Published in final edited form as:

J Dent Res. 2007 August ; 86(8): 775–779.

Accumulation of Topical Naproxen by Cultured Oral Epithelium

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Abstract

Topically administered non-steroidal anti-inflammatory drugs (NSAIDs) inhibit periodontal bone loss, but little is known about the mechanism by which they penetrate oral epithelium. Active transporters could potentially play a role in this process. In this study, we used a cell line derived from oral epithelium to investigate a role for transporters and to characterize conditions that enhance epithelial penetration. Using fluorescence to monitor uptake, we demonstrated that SCC-25 cell monolayers transport naproxen with a Michaelis constant (K_m) and maximum velocity (V_{max}) of 164 $\mu\text{g/mL}$ and 0.94 $\text{ng/min}/\mu\text{g}$ protein, respectively. At steady state, the intracellular/extracellular concentration ratio was 3.4. Naproxen accumulation was more efficient at acidic pH than under neutral or alkaline conditions. Small proportions of glycerol, Pluronic F-127, and glucosylceramide enhanced naproxen entry. The individual and combined effects of glycerol and Pluronic F-127 were of lesser magnitude than those obtained with glucosylceramide or at pH 6.3. Thus, SCC-25 cells possess transporters for naproxen.

Keywords

analgesic; inflammatory periodontitis

INTRODUCTION

Periodontitis is an inflammatory disorder that breaks down the supporting structures of the teeth. Some of the destructive aspects of this disease are mediated by arachidonic acid metabolism to prostaglandins by cyclooxygenase (COX). Prostaglandin E_2 (PGE_2) occurs at elevated levels in gingival crevicular fluid of individuals with gingivitis and periodontitis (Oringer, 2002), and is capable of inducing osteoclastic bone resorption and fibroblast production of interleukin-6 (which plays a role in bone resorption) (Tipton *et al.*, 2003). PGE_2 also enhances the secretion of destructive matrix metalloproteinases (MMPs) by infiltrating inflammatory cells and resident periodontal cells (Oringer, 2002). Non-steroidal anti-inflammatory drugs (NSAIDs) can inhibit the progression of periodontitis by inhibiting PGE_2 production *via* COX (Salvi *et al.*, 1997; Salvi and Lang, 2005).

Because of the potential for gastrointestinal upset, hepatic impairment, and other systemic complications, there are concerns about the use of systemic NSAIDs to treat periodontitis. However, topically administered NSAIDs offer a greater margin of safety. Previous studies have shown that PGE_2 concentrations in human gingival crevicular fluid are correlated with bone loss in periodontitis (Cavanaugh *et al.*, 1998). Clinical studies have demonstrated that topically administered 0.1% ketorolac tromethamine reduces PGE_2 levels in gingival crevicular fluid, and controls the increase of PGE_2 for 12 hrs (Preshaw *et al.*, 1998). When used over a six-month period, this rinse produced a significant, prolonged reduction of PGE_2

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in gingival crevicular fluid (Cavanaugh *et al.*, 1998). In individuals with periodontitis, twice-daily rinsing with 0.1% ketorolac was at least as effective in preserving alveolar bone as systemic flurbiprofen (50 mg twice daily) (Jeffcoat *et al.*, 1995). Studies utilizing the beagle model of periodontitis have shown that topically administered ketoprofen, flurbiprofen, and piroxicam can significantly reduce bone loss and slow the progression of periodontitis (Jeffcoat *et al.*, 1988; Howell *et al.*, 1991; Paquette *et al.*, 1997).

Although *in vitro* and *in vivo* studies have demonstrated the benefits of topical NSAIDs in the treatment of periodontitis, little is known about the mechanism by which these agents penetrate the oral epithelium. NSAIDs are organic anions, and many types of epithelium possess active transporters for these compounds (Kobayashi *et al.*, 2003). We hypothesize that active transporters play a role in the entry of NSAIDs into epithelial cells. The present study describes evidence to support this hypothesis.

MATERIALS & METHODS

SCC-25 cells (CRL-1628, American Type Culture Collection, Manassas, VA, USA), which were originally derived from oral mucosa, were seeded into 24-well tissue culture plates and cultured at 37°C in 5% CO₂ in 50% Dulbecco's modified Eagle's medium/50% Ham's F12 medium (Invitrogen Corp., Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum and 0.4 µg/mL hydrocortisone. For the experiments described below, the cells were seeded at a density of 9000/well and fed every third day until they formed a confluent monolayer. Cell protein was measured by the Bradford method (1976). We determined intracellular volume by equilibrating SCC-25 cells with [³H]-water (5 µCi/mL, NEN Life Science Products, Boston, MA, USA), as described by Brayton *et al.* (2002).

We assayed NSAID transport by measuring cell-associated naproxen fluorescence. Multiwell culture plates containing confluent cell monolayers were washed 4 times with Hanks' balanced salt solution (HBSS), overlaid with 0.2 mL/well HBSS, and warmed to 37°C prior to assay. To initiate the assay, we simultaneously added 0.2 mL of warm HBSS, containing twice the desired final naproxen concentrations (18 to 154 µg/mL), to each well, using multichannel pipettes. After incubation at 37°C for the indicated times, the naproxen solutions were quickly removed, and each well was rapidly washed 4 times with 0.5 mL phosphate-buffered saline solution. Cell monolayers underwent lysis by being scraped into 1 mL of 40 mM sodium phosphate, pH 6.8. After clarification by centrifugation (13,000 x g, 6 min), the fluorescence of the supernatant was measured as described by Sadecka *et al.* (2001).

To determine the affinity and maximal velocity of transport, we measured the kinetics of transport during the initial phase of transport (from 0 to 3 min) and analyzed it by the Lineweaver-Burk method. We used EnzPack for Windows (Biosoft, Cambridge, UK) to derive the Michaelis constant (K_m) and maximal transport velocity (V_{max}) values from least-squares regression lines obtained with the plotted data.

RESULTS

When incubated with naproxen at 37°C, SCC-25 cells accumulated naproxen in a saturable manner, attaining steady-state intracellular concentrations within 8 min. Analysis by the Lineweaver-Burk method confirmed that the kinetics of naproxen accumulation obeyed the Michaelis-Menten equation (Fig. 1, inset). At 37°C, the cells transported naproxen with a Michaelis constant (K_m) of 164 µg/mL and a maximum velocity (V_{max}) of 69 ng/min/well (0.94 ng/min/µg cell protein). Although the affinity of transport was relatively low, cells incubated with 10 µg/mL naproxen accumulated intracellular concentrations of naproxen that were 3.4-fold higher than at the extracellular level.

To determine the effect of pH on naproxen transport, we assayed the kinetics of transport over the range of pH 6.3 to 8.3. As assessed by the $V_{\max}/K_m$ ratio, pH had a significant effect on the efficiency of transport ($P < 0.001$, repeated-measures ANOVA, Fig. 2). Transport was significantly more efficient at pH 6.3 and 6.8 than at pH 7.3 ($P < 0.05$, Holm-Sidak test), while transport at pH 7.8 and 8.3 was similar in efficiency to pH 7.3 ($P > 0.05$).

We evaluated several buffer modifiers to determine whether they enhanced the penetration of oral epithelial cells by naproxen (Fig. 3). Ethanol (2 to 10%, v/v) significantly decreased the naproxen accumulation in a dose-dependent manner ($P < 0.001$, repeated-measures ANOVA). At a concentration of 10%, ethanol reduced naproxen content by nearly 60%. Over the range of 2% to 10% (v/v), glycerol significantly enhanced naproxen accumulation ($P < 0.001$). In the presence of 6% glycerol, naproxen content was enhanced by approximately 40% ($P < 0.05$, Holm-Sidak test). Pluronic F-127, a mild surfactant agent used in drug delivery, and glucosylceramide, a surface-active plant sphingolipid, also enhanced naproxen accumulation ($P < 0.001$). Pluronic F-127 enhanced naproxen content by approximately 30% at a concentration of 0.04% ($P < 0.05$), while glucosylceramide enhanced naproxen content by more than 2.2-fold at a concentration of 0.05% ($P < 0.05$).

The combination of glycerol and Pluronic F-127 produced additive enhancement of naproxen accumulation inside SCC-25 cells (Fig. 4). Compared with untreated control cells, naproxen content was 74% higher in cells treated with 5% glycerol and 0.05% Pluronic F-127 ($P < 0.05$, Holm-Sidak test). Individually, 5% glycerol and 0.05% Pluronic F-127 produced enhancements of approximately 48% and 21%, respectively ($P > 0.05$). These effects were of lesser magnitude than those obtained in the presence of 0.05% glucosylceramide or by adjustment of the buffer pH to 6.3, which enhanced naproxen content by 2.3-fold and 4.2-fold, respectively ($P < 0.05$, Holm-Sidak test).

DISCUSSION

Topically administered NSAIDs pose less risk of adverse side-effects than do systemic NSAIDs, and several studies suggest that they are equal or superior to systemic agents in slowing the progression of periodontitis. One clinical trial found no statistical differences between a regimen of topical ketoprofen gel (twice-daily rinse) and ketoprofen capsules (25 mg twice daily) with respect to their effects on the levels of PGE₂ and leukotriene B₄ in gingival fluid (Paquette *et al.*, 2000). Both produced a similar reduction in PGE₂ and LTB₄ relative to control samples, despite the lower dosage of the topical regimen (Paquette *et al.*, 2000). In a separate study, individuals rinsing twice daily for 6 mos with 0.1% ketorolac gained about 0.2 mm of bone height. Over the same period, those taking flurbiprofen tablets (50 mg *per day*) lost about 0.1 mm of bone, and control individuals lost about 0.6 mm (Jeffcoat *et al.*, 1995). Topical ketorolac yields gingival fluid ketorolac levels that are 22- to 49-fold higher than those obtained with a 10-mg capsule of ketoprofen, with only 15% of the systemic availability. The half-life of topical ketorolac in gingival fluid under these conditions was 5 hrs.

For topically administered NSAIDs to be absorbed, they must penetrate the oral epithelium. Our model for evaluating this process utilized a squamous cell carcinoma (SCC-25) cell line, which was derived from oral squamous cell carcinoma (Rheinwald and Beckett, 1981). These cells can be readily cultured without an underlying fibroblast feeder layer, which simplifies their use in transport assays. SCC-25 cells produce keratin and undergo many divisions in media, retaining much of the same physiology as normal oral epithelial cells. This system has previously been used for the study of the transport of topically applied antibiotics (Brayton *et al.*, 2002). Cultured SCC-25 cells attach to the plastic culture plates *via* their basal surface. This attachment impairs transport of nutrients across the basal surface, which is the route by which nutrients and systemically administered agents are taken up *in vivo*. Thus, the main route

of NSAID entry in this model is across the apical surface, which is the same surface topically applied NSAIDs would cross to enter oral epithelial cells *in vivo*. Our studies utilized naproxen, a non-selective COX inhibitor, as a proxy for other NSAIDs. Naproxen is highly fluorescent, so its movement into epithelial cells can be conveniently monitored by cell-associated fluorescence (Damiani *et al.*, 2002).

Movement of naproxen into SCC-25 cells was saturable, concentrative, and exhibited Michaelis-Menten kinetics, providing evidence that oral epithelial cells possess an active transport system for accumulating NSAIDs. Previous studies have shown that naproxen can also be actively transported by human gingival fibroblasts (Zavarella *et al.*, 2006). The Michaelis constant (127 $\mu\text{g/mL}$) and maximal transport velocity (1.42 $\text{ng/min}/\mu\text{g}$ cell protein) were reasonably similar to the values observed with SCC-25 cells in this study. These findings have therapeutic significance in periodontology, since topically applied NSAIDs must enter the gingival epithelium and distribute into the gingival connective tissue to inhibit COX effectively in the connective tissue. Although the affinity of naproxen transport by SCC-25 cells and gingival fibroblasts is somewhat low, the NSAID concentrations used in topical administration are relatively high.

While SCC-25 cells were capable of transporting naproxen over a wide pH range, naproxen transport was significantly enhanced under slightly acidic conditions. Naproxen is a weak organic acid, and is thought to be a substrate for organic anion transporters (You, 2004). It is possible that changes in the ionization of naproxen under acidic conditions make it a better substrate for transport. Moreover, studies of intestinal epithelial organic anion transporters have demonstrated that transport is proton-mediated and exhibits enhanced activity when the pH is decreased to 5.5 (Kobayashi *et al.*, 2003).

The addition of a small percentage of glycerol to the assay buffer significantly enhanced the movement of naproxen into SCC-25 cells. Glycerol is a hydrophilic emulsifier and surfactant agent that is used in cosmetics, soaps, foods, and creams. Glycerol can induce leakiness in renal epithelial tight junctions (Mullin and McGinn, 1988). Its ability to permeate the oral epithelial cell membrane and induce leakiness may have been partly responsible for the observed enhancement of intracellular naproxen content.

Pluronic F-127, a biocompatible surfactant polyol, significantly enhanced the entry of naproxen into SCC-25 cells. It has previously been used to facilitate the percutaneous absorption of NSAIDs (Takahashi *et al.*, 2002). Pluronic F-127 enhances the solubilization and cell permeability of lipophilic drugs, and is also capable of altering the properties of the plasma membrane. The range of Pluronic F-127 concentrations used in this study was selected to minimize the latter of these two effects.

Glucosylceramide, which consists of a long-chain sphingoid base linked to a fatty acid with an amide bond, significantly enhanced the transport of NSAIDs into the oral epithelial cells. Ceramides are one of the major sphingolipids of human epidermis (Takagi *et al.*, 2005), and they play a role in epidermal barrier function (Coderch *et al.*, 2003; Choi and Maibach, 2005). They exhibit a low degree of cytotoxicity (Vesper *et al.*, 1999). Previous studies have shown that glucosylceramide can penetrate the cell membrane, resulting in a reversible increase in permeability to NSAIDs and other drugs (Siskind *et al.*, 2002; Vavrova *et al.*, 2003). In contrast to glycerol, ceramides become embedded in the cell membrane, but are generally not capable of penetrating tight junctions. It is possible that the increased cell permeability of naproxen observed with glucosylceramide could have resulted from channel formation in the cell membrane by the ceramide (Siskind *et al.*, 2002).

Ethanol (2% to 10%) was the only modifier that significantly decreased the entry of naproxen into SCC-25 cells. Previous studies have shown that 5% to 15% ethanol significantly enhances

the permeability of the oral mucosa (Howie *et al.*, 2001). However, other studies have shown that combinations of ethanol and naproxen produced cytotoxic effects on intestinal mucosa (Oh *et al.*, 2005). It is possible that cytotoxicity was responsible for the decreased intracellular naproxen content observed in this study.

In conclusion, a cell line derived from oral epithelium possesses a system for actively transporting naproxen. Naproxen uptake and accumulation can be enhanced when the extracellular medium is acidified to pH 6.3. Incorporation of glycerol, Pluronic F-127, or glucosylceramide into the delivery buffer also enhances the cell-permeability of naproxen. Penetration of cells by naproxen appears to be impaired at alkaline pH and in the presence of ethanol. Topically applied NSAIDs have been used to relieve post-operative discomfort, and as adjunctive treatment to help control the inflammation and reduce the bone loss associated with periodontitis. They have the potential to provide oral inflammation reduction similar to, if not better than, that provided by systemic agents, with less risk of side-effects. NSAIDs could potentially be formulated into mouthrinses, toothpastes, gels, creams, or surgical dressings for topical application. The *in vitro* model described herein may be useful for optimizing the conditions for oral topical application to enhance the therapeutic effectiveness of NSAIDs.

ACKNOWLEDGMENTS

This investigation was supported by USPHS research grant R01 DE012601 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA.

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Figure 1.

Time-course of naproxen accumulation by cultured SCC-25 monolayers. Cells were incubated for 10 min at 37°C in HBSS prior to the addition of 50 $\mu\text{g/mL}$ naproxen. At the indicated times, the naproxen solution was removed, and extracellular naproxen was rapidly washed away. The data represent the mean of 6 experiments. **Inset:** Representative Lineweaver-Burk plot of naproxen transport by SCC-25 cells observed in a representative experiment conducted over a three-minute interval at 37°C.

Figure 2.

Effect of pH on accumulation of naproxen by confluent SCC-25 monolayers. Cell monolayers were washed and overlaid with HBSS adjusted to the indicated pH. Kinetic analysis was performed as shown in Fig. 1, and the $V_{\max}/K_m$ ratio was used as an index of transport efficiency. The data are presented as the mean $\pm$ SEM of 5 experiments. Relative to pH 7.3, transport efficiency was significantly higher at pH 6.3 and 6.8 ($P < 0.003$, repeated-measures ANOVA), but similar at pH 7.8 and 8.3 ($P > 0.3$).

Figure 3.

The effect of buffer modifiers on naproxen accumulation by SCC-25 cells. (**Upper panel**).

The effects of varied (from 0 to 10%) concentrations of ethanol and glycerol on naproxen accumulation by SCC-25 cells. Naproxen accumulation was monitored over 3 min in the presence of the indicated additions to HBSS, under experimental conditions similar to those used in Fig. 1. (**Lower panel**) The effects of glucosylceramide and Pluronic F-127 (from 0 to 0.1%) on naproxen accumulation by SCC-25 cells. Treatment effects by all 4 modifiers were significant ($P \leq 0.003$, repeated-measures ANOVA). Except for 2% ethanol, every individual treatment was significantly different compared with controls ($P < 0.05$, Holm-Sidak test). The data are presented as the mean $\pm$ SEM of 6 experiments.

Figure 4.

Comparison of the individual and combined effects of glycerol and Pluronic F-127 and the effects of glucocylceramide and acidic pH on naproxen accumulation by SCC-25 cells. The data represent the mean $\pm$ SEM of 5 experiments. Treatments significantly different from control are indicated by * ($P < 0.05$, Holm-Sidak post-test).