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Non-steroidal Anti-inflammatory Drugs Inhibit Calpain Activity and Membrane Localization of Calpain 2 Protease

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are used frequently worldwide for the alleviation of pain despite their capacity to cause adverse gastrointestinal (GI) side effects. GI toxicity, once thought to be the result of non-specific inhibition of cyclooxygenase (COX) enzymes, is now hypothesized to have multiple other causes that are COX independent. In particular, NSAIDs inhibit intestinal epithelial restitution, the process by which barrier function in intestinal mucosa is restored at sites of epithelial wounds within hours through cell spreading and migration. Accordingly, recent evidence indicates that the expression of calpain proteases, which play a key role in cell migration, is decreased by NSAIDs that inhibit cell migration in intestinal epithelial cells (IEC). Here, we examine the effect of NSAIDs on calpain activity and membrane expression in IEC-6 cells. Indomethacin, NS-398, and SC-560 inhibited calpain activity and decreased expression of calpain 2 in total membrane fractions and in plasma membranes involved in cell attachment to the substrate. Additionally, we demonstrated that inhibition of calpain activity by NSAIDs or ALLM, a calpain inhibitor, limits cell migration and *in vitro* wound healing of IEC-6 cells. Our results indicate that NSAIDs may inhibit cell migration by decreasing calpain activity and membrane-associated expression of calpain 2. Our results provide valuable insight into the mechanisms behind NSAID-induced GI toxicity and provide a potential pathway through which these negative side effects can be avoided in future members of the NSAID class.

Keywords

calpain activity; NSAIDs; cell migration; wound healing; intestinal epithelial cell

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs worldwide despite their well-known gastrointestinal (GI) side effects. Estimates indicate that as many as 50% of people taking NSAIDs daily show some symptoms of NSAID-induced GI toxicity (Wallace and Vong, 2008), and 2-4% of individuals develop serious problems

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requiring medical intervention, and even rarely death (Wolfe, et al., 1999). These effects, however, are not limited to humans, and GI toxicity is clinically recognized in companion animals, particularly horses, that are treated regularly with NSAIDs. Of particular interest is that lesions occur in anatomic locations similar to those seen in humans (Oren and Ligumsky, 1994; Israel, et al., 2001; Karcher, et al., 1990; Lichtenberger, 2001; MacAllister, et al., 1993; Neiger, 2003; MacAllister, 1983). As a result, NSAID-induced GI toxicity accounts for yearly socioeconomic losses of more than a billion dollars in direct (health care, pharmaceuticals, hospitalization) and indirect (work loss) costs (Lichtenberger, 2001; Delco, et al., 2004).

Historically, non-specific inhibition of cyclooxygenase (COX) isoforms has been blamed for GI toxicity following NSAID treatment. However, accumulating evidence indicates that inhibition of COX isoforms does not entirely explain the range of toxic effects seen with NSAID use (Lichtenberger, 2001). Accordingly, several other mechanisms of action, including uncoupling of oxidative phosphorylation (Mahmud, et al., 1996; Somasundaram, et al., 2000), decreases in rates of apoptosis (Ashton and Hanson, 2002), and inhibition of cell migration or epithelial restitution (Pai, et al., 2001; Rahgozar, et al., 2001; Raveendran, et al., 2008; Freeman, et al., 2007; Penney, et al., 1995; Schmassmann, et al., 1995), have been suggested to account for these discrepancies.

Epithelial restitution is the process by which physical wounds in the GI epithelia are closed and barrier function is restored (Dignass, 2001). Restitution occurs within a period of minutes to hours following wounding and relies upon cell migration, but with cell proliferation only secondarily re-establishing the normal cell density. Re-establishment of the barrier function of the GI is imperative to maintain the separation of the internal milieu from the external environment. Disruption of this process can lead to invasion of the epithelial by microbes and subsequent inflammation culminating in epithelial erosion.

Cell migration of intestinal epithelia is a complicated process that is mediated by a variety of cellular signaling pathways and effectors (Dignass, 2001; Pai, et al., 2001), but very little is known about how NSAIDs affect these pathways. Recently, microarray analysis of intestinal epithelial cells (IEC) revealed that treatment with NSAIDs with ulcerogenic potential inhibits the mRNA and/or protein expression of a family of intracellular limited proteases, called calpains (Raveendran, et al., 2008). Specifically, indomethacin and NS-398 caused significant decreases in the protein expression of calpains 1, 2, and 8. These findings are significant because calpain proteases play key roles in many cell processes, including proliferation, cytoskeletal organization, signal transduction, blood clotting cascades, and cell migration (Benyamin, 2006; Lebart and Benyamin, 2006; Glading, et al., 2002; Lokuta, et al., 2003; Perrin and Huttenlocher, 2002). However, as calpains are present in far excess to their regulated activity and undergo autoproteolytic degradation, the level of protein does not dictate the activity in the cell.

Therefore, we investigated the effects of NSAIDs with variable ulcerogenic potential on calpain activity in IEC cells and compared this to the effects of ALLM, a calpain inhibitor. As the calpain activity involved in cell migration is actuated by membrane-associated calpains (Glading, et al., 2001), we also examined the expression of calpain 2 protein in membrane fractions and membrane footprints and correlated the membrane expression of calpain 2 and the activity of calpain proteases with the inhibition of migration by NSAIDs with ulcerogenic potential or ALLM. We show that calpain activity and membrane expression are affected by treatment with indomethacin, NS-398, and SC-560, and that inhibition of cell migration is associated with inhibition of calpain activity at the membrane attached to the substrate.

Materials and Methods

Reagents

Cell culture medium was obtained from American Type Culture Collection (ATCC, Manassas, VA). Serum was obtained from Invitrogen (Calsbad, CA) and chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise. Matrigel basement membrane was purchased from BD Biosciences (Bedford, MA). SC-560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole) and NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) were obtained from Cayman Chemical (Ann Arbor, MI).

Cell Culture

The IEC-6 cell line, developed by Quaroni *et al.* (Quaroni, et al., 1979), was purchased from ATCC, (Manassas, VA). IEC-6 culture conditions were similar to those described previously (Freeman, et al., 2007). The basic culture medium consisted of DMEM supplemented with heat-inactivated fetal bovine serum (FBS, 5%), insulin (10 µg/ml) and gentamicin (50 µg/ml). Cells were maintained in 75 cm² tissue culture flasks at 37 C in a humidified atmosphere of 5% CO₂ in air. Cell passages 16-20 of IEC-6 were used for all experiments to minimize the effects of passage.

Calpain activity

Calpain activity was assessed using the *tert*-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin (BOC-LM-CMAC, Invitrogen) assay (Rosser, et al., 1993) using a previously published procedure (Glading, et al., 2000). IEC-6 cells were seeded onto 35 mm optical quality plates coated with collagen (Matek, Ashland, MA) 96 h prior to imaging. Cells were then treated with NSAIDs (100 µM indomethacin, 100 µM NS-398, or 1 µM SC-560) 6, 12, 24, 48, or 72 h prior to assay or with various concentrations of N-acetyl-Leu-Leu-methioninal (ALLM, EMD Biosciences, Gibbstown, NJ) 24 h prior to assay. Cells were incubated in 15 µM BOC-LM-CMAC for 15 min prior to imaging. Calpain activity was quantified by measuring the mean fluorescence of cells using the ImageJ software (NIH, city, state) from at least three fields from two or more plates for each treatment group.

Membrane preparations

For membrane protein expression analysis, lysates were collected and homogenized in a sucrose isolation buffer (0.3 M sucrose, 10 mM imidazole, and a 1:500 dilution of protease inhibitors; Sigma, St. Louis MO). Following centrifugation (11,000 g for 14 min), supernatants were saved (on ice) whereas pellets were re-homogenized in sucrose isolation buffer. The re-homogenized pellets were centrifuged again, and the supernatant was pooled with the previous supernatant (on ice). These were centrifuged again (100,000 g for 90 min). Pellets following ultracentrifugation were resuspended in a commercially available isolation buffer (RIPA, Santa Cruz Biotechnology, Santa Cruz, CA) and centrifuged (16,000 g for 15 min) to remove any remaining undissolved debris. Supernatants were then aliquoted and stored at -80 C until analyzed by gel electrophoresis and Western blotting.

Western Blot Analysis

Whole cell lysates were collected using a commercially available isolation buffer (RIPA, Santa Cruz Biotechnology, Santa Cruz, CA) and protein content was determined by Micro BCA Protein Assay (Pierce, Rockford IL). Lysates from membrane preparations were separated by SDS-PAGE, transferred to membranes, and probed sequentially with appropriate primary and secondary antibodies. Primary antibodies for calpain 2 were obtained commercially (Abcam, Cambridge, MA) and used at a dilution of 1:1000.

Antibodies were diluted into blocking buffer [5% non-fat milk in Tris-buffered saline (TBS) with Tween-20 (0.1%)]; TBS was made from a commercially available 10X stock (Bio-Rad). Membranes were incubated in primary antibody overnight at 4 C. Stabilized goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted into the blocking buffer, and membranes were exposed to secondary antibody for 1 h at room temperature. Immunocomplexes were visualized using an enhanced chemiluminescence detection system (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce). Equal loading of protein was confirmed by stripping and reprobing membranes with antibody directed against actin (Sigma). Our previous work validated the use of actin as a loading control in experiments that included NSAID-treated IEC-6 monolayers (Freeman, et al., 2007). Densitometry was performed using imaging software (AlphaEaseFC, AlphaInnotech, San Leandro, CA). The expression of calpain 2 was normalized to actin for comparisons between treatment groups. Summary data shown is based on Western blots performed using cell lysates from at least four independent experiments employing all treatment groups.

Cell Footprinting

IEC-6 cells were plated onto glass coverslips and treated with NSAIDs (100 μ M indomethacin, 100 μ M NS-398, or 1 μ M SC-560) for 48 h. Apical membranes of IEC-6 cells were then removed using a previously published procedure (Beer-Stolz and Jacobson, 1992; Shao, et al., 2006). All procedures were carried out on ice, and all solutions were kept at 4 C. Briefly, cells were treated with morpholineethanesulfonic acid-buffered saline (MBS, 20 mM morpholineethanesulfonic acid [pH 5.5], 135 mM NaCl, 0.5 mM CaCl_2 , 1mM MgCl_2) to prepare them for coating. Then cell membranes were coated with a 1% cationic colloidal silica (provided by Donna Beer Stolz, University of Pittsburgh, Pittsburgh, PA and diluted into a 30% stock solution; Chaney and Jacobson, 1983). Silica was then washed off, and cells were treated with a 1% polyacrylic acid solution followed by a hypotonic lysis buffer (2.5 mM imidazole [pH 7.0] supplemented with protease inhibitors [1:1000 dilution, mammalian protease inhibitor cocktail, Sigma]). Cells were deroofed by washing coverslips with lysis buffer using a 10 mL syringe and 18 gauge needle. Cells were then fixed with 2% paraformaldehyde and blocked with 5% non-immune goat serum. Footprints were probed with a monoclonal antibody to calpain 2 (1:1000 dilution, EMD Biosciences) followed by a secondary antibody (Alexafluor 488 goat anti-mouse, 1:500 dilution, Invitrogen). F-actin was labeled with rhodamine phalloidin (1:250 dilution, Invitrogen) and nuclei were stained with DAPI (1:12000 dilution, Sigma). Gelvatol was used to fix coverslips to slides. Stained samples were then viewed on an Olympus Bx51 fluorescence microscope (Olympus, Malvern, PA) equipped with an Olympus color video camera. Images were analyzed using the ImageJ software to measure mean fluorescence of calpain expression in IEC-6 cells.

Migration Assays

Cell migration of IEC-6 cells treated with NSAIDs was assessed using an established cell migration assay (Freeman, et al., 2007). Briefly, confluent monolayers grown on a Matrigel basement membrane and treated with NSAIDs for 24 or 48 h with indomethacin (100 μ M), NS-398 (100 μ M), or SC-560 (1 μ M), or ALLM (25 μ M) were wounded by drawing a razor blade sharply across the dish and removing approximately one-third of the monolayer, creating a border or scratch line. Micrographs (Nikon ACT-1; Nikon, Tokyo, Japan) were captured after creation of this defect and 4, 6, and 24 h later. Cell migration was measured using analysis software to document restitution (AlphaEase, Cell Biosciences, Inc, Santa Clara, CA). A standardized rectangle of interest was created and positioned at the scratch line, and the percentage of this region occupied by migrating cells was measured over time. Two fields were examined and measured on three different monolayers per treatment with NSAIDs.

Statistics

Statistical analysis was performed using a one-way *ANOVA* with a *post hoc* least significant difference test to determine significance ($p < 0.05$) with Statistix 7 software (Analytical Software, Tallahassee, FL).

Results

NSAIDs inhibit calpain activity

Previous experiments had demonstrated that total protein expression of calpains 1, 2, and 8 in IEC-6 cells were decreased following 72 h of treatment with indomethacin or NS-398 (Raveendran, et al., 2008). Therefore, we examined calpain activity following treatment with NSAIDs by measuring the fluorescence of the calpain-specific substrate, BOC-LM-CMAC. Figure 1A shows photomicrographs taken of BOC-LM-CMAC fluorescence in IEC-6 cells treated with vehicle control (0.1% DMSO), indomethacin (100 μ M), NS-398 (100 μ M), or SC-560 (1 μ M) for 48 h prior to analysis. A qualitative examination of the micrographs indicates that both indomethacin and NS-398 decrease BOC-LM-CMAC fluorescence, and calpain activity, after 48 h of treatment.

Subsequently, we performed quantitative analysis of the mean fluorescence of IEC-6 cells treated with NSAIDs for 6 (B), 12 (C), 24 (D), 48 (E), or 72 h (F). Treatment with NS-398 caused a significant decrease in calpain activity at all time points. Inhibition seemed to increase with increasing lengths of treatment with the drug (see Table 1 for summary fluorescence data). In contrast, indomethacin (Indo) initially inhibited calpain activity at 6 h, but IEC-6 cells seemed to recover by 12 h increasing calpain activity to higher than control levels. Calpain activity in the presence of indomethacin then decreased and hit its minimum somewhere between 24 and 48 h before returning to slightly higher than control levels at 72 h. Surprisingly, SC-560, despite previously having no effect on IEC-6 cell migration at 72 h (Raveendran, et al., 2008; Freeman, et al., 2007), significantly inhibited calpain activity as early as 6 h. In fact, at this time point, SC-560 caused greater inhibition of calpain activity than either indomethacin or NS-398, the two NSAIDs in this study that have significant ulcerogenic potential. Though activity was still less than that of control, fluorescence in the presence of SC-560 seemed to increase between the time points of 12 and 48 h, eventually recovering to activity levels higher than those of control at 72 h.

Similarly, we also examined the effects of ALLM, a known calpain inhibitor on calpain activity. Figure 2 shows the effects on calpain activity of applying concentrations (ranging from 1 to 100 μ M) of ALLM to IEC-6 cells grown on collagen for 24 h prior to assay. Our results showed that 1 μ M ALLM actually had a stimulatory effect on calpain activity, whereas 10, 50, and 100 μ M ALLM showed significantly increasing levels of inhibition of calpain activity.

NSAIDs decrease protein expression of calpain 2 in membranes at the substrata

The effects of indomethacin, NS-398, and SC-560 on calpain activity in IEC-6 cells led us to question where the deficits in calpain activity were occurring. Accordingly, we prepared membrane preparations of IEC-6 cells treated with vehicle control (0.1% DMSO), indomethacin (Indo, 100 μ M), or NS-398 (100 μ M) for 72 h. Probing of Western blots with antibodies specific to calpain 2 revealed that treatment with either indomethacin or NS-398 resulted in an ~40% decrease in calpain 2 protein expression in membrane fractions from IEC-6 cells (Fig. 3).

Decreases in calpain 2 protein expression in membrane fractions indicates that calpain 2 expression is reduced by NSAID exposure but does not identify any specific fraction which

may be particularly deficient in calpain 2 protein. Therefore, we used a cell de-roofing protocol that has been previously developed (Beer-Stolz and Jacobson, 1992) to remove the portion of the cell membrane not attached to the substrate. In this fashion, we could directly probe membranes involved in cell attachment for calpain 2 protein expression. Figure 4A shows photographs of IEC-6 cell footprints following the de-roofing procedure. Qualitative assessment of the footprints suggests that calpain 2 protein expression (green) is reduced by indomethacin and NS-398, but not SC-560 after 48 h of treatment. Furthermore, indomethacin and NS-398 also seem to alter the arrangement of actin filaments (orange) within the IEC-6 cells when compared with control. Quantitative analysis of calpain 2 fluorescence in footprints of IEC-6 cells (Fig. 4B) showed that indomethacin and NS-398 decrease calpain 2 expression by ~70%, whereas SC-560, surprisingly, decreases calpain 2 protein expression by ~30%.

NSAIDs inhibit cell migration

Our results demonstrated that NSAIDs and ALLM inhibit calpain activity in IEC-6 cells and NSAIDs decreased calpain 2 expression in membrane fractions. Accordingly, we wanted to relate these effects with inhibition of cell migration. Previous experiments established that indomethacin and NS-398, but not SC-560 inhibited cell migration after 72 h of treatment. Therefore, we examined the effects of these NSAIDs on cell migration after 24 (Fig. 5A) or 48 h (Fig. 5B) of treatment with indomethacin (100 μ M), NS-398 (100 μ M), SC-560 (1 μ M), or ALLM (25 μ M, 24 h treatment only). Cells were wounded and then migration was assessed for migration at 4 and 6 h post wounding (only data for 4 h is shown). Indomethacin or NS-398 each significantly inhibited cell migration at 4 and 6 h post wounding following 24 and 48 h of treatment with drug. ALLM also significantly inhibited cell migration after 24 h of treatment. The concentration of ALLM, 25 μ M, was chosen for this experiment because it should cause similar levels of inhibition of calpain activity as 100 μ M NS-398 at 24 h. Not surprisingly, the inhibition of cell migration caused by ALLM was significantly less than that of NS-398 in this experiment.

Surprisingly, SC-560 also caused significant inhibition of cell migration at both 24 and 48 h. This result is in line with our data showing SC-560 reduced calpain activity and calpain protein expression in IEC-6 footprints. However, it appears that the diminished cell migration caused by SC-560 is transient as cells treated with SC-560 for 48 h migrated farther than cells treated for 24 h. Furthermore, previous reports show that SC-560 has no effect on cell migration after 72 h of treatment (Raveendran, et al., 2008).

Discussion

Widespread use of NSAIDs in clinical settings to ameliorate pain and inflammation is accompanied by a range of negative side effects, most notably in the GI tract (Wallace and Vong, 2008; Wolfe, et al., 1999; Lichtenberger, 2001; Delco, et al., 2004). Numerous mechanisms of action have been proposed to mediate the toxic effects of NSAIDs on GI epithelia (Mahmud, et al., 1996; Somasundaram, et al., 2000; Ashton and Hanson, 2002), but of particular interest to our laboratory is the inhibition of epithelial wound healing by NSAIDs (Pai, et al., 2001; Rahgozar, et al., 2001; Raveendran, et al., 2008; Freeman, et al., 2007; Penney, et al., 1995; Schmassmann, et al., 1995). Previously, our laboratory showed that mRNA and total protein expression of calpains, a family of intracellular limited proteases, is reduced. Therefore, we sought to determine if calpain activity and membrane-associated expression of calpain 2 were affected by treatment with NSAIDs, providing a possible physiological mechanism through which these drugs inhibit cell migration and ultimately epithelial restitution in the GI tract.

NSAID-mediated toxicity appears to be related to the inhibition of migration of the GI epithelial lining thus preventing the restitution of small wounds. Evidence has accumulated that this is COX-independent, and correlated to changes in levels of the calpain proteases. Previously, we demonstrated that calpain mRNA and total protein expression were decreased by 72 h treatment with indomethacin or NS-398, but not SC-560, and that this was consistent with inhibition of cell migration by these drugs (Raveendran, et al., 2008). Furthermore, other laboratories had also demonstrated that NSAIDs reduce calpain protein levels (Fushimi, et al., 2004; Banik, et al., 2000). But as calpains have pleiotrophic effects on cells and distinct subcellular pools contribute to these, global assessments of calpains can be misleading. Thus we focused on the calpain fraction and isotype that affect cell motility. To our knowledge, this is the first report of NSAIDs affecting calpain activity and/or membrane-associated expression of calpain protein. Our results conclusively showed that NS-398 inhibited both calpain activity (acutely and chronically) and calpain expression in membrane fractions and cell footprints at all time points examined. Furthermore, this inhibition was also correlated with a decrease in cell migration observed at 24 and 48 h and previously at 72 h (Raveendran, et al., 2008).

Similarly, indomethacin decreased membrane-associated protein expression of calpain 2 following either 48 or 72 h of treatment and inhibited cell migration at 24 and 48 h and previously at 72 h (Raveendran, et al., 2008). Surprisingly, however, calpain activity was not consistently suppressed by indomethacin treatment. Instead, calpain activity varied from about 70% of control at 24 and 48 h to 110% of control at 72 h. The BOC-LM-CMAC method for measuring calpain activity is not specific to any one calpain isoform and is commonly thought to measure the activities of both calpain 1 and calpain 2 proteases. Thus, the discrepancies observed between migration and calpain 2 protein expression data compared to calpain activity may be associated with increased activation/expression of calpain 1 proteases as a result of downregulation of calpain 2 protein expression. Alternatively, it is possible that membrane-associated protein expression, not activity, is the primary limiting factor associated with inhibition of cell migration by indomethacin, even though treatment with ALLM and inhibition of calpain activity alone appears to be sufficient to inhibit cell migration.

Based on previous data, we did not expect SC-560 to suppress calpain activity or protein expression in our experiments (Raveendran, et al., 2008). Surprisingly, SC-560 was an effective inhibitor of calpain activity, membrane-associated calpain protein expression, and cell migration. The ability of SC-560 to cause these effects in IEC-6 cells, however, seems to be transient. Calpain activity, though significantly decreased compared to control from 6 to 48 h, appeared to recover throughout the experiment resulting in completely restored activity at 72 h. These results are consistent with both our migration data, showing inhibition of migration of IEC-6 cells after 24 and 48 h of treatment, and with previously published data showing no effect of SC-560 on cell migration after 72 h of treatment (Raveendran, et al., 2008). These data represent a significant difference between the NSAIDs with ulcerogenic potential (indomethacin and NS-398) and those without (SC-560) and may point the way to therapeutic strategies that can alleviate or avoid altogether GI complications associated with daily NSAID use.

The importance of calpains to the process of cell migration is well established. Calpain activity is controlled by a variety of signaling pathways and intracellular conditions and results in perpetration of signaling, rather than a degradative process (Glading, et al., 2002). Activation of calpain 2, in particular, is a complex and multifaceted process that may involve steps as diverse as phospholipid binding, autolysis, removal of calpastatin, and phosphorylation (Glading, et al., 2002; Perrin and Huttenlocher, 2002). Left to its own devices, calpain 2 requires millimolar concentrations of Ca^{2+} *in vitro* for activation (Satish,

et al., 2005; Glading, et al., 2002), which are not attainable intracellularly, except in cells in which apoptosis has been initiated. Instead, calpain 2 requires at least 3 important steps to occur prior to activation during cell migration: phosphorylation, membrane translocation, and binding to phospholipid. Calpain 2 is phosphorylated at serine 50 by activation of plasma membrane-bound EGF receptors (EGFR), and other receptor tyrosine kinases, and subsequent activation of the ERK/MAP kinase signaling cascade (Glading, et al., 2000; Glading, et al., 2001; Glading, et al., 2004; Smith, et al., 2003). The need for membrane bound EGFR, and membrane-localized ERK, highlights the necessary translocation of calpain 2 to the plasma membrane. Translocation of calpain 2 is accomplished by specific binding of calpain 2 to PIP₂ which is localized to the rear of cells during migration (Arthur and Crawford, 1996; Suzuki, 1992; Shao, et al., 2006; Chou, et al., 2002). Calpain 2 is then able to aid in cell attachment turnover, permitting rear retraction and forward locomotion. Incidentally, these and previous experiments examining cell migration following treatment with NSAIDs have shown that the morphology of migrating cells is consistent with retained rear attachments (Raveendran, et al., 2008; Freeman, et al., 2007). It would be of interest therefore, to determine the molecular mechanism by which NSAIDs reduce calpain activity and what effects these deficits have on the cleavage of targets of calpain activity, particularly those involved with cell adhesion.

In conclusion, our results indicate that calpain activity and membrane-associated calpain 2 protease expression are decreased as a result of treatment with NSAIDs. Furthermore, our results also indicate that chronic depression of calpain activity and/or membrane-associated calpain 2 protease expression may be a mechanism through which NSAIDs with ulcerogenic potential cause GI toxicity.

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References

- Arthur JSC, Crawford C. Investigation of the interaction of m-calpain with phospholipids: calpain-phospholipid interactions. *Acta Bioch Bioph.* 1996; 1293:201–06.
- Ashton M, Hanson PJ. Disparate effects of non-steroidal anti-inflammatory drugs on apoptosis in guinea pig gastric mucous cells: inhibition of basal apoptosis by diclofenac. *Br J Pharmacol.* 2002; 135:407–16. [PubMed: 11815376]
- Banik NL, Matzelle D, Terry E, Gantt-Wilford G, Hogan EL. Inhibition of proteolysis by a cyclooxygenase inhibitor, indomethacin. *Neurochem Res.* 2000; 25:1509–15. [PubMed: 11071371]
- Beer-Stolz D, Jacobson BS. Examination of transcellular membrane protein polarity of bovine aortic endothelial cells in vitro using the cationic colloidal silica microbead membrane-isolation procedure. *J Cell Sci.* 1992; 103:39–51. [PubMed: 1331135]
- Benyamin Y. The structural basis of calpain behavior. *FEBS J.* 2006; 273:3413–14. [PubMed: 16884486]
- Chaney LK, Jacobson BS. Coating cells with colloidal silica for high yield isolation of plasma membrane sheets and identification of transmembrane proteins. *J Biol Chem.* 1983; 258:10062–72. [PubMed: 6309765]
- Chou J, Beer-Stolz D, Burke NA, Watkins SC, Wells A. Distribution of gelsolin and phosphoinositol 4,5-bisphosphate in lamellipodia during EGF-induced motility. *Int J Biochem Cell B.* 2002; 34:776–90.
- Delco F, Michetti P, Beglinger C, Fried M, Szucs TD. Health care resource utilization and costs of NSAID-induced gastrointestinal toxicity. *Digestion.* 2004; 69:10–19. [PubMed: 14755148]
- Dignass AU. Mechanisms and modulation of intestinal epithelial repair. *Inflamm Bowel Dis.* 2001; 7:68–77. [PubMed: 11233665]

- Freeman LC, Narvaez DF, McCoy A, von Stein FB, Young S, Silver K, Ganta S, Koch D, Hunter R, Gilmour RF, Lillich JD. Depolarization and decreased surface expression of K⁺ channels contribute to NSAID-inhibition of intestinal restitution. *Biochem Pharmacol.* 2007; 74:74–85. [PubMed: 17499219]
- Fushimi K, Nakashima S, Banno Y, Akaike A, Takigawa M, Shimizu K. Implication of prostaglandin E(2) in TNF alpha-induced release of m-calpain from HCS-2/8 chondrocytes: inhibition of m-calpain release by NSAIDs. *Osteoarthritis Cartilage.* 2004; 12:895–903.
- Glading A, Bodnar RJ, Reynolds IJ, Shiraha H, Satish L, Potter DA, Blair HC, Wells A. Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. *Mol Cell Biol.* 2004; 24:2499–512. [PubMed: 14993287]
- Glading A, Chang P, Lauffenburger DA, Wells A. Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. *J Biol Chem.* 2000; 275:2390–98. [PubMed: 10644690]
- Glading A, Lauffenburger DA, Wells A. Cutting to the chase: calpain proteases in cell motility. *Trends Cell Biol.* 2002; 12:46–54. [PubMed: 11854009]
- Glading A, Uberall F, Keyse S, Lauffenburger DA, Wells A. Membrane Proximal ERK signaling is required for m-calpain activation downstream of epidermal growth factor receptor signaling. *J Biol Chem.* 2001; 276:23341–48. [PubMed: 11319218]
- Israel LH, Koea JB, Stewart ID, Wright CL, Frankish PD. Nonsteroidal anti-inflammatory drug-induced strictures of the colon. *Dis Colon Rectum.* 2001; 44:1362–64. [PubMed: 11584216]
- Karcher LF, Dill SG, Anderson WI, King JM. Right dorsal colitis. *J Vet Intern Med.* 1990; 4:247–53. [PubMed: 2262926]
- Lebart M-C, Benyamin Y. Calpain involvement in the remodeling of cytoskeletal anchorage complexes. *FEBS J.* 2006; 273:3415–26. [PubMed: 16884487]
- Lichtenberger LM. Where is the evidence that cyclooxygenase inhibition is the primary cause of nonsteroidal anti-inflammatory drug (NSAID)-induced gastrointestinal injury? Topical injury revisited. *Biochem Pharmacol.* 2001; 61:631–37. [PubMed: 11266647]
- Lokuta MA, Nuzzi PA, Huttenlocher A. Calpain regulates neutrophil chemotaxis. *Proc Nat Acad Sci.* 2003; 100:4006–11. [PubMed: 12649322]
- MacAllister CG. Effects of toxic doses of phenylbutazone in ponies. *Am J Vet Res.* 1983; 44:2277–79. [PubMed: 6660616]
- MacAllister CG, Morgan SJ, Borne AT, Pollet RA. Comparison of adverse effects of phenylbutazone, flunixin meglumine, and ketoprofen in horses. *J Am Vet Med Assoc.* 1993; 202:71–77. [PubMed: 8420909]
- Mahmud T, Rafi SS, Scott DL, Wrigglesworth JM, Bjarnason I. Nonsteroidal antiinflammatory drugs and uncoupling of mitochondrial oxidative phosphorylation. *Arthritis Rheum.* 1996; 39:1998–2003. [PubMed: 8961904]
- Neiger R. NSAID-induced gastrointestinal adverse effects in dogs - can we avoid them? *J Vet Intern Med.* 2003; 17:259–61. [PubMed: 12774964]
- Oren R, Ligumsky M. Indomethacin-induced colonic ulceration and bleeding. *Ann Pharmacother.* 1994; 28:883–85. [PubMed: 7949507]
- Pai R, Szabo IL, Giap AQ, Kawanaka H, Tarnawski AS. Nonsteroidal anti-inflammatory drugs inhibit re-epithelialization of wounded gastric monolayers by interfering with actin, Src, FAK, and tensin signaling. *Life Sci.* 2001; 69:3055–71. [PubMed: 11758831]
- Penney AG, Malcontenti-Wilson C, O'Brien PE, Andrews FJ. NSAID-induced delay in gastric ulcer healing is not associated with decreased epithelial cell proliferation in rats. *Digest Dis Sci.* 1995; 40:2684–93. [PubMed: 8536532]
- Perrin BJ, Huttenlocher A. Calpain. *Int J Biochem Cell B.* 2002; 34:722–25.
- Rahgozar M, Pazokitoroudi H, Bakhtiarian A, Djahanguiri B. Diazoxide, a K_ATP opener, accelerates restitution of ethanol or indomethacin-induced gastric ulceration in rats independent of polyamines. *J Gastroen Hepatol.* 2001; 16:290–96.
- Raveendran NN, Silver K, Freeman LC, Narvaez DF, Weng K, Ganta S, Lillich JD. Drug-induced alterations to gene and protein expression in intestinal epithelial cell 6 cells suggest a role for

- calpains in the gastrointestinal toxicity of nonsteroidal anti-inflammatory agents. *J Pharmacol Exp Ther.* 2008; 325:389–99. [PubMed: 18281595]
- Rosser BG, Powers SP, Gores GJ. Calpain activity increases in hepatocytes following addition of ATP: Demonstration by a novel fluorescent approach. *J Biol Chem.* 1993; 268:23593–600. [PubMed: 8226886]
- Satish L, Blair HC, Glading A, Wells A. Interferon-inducible protein 9 (CXCL11)-induced cell motility in keratinocytes requires calcium flux-dependent activation of μ -Calpain. *Mol Cell Biol.* 2005; 25:1922–41. [PubMed: 15713646]
- Schmassmann A, Tarnawski AS, Peskar BM, Varga L, Flogerzi B, Halter F. Influence of acid and angiogenesis on kinetics of gastric ulcer healing in rats: interaction with indomethacin. *Am J Physiol.* 1995; 268:G276–G85. [PubMed: 7532364]
- Shao H, Chou J, Baty C, Burke NA, Watkins SC, Beer-Stolz D, Wells A. Spatial localization of m-calpain to the plasma membrane by phosphoinositide biphosphate binding during epidermal growth factor receptor-mediated activation. *Mol Cell Biol.* 2006; 26:5481–96. [PubMed: 16809781]
- Smith SD, Jia Z, Huynh KK, Wells A, Elce JS. Glutamate substitutions at a PKA consensus site are consistent with inactivation of calpain by phosphorylation. *FEBS Lett.* 2003; 542:115–18. [PubMed: 12729909]
- Somasundaram S, Sigthorsson G, Simpson RJ, Watts J, Jacob M, Tavares IA, Rafi S, Roseth A, Foster R, Price AB, Wrigglesworth JM, Bjarnason I. Uncoupling of intestinal mitochondrial oxidative phosphorylation and inhibition of cyclooxygenase are required for the development of NSAID-enteropathy in the rat. *Aliment Pharm Ther.* 2000; 14:639–50.
- Suzuki K. Modulation of cellular signals by calpain. *Ann Proc NY Acad Sci.* 1992; 674:218–27.
- Wallace JL, Vong L. NSAID-induced gastrointestinal damage and the design of GI-sparing NSAIDs. *Curr Opin Invest Dr.* 2008; 9:1151–56.
- Wolfe MM, Lichtenstein DR, Singh G. Gastrointestinal toxicity of nonsteroidal anti-inflammatory drugs. *New Engl J Med.* 1999; 341:1397–99.

Abbreviations

NSAIDs	non-steroidal anti-inflammatory drugs
GI	gastrointestinal
COX	cyclooxygenase
IEC	intestinal epithelial cells
SC-560	5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole
NS-398	N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide
BOC-LM-CMAC	<i>tert</i> -butoxycarbonyl-Leu-Met-chloromethylaminocoumarin
ALLM	N-acetyl-Leu-Leu-methioninal
DMSO	dimethylsulfoxide

Figure 1.

Inhibition of calpain activity by NSAIDs. Micrographs were taken of BOC-LM-CMAC fluorescence in IEC-6 cells cultured on collagen following 48 h of NSAID treatment (A). Calpain activity was assessed in IEC-6 cells following 6 (B), 12 (C), 24 (D), 48 (E), or 72 h (F) of treatment with vehicle control (0.1% DMSO), indomethacin (Indo, 100 μ M), NS-398 (100 μ M), or SC-560 (1 μ M). * indicates a statistically significant difference from control ($p < 0.05$).

Figure 2. Calpain activity measured by BOC-LM-CMAC fluorescence in IEC-6 cells treated with a range of concentrations of the calpain inhibitor, ALLM (24 h). * indicates a statistically significant difference from control ($p < 0.05$).

Figure 3.

Calpain 2 protein expression in membrane fractions isolated from IEC-6 cells treated for 72 h with vehicle control (0.1% DMSO), indomethacin (Indo, 100 μ M), NS-398 (100 μ M), or SC-560 (1 μ M). * indicates a statistically significant difference from control ($p < 0.05$).

Figure 4.

Calpain 2 protein expression in IEC-6 cell footprints. Micrographs (A) of cells treated for 48 h with vehicle control (0.1% DMSO), indomethacin (Indo, 100 μ M), NS-398 (100 μ M), or SC-560 (1 μ M). Calpain 2 is in green, actin is in orange, and cell nuclei were stained with DAPI (blue). Absence of blue fluorescence confirms that cells were properly de-roofed. Photos were analyzed for calpain 2 expression (B). * indicates a statistically significant difference from control ($p < 0.05$).

Figure 5.

Inhibition of IEC-6 cell migration by inhibitors of calpain activity. IEC-6 cells were treated with indomethacin (100 μ M), NS-398 (100 μ M), SC-560 (1 μ M) or ALLM (50 mM) for 24 (A) or 48 h (B) prior to wounding. Migrating cells were then photographed at 4 h post wounding and analyzed by measuring the fraction of a specified area covered by migrating cells. Results were normalized to control and presented as mean $\pm$ se. * indicates a statistically significant difference from control ($p < 0.05$).

Table 1

Effects of NSAIDs on calpain activity in IEC-6 cells.

	6 h	12 h	24 h	48 h	72 h
Control	1.0 ± 0.02	1.0 ± 0.02	1.0 ± 0.02	1.0 ± 0.01	1.0 ± 0.02
Indo	0.86 ± 0.02 *	1.15 ± 0.01 *	0.70 ± 0.01 *	0.76 ± 0.02 *	1.04 ± 0.02 *
NS-398	0.78 ± 0.01 *	0.62 ± 0.01 *	0.52 ± 0.01 *	0.56 ± 0.01 *	0.52 ± 0.01 *
SC-560	0.64 ± 0.01 *	0.81 ± 0.01 *	0.75 ± 0.01 *	0.94 ± 0.02 *	1.24 ± 0.02 *

Values are presented as relative fluorescence units ± se.

* denotes a significant difference ($p < 0.05$) from control