Published in final edited form as:

J Dent Res. 2006 May ; 85(5): 452–456.

Accumulation of Non-steroidal Anti-inflammatory Drugs by Gingival Fibroblasts

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to manage pain and inflammatory disorders. We hypothesized that gingival fibroblasts actively accumulate NSAIDs and enhance their levels in gingival connective tissue. Using fluorescence to monitor NSAID transport, we demonstrated that cultured gingival fibroblasts transport naproxen in a saturable, temperature-dependent manner with a K_m of 127 $\mu\text{g/mL}$ and v_{max} of 1.42 ng/min/ μg protein. At steady state, the intracellular/extracellular concentration ratio was 1.9 for naproxen and 7.2 for ibuprofen. Naproxen transport was most efficient at neutral pH and was significantly enhanced upon cell treatment with TNF- α . In humans, systemically administered naproxen attained steady-state levels of 61.9 $\mu\text{g/mL}$ in blood and 9.4 $\mu\text{g/g}$ in healthy gingival connective tissue, while ibuprofen attained levels of 2.3 $\mu\text{g/mL}$ and 1.5 $\mu\text{g/g}$, respectively. Thus, gingival fibroblasts possess transporters for NSAIDs that are up-regulated by an inflammatory mediator, but there is no evidence that they contribute to elevated NSAID levels in healthy gingiva.

Keywords

naproxen; ibuprofen; analgesic; inflammatory periodontitis

INTRODUCTION

Prostaglandins (PGs) play several important roles in physiological and pathological conditions. PGE₂ is a major mediator of pain and is thought to be involved in the pathogenesis of periodontal disease, due to its association with bone resorption and attachment loss (Offenbacher *et al.*, 1986, 1993). PGE₂ is produced by fibroblasts, neutrophils, monocytes, and epithelial cells in response to injury, dental plaque, and plaque by-products, which produce an inflammatory response in the gingiva. Non-steroidal anti-inflammatory drugs (NSAIDs) are a versatile class of agents that inhibit cyclo-oxygenase, the rate-limiting enzyme in the formation of PGE₂ from arachidonic acid. They are widely prescribed to manage pain and swelling and have also been used adjunctively in periodontal therapy. Studies in animals and humans suggest that the progression of periodontitis can be slowed by NSAIDs (Williams *et al.*, 1985, 1989; Offenbacher *et al.*, 1992; Jeffcoat *et al.*, 1995). Despite their extensive use, little is known about the factors that govern the distribution of systemic NSAIDs to the tissues of the periodontium. Gingival fibroblasts have been shown to take up tetracyclines and enhance their redistribution from systemic circulation to gingiva (Yang *et al.*, 2002). Fibroblasts are the most prevalent PGE₂-producing cell in gingival connective tissue and are a logical target for

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NSAIDs. We hypothesized that gingival fibroblasts possess active transporters that allow them to accumulate NSAIDs, thereby enhancing their distribution to gingival connective tissue. The aims of this study were to determine whether cultured gingival fibroblasts transport and accumulate naproxen, and to determine whether this process could potentially contribute to the attainment of higher steady-state NSAID levels in gingival connective tissue than in blood serum.

MATERIALS & METHODS

Isolation and Culture of Gingival Fibroblasts

Fibroblasts were isolated from explants obtained from healthy adult interproximal papillae as previously described (Mariotti and Cochran, 1990). Informed consent was obtained from the donors under a protocol approved by the Institutional Review Board. Cells were used between passage numbers 4 and 15 and were cultured at 37°C in 5% CO₂ in minimal essential medium (MEM, Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM glutamine and 10% fetal bovine serum. For the experiments described below, fibroblasts were seeded into 24-well cell culture plates and fed every 3 days until they formed a confluent monolayer. Cell protein was measured with the Bradford method (1976).

Assay of Naproxen Transport

Transport was assayed by the measurement of cell-associated naproxen or ibuprofen fluorescence. Multiwell culture plates containing confluent cell monolayers were washed 4x with Hanks' balanced salt solution (HBSS), overlaid with 0.2 mL/well HBSS, and warmed to 37°C prior to assay. In the naproxen transport assay, 0.2 mL of warm HBSS containing twice the desired naproxen concentration was simultaneously added to each well with multichannel pipettes. After incubation at 37°C for the indicated times, the naproxen solution was quickly removed. The cells were rapidly washed 4x with 0.5 mL/well phosphate-buffered saline and subjected to lysis by being scraped into 1 mL of 40 mM sodium phosphate (pH 6.85). After cells were briefly centrifuged at 14,000 × g, NSAID fluorescence was measured as described by Sadecka *et al.* (2001).

To determine the affinity and velocity of transport, we assayed the kinetics of transport over a three-minute period after the addition of naproxen and analyzed it by the Lineweaver-Burk method. We used EnzPack for Windows (Biosoft, Ferguson, MO, USA) to derive the Michaelis constant (K_m) and maximal transport velocity (V_{max}) values from regression lines obtained with the plotted data. Several organic anions inhibited naproxen transport and altered the Lineweaver-Burk plot intercepts. We used the pattern of alterations produced by these agents to determine the mechanism of inhibition and the inhibition constant (K_i). We measured intracellular volume by equilibrating fibroblast monolayers with [³H]-water (5 µCi/mL, NEN Life Science Products) exactly as described by Yang *et al.* (2002).

Human Studies

Two groups of six subjects with good systemic and periodontal health were recruited from an Ohio State University College of Dentistry clinic population that had been treatment-planned for pre-prosthetic surgery or soft-tissue grafting. Pregnant subjects and individuals taking NSAIDs or any other medications were excluded. Informed consent was obtained under a protocol approved by the Institutional Review Board. Subjects were issued a seven-day supply of naproxen (375 mg every 12 hrs, group 1) or ibuprofen (400 mg every 8 hrs, group 2), along with detailed instructions. Subjects were asked to record the times and dates they took the medications. At the time of the surgical procedure (which was intentionally scheduled 6–10 hrs after the final dose), samples of peripheral blood (3 mL) and gingival connective tissue (15–35 mg) were obtained. Gingival tissue samples were blotted, weighed, cooled on ice, and

processed for high-performance liquid chromatography (HPLC) exactly as described by Dominkus *et al.* (1996).

Measurement of NSAIDs in Serum and Tissue Samples

Ibuprofen and naproxen content was measured by isocratic reverse-phase HPLC (Farrar *et al.*, 2002). The samples were analyzed by chromatography on a C18 column (NovaPak, 5 × 100 mm, Waters Corp, Milford, MA, USA). The mobile phase consisted of acetonitrile:100 mM sodium dihydrogen phosphate adjusted to pH 2.6 (60:40 v/v for naproxen analysis and 65:35 for ibuprofen). Sample elution was monitored by ultraviolet absorbance (230 nm) and fluorescence (with a 280-nm excitation wavelength and a 340 emission filter). We calculated serum NSAID levels by dividing drug content of each sample by sample volume. Tissue NSAID content was normalized to wet sample weight.

RESULTS

Gingival fibroblasts accumulated naproxen in a saturable and temperature-dependent manner (Fig. 1). At 37°C, the kinetics of naproxen transport yielded linear plots when analyzed by the Lineweaver-Burk method. Naproxen was transported with a K_m of 127 ± 9.4 µg/mL and a V_{max} of 1.42 ± 0.14 ng/min/µg cell protein. Fibroblasts accumulated relatively high levels of naproxen as well as ibuprofen. Incubation with extracellular naproxen concentrations of 5 µg/mL and 10 µg/mL was associated with a ratio of intracellular to extracellular concentrations (C/E) of 3.6 ± 0.4 and 1.9 ± 0.2 , respectively. Incubation with 10 µg/mL ibuprofen resulted in a C/E of 7.2 ± 0.9 (data not shown). Uptake of naproxen was inhibited when temperature was reduced from 37° to 3°C (Fig. 1).

Transport of naproxen was most efficient at neutral pH. At pH 7.3 and 7.8, the K_m for naproxen transport approximated 130 and 187 µg/mL, respectively. At pH 6.3, 6.8, and 8.3, K_m exceeded 400 µg/mL (Fig. 2, upper panel). Since NSAIDs are weak organic acids, it is feasible that a member of the organic anion transporter family plays a role in their accumulation by fibroblasts. Consistent with this possibility, the well-known organic anion substrates phenol red and penicillin both inhibited naproxen accumulation by fibroblasts ($K_i = 0.45 \pm 0.14$ mM and 2.4 ± 0.45 mM, respectively). The pattern of inhibition produced by phenol red was competitive with respect to naproxen (Fig. 2, lower panel).

Protein kinase C plays a role in regulating many transport processes. To begin to characterize the mechanisms by which NSAID transport is modulated, we pre-treated fibroblast monolayers with 100 nM phorbol myristate acetate, a potent receptor-independent activator of protein kinase C. This agent enhanced the accumulation of naproxen and ibuprofen by nearly 60%, and its effects were dose-dependent (Fig. 3, upper panel). Phorbol ester treatment decreased the K_m of naproxen transport from 127 to 68.7 µg/mL without changing V_{max} (data not shown). In addition, cell exposure to physiological concentrations of TNF-α (from 3 to 30 ng/mL) significantly enhanced fibroblast naproxen transport in a dose-dependent manner ($P = 0.002$ after 1 hr, $P = 0.001$ after 3 or 6 hrs, repeated-measures analysis of variance, Fig. 3, lower panel). TNF produced > 30% enhancement after 1 hr, 53% enhancement after 3 hrs, and 102% enhancement after 6 hrs ($P > 0.05$ for all, Dunnett's test).

To test the hypothesis that fibroblasts enhance the distribution of NSAIDs to the gingiva, we measured steady-state naproxen and ibuprofen levels and compared them in serum and gingival connective tissue in subjects who had undergone 7 days of treatment with naproxen or ibuprofen. Blood and tissue samples were obtained approximately 6.8 hrs after subjects completed the seven-day course of naproxen and 6.2 hrs after they completed the ibuprofen regimen (Table). Naproxen levels averaged 61.9 ± 8.5 µg/mL in serum and 9.4 ± 0.9 µg/g in

gingival connective tissue (difference significant, $P = 0.001$, paired t test). Ibuprofen levels were $2.3 \pm 0.6 \mu\text{g/mL}$ in serum and $1.5 \pm 0.6 \mu\text{g/g}$ in gingival connective tissue ($P = 0.285$).

DISCUSSION

NSAIDs are among the most commonly prescribed medications in dentistry. Their efficacy is strongly associated with the inhibition of arachidonic acid metabolism by cyclo-oxygenase. Prostaglandins produced by this pathway mediate pain and contribute to enhanced osteoclast activity during periods of active periodontal disease. Following periodontal surgery, ibuprofen decreases pain intensity and reduces PGE_2 levels in gingival tissue by over 95% (O'Brien *et al.*, 1996). In a randomized, placebo-controlled clinical trial, administration of an 18-month course of flurbiprofen significantly reduced the rate of periodontal bone loss in subjects with severe chronic periodontitis (Williams *et al.*, 1989). Gingival fibroblasts express cyclo-oxygenase and produce prostaglandins, so they are a logical target for the treatment of gingival pain and inflammatory periodontitis. Fibroblasts are the predominant cell in healthy gingival connective tissue, comprising approximately 65% of the total cell population (Lindhe *et al.*, 2003) and 5.2% of the volume (Schroeder *et al.*, 1973). In inflamed gingival connective tissue, fibroblasts comprise 14.5% of the volume.

The present study demonstrates that gingival fibroblasts possess active transporters that can take up and accumulate NSAIDs. Fibroblasts transport naproxen in a saturable, temperature-dependent manner, with kinetics that yield a linear Lineweaver-Burk plot. In spite of the relatively high K_m for naproxen transport, fibroblasts were capable of accumulating a considerable amount of drug. The observed cellular/extracellular concentration (C/E) ratios exceeded 1.9 for naproxen and 7.2 for ibuprofen, demonstrating that fibroblasts accumulate these drugs against a concentration gradient. The lowest K_m value for naproxen transport was observed around pH 7.3, an indication that the transporter has its highest affinity for substrate around neutral pH.

NSAIDs are weak organic acids and are thought to be substrates for organic anion transporters, which interact with a wide variety of anionic drugs (You, 2004). Consistent with this possibility, naproxen transport was competitively inhibited by the organic anion transporter substrates phenol red and penicillin. The activity of some organic anion transporters is regulated by the protein kinase C signal transduction pathway. Roelofsen *et al.* (1991) showed that the activity of a multi-specific organic anion transporter is stimulated by PMA (which directly activates protein kinase C) and reduced by an inhibitor of protein kinase C. In the present study, PMA decreased the K_m of fibroblast naproxen transport by cultured gingival fibroblasts and significantly enhanced intracellular accumulation of naproxen and ibuprofen. This suggests that protein kinase C plays a role in signaling for increased NSAID transport activity by gingival fibroblasts.

With its ability to stimulate matrix metalloproteinase and prostaglandin production by fibroblasts, TNF- α is an important mediator of the inflammatory response (Birkedal-Hansen, 1993). Interestingly, protein kinase C mediates some of its effects (Gorospe *et al.*, 1993). TNF- α significantly enhanced the transport of naproxen by gingival fibroblasts within 1 hr, and these effects were sustained for at least 6 hrs. Transport activity was presumably enhanced by up-regulation of existing transporters, since there was little potential for induction of new transporter gene expression or an increase in cell number over this time course. This enhancement by TNF could potentially contribute to enhanced NSAID accumulation by fibroblasts in inflamed gingiva. A similar pattern of enhancement by TNF- α has been reported with respect to minocycline transport by gingival fibroblasts (Walters *et al.*, 2005).

Previous studies have shown that gingival fibroblasts take up and accumulate tetracyclines and fluoroquinolones (Yang *et al.*, 2002). In this capacity, fibroblasts could potentially have a favorable therapeutic impact by acting as reservoirs to sustain antimicrobial levels in the gingiva. This could explain why systemically administered doxycycline and ciprofloxacin can attain higher concentrations in gingival crevicular fluid and gingival connective tissue than in blood serum (Lavda *et al.*, 2004). In contrast to these two antimicrobials, the present study provides no evidence that naproxen and ibuprofen reach higher levels in gingival connective tissue than in blood serum. Naproxen and ibuprofen both exhibit extensive (> 99%) binding to serum proteins, most notably albumin (Lin *et al.*, 1987). This may limit the availability of NSAIDs for uptake by fibroblasts at peripheral sites. With its low affinity for substrate, fibroblast NSAID transport may be inefficient under these conditions. Only 800 μ M (0.2 μ g/mL) naproxen and 6.0 mM (1.2 μ g/mL) ibuprofen are required to produce 50% inhibition of PGE₂ production by cyclo-oxygenase (Cushman and Cheung, 1976). Thus, the observed tissue levels of naproxen and ibuprofen (9.4 μ g/g and 1.5 μ g/g, respectively) exceed the concentrations needed to inhibit PGE₂ synthesis.

In summary, gingival fibroblasts possess a transport system that takes up and concentrates NSAIDs inside these cells. Our findings suggest that this transporter could be a member of the organic anion transporter family. Its activity is significantly up-regulated by TNF- α , through a mechanism that could potentially involve protein kinase C. Fibroblasts comprise a relatively small fraction of healthy gingival connective tissue, and the K_m of NSAID transport is relatively high. Thus, the impact of this transport system on peak NSAID levels may be somewhat limited in healthy gingiva. In inflamed gingiva, however, the volume of the fibroblast compartment is nearly three times larger, and NSAID accumulation by fibroblasts may be significantly up-regulated by TNF- α . It is therefore feasible that fibroblast NSAID transport could materially influence NSAID levels attained at inflamed periodontal sites.

Acknowledgements

The authors are grateful to Dr. Angelo Mariotti for providing human gingival fibroblasts. This investigation was supported by USPHS research grants R01 DE012601 and T32 DE014320 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA.

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Figure 1.

Naproxen accumulation by cultured gingival fibroblast monolayers. Cells were pre-incubated in a balanced salt solution at 37°C or 3°C for 10 min prior to the addition of 40 μg/mL naproxen. At the indicated times, the naproxen solution was removed, and extracellular naproxen was rapidly washed away. Where indicated, the cells were pre-treated for 15 min with 100 nM phorbol myristate acetate. Data are expressed as mean ± SEM of 6 experiments.

Figure 2.

Characteristics of naproxen transport by cultured gingival fibroblasts. **Upper panel:** pH dependence of naproxen transport. Data are expressed as mean $\pm$ SEM of 5 experiments. At pH 6.3, 6.8, and 8.3, the K_m of transport was significantly higher than at pH 7.3 ($P < 0.05$, Dunnett's test). **Lower panel:** Lineweaver-Burk plot of naproxen transport kinetics, showing competitive inhibition in the presence of 1 mM phenol red. The data are representative of 4 experiments.

Figure 3.

Stimulation of fibroblast naproxen accumulation by phorbol myristate acetate and TNF- α . **Upper panel:** Enhancement of fibroblast naproxen and ibuprofen transport by phorbol myristate acetate. Confluent fibroblast cultures were treated for 15 min with the indicated phorbol ester concentrations prior to incubation with naproxen or ibuprofen. The treatment effects of phorbol myristate acetate were significant for both NSAIDs ($P < 0.001$, analysis of variance, $n = 6$ experiments). **Lower panel:** Time- and dose-dependent enhancement of naproxen transport by TNF- α . Confluent fibroblasts were starved for 16 hrs in medium containing 0.5% fetal bovine serum and treated with the indicated concentrations of TNF for

1, 3, or 6 hrs prior to assay of naproxen transport. TNF produced a significant treatment effect at 1, 3, and 6 hrs ($P \leq 0.002$, analysis of variance, $n = 7$ experiments).

Table
Naproxen and Ibuprofen Content of Serum and Gingival Connective Tissue

NSAID	Time Since Last Dose ¹	Serum Level ²	Tissue Level ³
Naproxen	6.8 ± 0.7	61.9 ± 8.5	9.4 ± 0.9 ⁴
Ibuprofen	6.2 ± 0.5	2.3 ± 0.6	1.5 ± 0.6

¹ Mean (± SEM) time in hrs between last NSAID dose and sample acquisition.

² Results expressed as mean ± SEM in µg/mL.

³ Results expressed as mean ± SEM in µg/g tissue wet weight.

⁴ Result is significantly different from serum level (P = 0.001, paired *t* test).