonic acid (AA) in the absence or presence of benzydamine (1.0 μM) on PGE_2 production in 24 h cultures of gingival fibroblasts (N – 34). Cell density was 1.4×10^4 cells/cm^2. Mean value of triplicates ± SD

	PGE_2 (pg/10^3 cells)	
	– Benzydamine	+ Benzydamine
Control	5.4 ± 0.15	4.7 ± 0.40[a]
AA (5 μM)	29.0 ± 2.6	19.3 ± 3.8[a]
AA (10 μM)	100.4 ± 7.8	77.2 ± 10.6[a]

[a] Significantly different from cells not treated with benzydamine ($P < 0.05$)

Table 3. Effect of IL-1β (300 pg/mL) and TNFα (10 ng/mL) in the absence or presence of lidocaine in 24 h of cultures on PGE_2 production in human gingival fibroblasts ($n = 34$). Cell density was 1.4×10^4 cells/cm^2. Mean ± SD of triplicate determinations

	PGE_2 (pg/10^3 cells)		
	– Lidocaine	+ Lidocaine (1.0 μM)	+ Lidocaine (10 μM)
Control	0.7 ± 0.3	0.8 ± 0.3	0.8 ± 0.1
IL-1β	173 ± 33	293 ± 12[a]	299 ± 36[b]
TNFα	17 ± 3	35 ± 7[b]	33 ± 11

[a, b] Significantly different from cells not treated with lidocaine a; ($P < 0.01$), b; ($P < 0.05$)

(300 pg/mL), the drug significantly reduced the release of ^{3}H-AA induced by the cytokine (Fig. 4A). Similarly, benzydamine also reduced the release of ^{3}H-AA from gingival fibroblasts stimulated by TNFα (20 ng/ml) (Fig. 4B).

In the next series of experiments, we investigated whether the inhibitory effect of benzydamine on PGE_2 production was due to involvement of the enzyme COX. The addition of exogenous unlabeled AA (5 and 10 μM) to the cells resulted in an enhanced PGE_2 production in gingival fibroblasts (Table 2). Moreover, when the cells were treated simultaneously with benzydamine and exogenous AA, the drug benzydamine (1.0 μM) significantly reduced the PGE_2 production induced by AA (Table 2).

The effect of benzydamine on cell proliferation was assessed as incorporation of [^{3}H]-thymidine into DNA during a 24 h pulse. The agent benzydamine, 1.0 μM (the highest concentration used), neither alone nor in combination with IL-1β or AA affected the cell proliferation compared to control cells (data not shown).

We also investigated the effect of lidocaine, a membrane stabilizatory agent, on cytokine induced PGE_2 production. Lidocaine (1 and 10 μM) exhibited a stimulatory effect on the production of PGE_2 induced by IL-1β or TNFα (Table 3).

Discussion

Using benyzdamine in the management of inflammatory oropharyngeal conditions including mucosites in the oral mucosa during radiation therapy demonstrates encouraging clinical results (18, 19). Although several in vitro studies have demonstrated that benzydamine has an anti-inflammatory capacity, the mechanism of the agent is not fully understood.

The results presented here demonstrate that benzydamine reduces the production of PGE_2 and PGI_2 in gingival fibroblasts challenged with IL-1β or TNFα. The agent also reduces the basal level of prostaglandin formation in unstimulated cells.

Our finding that benzydamine reduces prostaglandin formation is in line with the findings reported by Segre & Hammarström (8), but in contrast to Goppelt-Strübe et al. (20), who demonstrated enhanced PGE_2 synthesis in macrophages in the presence of benzydamine. However, the concentration of benzydamine used by Goppelt-Strübe et al. (20) was approximately 100 times higher than we used in the cell culture experiments. In our experience, that concentration of the agent is cytotoxic for gingival fibroblasts, resulting in enhanced PGE_2 formation (unpublished observation).

Previously, we reported that the cytokines IL-1β and TNFα stimulate the release of AA from prelabeled gingival fibroblasts at the level of PLA_2 and subsequently the formation of PGE_2 (15, 21). The finding that benzydamine reduces the release of ^{3}H-AA in the presence of IL-1β or TNFα indicates that the inhibitory effect of the agent on prostaglandin formation is partly mediated at the level of PLA_2, suggesting that benzydamine reduces the prostaglandin production by diminishing the liberation of arachidonic acid from phospholipids. The mechanism whereby benzydamine reduces prostaglandin formation in unstimulated cells is probably also due to a reduction in the AA release based on the fact that the basal level of ^{3}H-AA release was reduced. The inhibitory effect of benzydamine on the release of ^{3}H-AA in the presence of IL-1β or TNFα seems not to be due to a specific interaction between the agent and the cytokine on the receptor level. This assumption is based on the finding that benzydamine reduced not only the stimulatory effect of IL-1β but also the action of TNFα on PGE_2 formation. The fact that benzydamine reduced both the effect of IL-1β and TNFα on PGE_2 synthesis supports the view that the agent interferes with one or more second messengers involved in the signal transduction pathway of IL-1β and TNFα. This suggestion is compatible with the finding that the inhibitory effect of benzydamine on prostaglandin formation, induced by IL-1β or TNFα, was more pronounced compared to untreated cells. The inhibitory effect of benzydamine on prostaglandin production in gingival fibroblasts seems not to be due to a direct toxic effect since benzydamine treatment, in the concentrations

used, did not reduce the incorporation of ^{3}H-thymidine into DNA.

The amount of PGE_2 production determined after the addition of exogenous AA is presumably a result of COX-1 and/or COX-2 activity. The finding that benzydamine reduces the PGE_2 synthesis induced by exogenous AA indicates that the enzymes COX may also be involved in the inhibitory action of benzydamine. In light of the fact that both IL-1β and TNFα induce the gene expression of COX-2 in human gingival fibroblasts (21, 22), we hypothesize that COX-2 directly or indirectly may be involved in the inhibitory action of benzydamine.

It is suggested that benzydamine belongs to the family of nonsteroidal anti-inflammatory drugs (NSAIDs) (5). We have earlier demonstrated that the NSAID drug, indomethacin, a COX inhibitor, completely abolishes the PGE_2 production induced by inflammatory mediators such as IL-1β and TNFα in gingival fibroblasts (23). However, the effect of benzydamine (1 μM) on PGE_2 formation seems to be weaker than the corresponding concentration of indomethacin in gingival fibroblasts (23). The finding that benzydamine, in contrast to other NSAID agents, does not exhibit ulcerogenic effects (4) may be related to a weaker capacity of the drug to reduce prostaglandin formation. The results that benzydamine reduces prostaglandin production, induced by IL-1β or TNFα, in gingival fibroblasts may explain the anti-inflammatory effect of benzydamine used in the management of inflammatory oropharyngeal conditions.

It has been reported that the anti-inflammatory agent benzydamine stabilizes cellular membranes (5). Therefore, we tested the effect of lidocaine, an agent with stabilizing properties, on cytokine-induced PGE_2 production. In contrast to benzydamine, the agent lidocaine enhanced the stimulatory effect of IL-1β and TNFα on PGE_2 synthesis in gingival fibroblast. This stimulatory effect of lidocaine on PGE_2 formation may be due to an upregulation of the activity of COX, as reported previously (24).

The level of the inflammatory mediator, PGE_2, is enhanced in gingival crevicular fluid from patients with chronic inflammatory lesions (25, 26). Furthermore, there is strong evidence that PGE_2 plays an important role in the pathogenesis of periodontitis (27). Interestingly, it has also been reported that the use of flurbiprofen (NSAID) reduces the progression of alveolar bone loss in patients with periodontitis (27). In light of the fact that benzydamine reduces the production of prostaglandins, it is worth testing whether the use of this drug may also be of benefit in the management of patients with periodontal diseases.

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