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Suppression of Calpain Expression by NSAIDs is Associated with Inhibition of Cell Migration in Rat Duodenum

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the alleviation of pain and inflammation, but these drugs are also associated with a suite of negative side effects. Gastrointestinal (GI) toxicity is particularly concerning since it affects an estimated 70% of individuals taking NSAIDs routinely, and evidence suggests the majority of toxicity is occurring in the small intestine. Traditionally, NSAID-induced GI toxicity has been associated with indiscriminate inhibition of cyclooxygenase isoforms, but other mechanisms, including inhibition of cell migration, intestinal restitution, and wound healing, are likely to contribute to toxicity. Previous efforts demonstrated that treatment of cultured intestinal epithelial cells (IEC) with NSAIDs inhibits expression and activity of calpain proteases, but the effects of specific inhibition of calpain expression *in vitro* or the effects of NSAIDs on intestinal cell migration *in vivo* remain to be determined. Accordingly, we examined the effect of suppression of calpain protease expression with siRNA on cell migration in cultured IECs and evaluated the effects of NSAID treatment on epithelial cell migration and calpain protease expression in rat duodenum. Our results show that calpain siRNA inhibits protease expression and slows migration in cultured IECs. Additionally, NSAID treatment of rats slowed migration up the villus axis and suppressed calpain expression in duodenal epithelial cells. Our results are supportive of the hypothesis that suppression of calpain expression leading to slowing of cell migration is a potential mechanism through which NSAIDs cause GI toxicity.

Keywords

NSAID; cell migration inhibition; bromodeoxyuridine; calpain; duodenum

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the alleviation of pain and inflammation associated with injury or disease. Millions of prescriptions are written and

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billions of tablets are sold over the counter every year (Wolfe et al. 1999) with as many as 60 million people in the United States taking NSAIDs regularly (Dai et al. 2005). Unfortunately, the widespread use of NSAIDs is also associated with a suite of negative side effects, particularly renal, cardiac, or gastrointestinal (GI) toxicity.

NSAID-induced GI toxicity is of particular concern given the rate at which NSAID users suffer symptoms ranging from dyspepsia to erosion and ulceration or even death (Israel et al. 2001; Karcher et al. 1990; Lichtenberger 2001; MacAllister et al. 1993). Estimates of the incidence of significant NSAID-induced GI toxicity range from 50 to 70% of chronic NSAID users, with the vast majority of individuals having subclinical toxicity (Bjarnason et al. 1993; Graham et al. 2005; Maiden et al. 2005; Park et al. 2011). Most of the clinical and experimental efforts have traditionally focused on ameliorating gastroduodenal damage, but significant toxicity also occurs in the small intestine even when common gastroduodenal protective measures, such as using NSAIDs selective for inhibition of isoform 2 of cyclooxygenase (COX), co-treatment with proton pump inhibitors (PPI), pro-NSAIDs, etc., are employed (Kanbayashi and Konishi 2015; McCarthy 2009; Satoh et al. 2012, 2014; Wallace 2013). In fact, use of some of these protective measures are associated with increased damage in the small intestine (Wallace 2013), and newer protective modalities, such as inhibitors of monoacylglycerol lipase, fatty acid amide hydrolase, and soluble epoxide hydrolase (Goswami et al. 2016; Kinsey et al. 2011; Sasso et al. 2015), are under development.

The ability of NSAIDs to attenuate inflammation has traditionally been attributed to inhibition of one or both of the isoforms of COX, COX 1 or COX 2 (the former being constitutive and the latter being inducible, particularly during inflammation; Lichtenberger 2001; Radi 2009). COX enzymes are necessary components of the arachidonic acid pathway, converting arachidonic acid into prostaglandins (PG), which not only mediate inflammation but also are necessary for cellular homeostasis and exert a protective effect on the GI (Radi 2009). NSAIDs are typically characterized as either selective to one COX isoform or the other, or non-selective (Fendrick and Greenberg 2009), and it has traditionally been held that non-selective inhibition of COX enzymes is responsible for observed GI toxicity (Fendrick and Greenberg 2009; Lichtenberger 2001). However, as has been noted above, toxicity as a result of NSAID treatment is observed in the small intestine even with the use of COX-2 selective NSAIDs (Kanbayashi and Konishi 2015; McCarthy 2009). Further, mice genetically engineered to silence COX 1 expression are perfectly healthy under normal conditions, not showing any untoward gastric toxicity (Langenbach et al. 1995). Though inhibition of COX remains important in evaluating the toxic effects of NSAIDs on GI epithelia, other mechanisms have also been proposed to contribute to GI toxicity that may be independent of inhibition of COX activity, including altering intestinal microflora, decreasing apoptosis, depolarizing membrane potential, uncoupling of oxidative phosphorylation (mitochondrial toxicity), interfering with neutrophil recruitment, and inhibiting cell migration/epithelial restitution (Ashton and Hanson 2002; Freeman et al. 2007; Mahmud et al. 1996; Pai et al. 2001; Penney et al. 1995; Raveendran et al. 2008; Schmassmann et al. 1995; Silver et al. 2012; Silver et al. 2010; Silver et al. 2015; Somasundaram et al. 2000).

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Epithelial restitution is an important repair modality in the GI tract that is wholly dependent on cell migration (initially) and functions to restore wounds in the epithelial barrier within minutes or hours of the damaging event (Dignass 2001; Sturm and Dignass 2008). In addition to wound healing, cell migration also permits movement of maturing cells from the intestinal crypts, where proliferation occurs, up the long axis of the villi to the apex to replace cells that have undergone apoptosis and sloughed into the lumen (Han et al. 1993; Onishi et al. 2007; Qi et al. 2009; Shibahara et al. 1995; Solanas and Batlle 2011; Takeuchi et al. 1998, 1999). Cell migration in cultured intestinal epithelial cells (IEC) is inhibited by treatment with NSAIDs with ulcerogenic potential through a variety of affected targets or pathways, including depolarizing membrane potential, inhibiting voltage-gated potassium channel expression, and suppressing calpain protease expression and activity (Freeman et al. 2007; Pai et al. 2001; Penney et al. 1995; Rahgozar et al. 2001; Raveendran et al. 2008; Silver et al. 2012; Silver et al. 2010; Silver et al. 2015). Interestingly, in these experiments, an NSAID with low ulcerogenic potential, SC-560, had no inhibitory effect on IEC migration.

Calpains are a family of cysteine proteases that play diverse physiological roles, including in proliferation, signal transduction, blood clotting cascades, rearranging cytoskeletal components, and cell migration (Benyamin 2006; Glading et al. 2002; Gora and Latajka 2015; Lebart and Benyamin 2006; Leloup and Wells 2011; Lokuta et al. 2003; Moretti et al. 2014; Perrin and Huttenlocher 2002). Most relevant to the current study, previous efforts show that NSAID treatment inhibits calpain protease activity by suppressing total and plasma membrane protein expression and/or depolarizing membrane potential in cultured rat intestinal epithelial cells (IEC-6; Raveendran et al. 2008; Silver et al. 2010; Silver et al. 2015). Furthermore, inhibition of calpain activity by ALLM, or calpain inhibitor II (N-acetyl-L-leucyl-N-[(1S)-1-formyl-3-(methylthio)propyl]-L-leucinamide), results in a dose-dependent inhibition of cell migration in rat IEC-6 cells (Silver et al. 2010). Interestingly, the effects of specific inhibition of expression of calpain 1 or 2 proteases (pharmacological interventions with NSAIDs or ALLM have wider effects than just on calpain proteases) on IEC migration and wound healing is yet to be determined.

Similarly, though the effects of NSAIDs on cell migration in cultured cells or in *ex vivo* epithelial layers has been examined, there is currently still a lack of information on the effects of NSAID treatment on migration of epithelial cells *in vivo* in intact small intestine. Accordingly, this study was designed to fill these gaps in our collective knowledge. Rates of cell migration in small intestine up the intestinal villi have only recently been characterized in the rat by pulse labeling proliferating enterocytes with bromodeoxyuridine (BrdU; Qi et al. 2009), and we employed this information in our experimental design. Rats were orally treated with indomethacin or NS-398 for 72 h. In addition, rats were also pulsed once with BrdU at specific time points (12, 18, or 24 h) prior to euthanasia. Analysis of sections of duodenum revealed slight morphometrical changes in villus structure following treatment with indomethacin as well as significant inhibition (20 – 25%) of cell migration up the villus axis following treatment with either indomethacin or NS-398. Further, samples of duodenal mucosa subjected to gene and protein expression analysis revealed that expression of calpain 1 or 2 proteases is significantly reduced by NSAID treatment. Also, use of siRNA techniques to specifically suppress expression of calpain 1 or 2 proteases in cultured IEC-6

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cells inhibited cell migration in a traditional scratch assay in addition to suppressing calpain expression. Our results show that inhibition of calpain 1 or 2 protease expression slows cell migration in IEC-6 cells, and that treatment with NSAIDs suppresses migration of epithelial cells up the villus axis in rat duodenum, an effect that is associated with reductions in the expression of calpain 1 and 2 proteases. Our results provide a potential mechanism through which this suppression of migration may occur (through inhibition of calpain protease expression and/or activity) which is consistent with our other results *in vitro* and previously published research on the effects of NSAIDs in cultured intestinal epithelial cells (Raveendran et al. 2008; Silver et al. 2010; Silver et al. 2015).

2. Materials and Methods

2.1 Reagents

Reagents necessary for these experiments were acquired from commercial sources. Dulbecco's Modified Eagles Media (DMEM) and the IEC-6 cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Fetal bovine serum, gentamicin, and insulin used to supplement the DMEM as well as BOC-LM-CMAC (7-amino-4-chloromethylcoumarin, tert-butoxycarbonyl-L-leucyl-L-methionine amide) for calpain activity assays were purchased from Life Technologies (Grand Island, NY, USA). NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) and ALLM (N-acetyl-L-leucyl-N-[(1S)-1-formyl-3-(methylthio)propyl]-L-leucinamide) were purchased from Cayman Chemical (Ann Arbor, MI, USA), whereas indomethacin and bromodeoxyuridine (BrdU) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Animal studies

Sixty 12-week old male Wistar rats (~350 g each) were kept under standard laboratory conditions with a 12 hour light/dark cycle and free access to food and water. Rats were randomly divided into three treatment groups (control, indomethacin, or NS-398) and then further subdivided into groups of 5 individuals each per treatment group per time point. Indomethacin and NS-398 were dissolved in dimethyl sulfoxide (DMSO), which was then diluted in saline to the appropriate concentration prior to use. Rats were weighed treated with vehicle control (DMSO in saline) or 10 mg/kg indomethacin or NS-398 by gavage once daily for 72 h. At specific time intervals prior to euthanasia (6, 12, 18, or 24), rats were given an intraperitoneal injection of BrdU (50 mg/kg) in saline as has previously been described and employed for measuring migration of enterocytes (Qi et al. 2009). Rats were euthanized by asphyxiation with CO_2 and small intestinal tissue samples were taken from duodenum. These experiments were performed in accordance with and with approval from the Institutional Animal Care and Use Committee at Kansas State University.

2.3 Histopathology and Histomorphometry

Whole round sections of small intestine were collected from duodenum and fixed in 10% formalin. Additionally, sections of duodenum were also removed from rats, sliced open with surgical scissors, stapled to a rigid support, and fixed in 10% formalin. These samples were transferred to the Kansas State University Veterinary Diagnostic Laboratory (KSVDL) for routine histological processing and paraffin embedding. Deparaffinized sections were

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stained with haematoxylin and eosin. Immunohistochemical staining was performed on formalin fixed paraffin-embedded tissues that were sectioned at 4 μm thickness onto positively charged slides. Slides were stained using the Leica Bond-Max autostainer (Leica Biosystems, Buffalo Grove, IL) with the Polymer Refine Detection kit (Leica Biosystems). The anti-BrdU primary antibody was diluted with Bond Primary Antibody Diluent (tris-buffered saline) to 1:100. Epitope retrieval was done with a proteinase K enzyme digestion at 37°C for 10 minutes. Tissue sections were incubated with the primary antibody for 15 min at ambient temperature. Polymerization was performed with Powervision Poly-HRP α-Mouse polymer (Leica Biosystems) for 25 min at ambient temperature. Visualization was done with DAB for the chromogen and counterstained with hematoxylin.

For measurement of crypt and villus length, 10 complete crypts or villi from at least two slides per animal were measured. Individual measurements from each animal were averaged to provide a median crypt or villus length per animal. These average crypt or villus lengths from 16 to 19 individual animals per NSAID treatment group were combined to generate an overall mean length of crypts and villi for each treatment. Cell migration was evaluated by measuring the maximum distance anti-BrdU stained cells had migrated up the crypt to villus axis on 10 individual villi from at least two slides per animal. The fractional migration distance was then calculated as the ratio of the average maximum distance cells had traveled up the crypt to villus axis versus the average crypt to villus length (calculated as the sum of the average crypt and villus lengths for each treatment group).

2.4 Gene Expression

Mucosal scrapings were taken from duodenum sections using a scalpel blade and stored in RNALater (Qiagen, Valencia, CA, USA) at −80 °C until time to harvest RNA. RNA was isolated (five rats per treatment group) using TRIzol reagent (Life Technologies) in conjunction with a commercial kit (Qiagen). DNase I was used to treat samples and ensure that no genomic DNA was contaminating our samples. RNA samples were quantified on a Nanodrop Spetrophotometer (Fisher Scientific, Pittsburgh, PA, USA) and subject to one step quantitative RT-PCR on a StepOne Plus real-time PCR system (Fisher Scientific) using SYBR green as the reporter dye (Superscript III Platinum Taq one-step qRT-PCR kit, Fisher Scientific). Gene specific primers for calpain 1 and 2 (used previously; Raveendran et al. 2008), 18S, and GAPDH were as follows: calpain 1 forward 5′-tatcctccaccgagtggttc-3′ and reverse 5′-tccttggtgggtagcaaatc-3′; calpain 2 forward 5′-tgccccagctggaacacg-3′ and reverse 5′-caggtgagggtgtccgg-3′; 18S forward 5′-tcgctccaccaactaagaac-3′ and reverse 5′-gaggttcgaagacgatcaga-3′; and GAPDH forward 5′-gacatgccgcctggagaaac-3′; and reverse 5′-agcccaggatgccctttagt-3′. Thermal cycling conditions were: 50 °C for 30 min, 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 78 °C for 30 s, with fluorescence recorded during the 78 °C step. Relative expression of calpain 1 or 2 mRNA was then calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001) using 18S and GAPDH as controls. Amplification efficiencies were calculated for all primer pairs using serial dilutions of template and found to be similar (~95%). The geometric mean of the resulting relative fold changes in expression versus 18S and GAPDH were then calculated and presented as the fold change in expression for calpain 1 or 2. Products from each primer pair were examined after separation on an agarose gel and sequenced to confirm the

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presence of a single PCR product of the correct size and sequence. qRT-PCR experiments were performed using four to five biological replicates (rats) per treatment and two technical replicates per sample.

2.5 Protein Expression

Mucosal scrapings from rat duodenum were collected and stored at −80 °C until isolation of whole cell lysates. At harvest time, scrapings were immersed in cold commercial lysis buffer containing protease inhibitors (RIPA lysis buffer, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and sonicated. Lysate concentrations were assayed by measuring absorbance at 562 nm using a standard BCA assay (Thermo Scientific). SDS acrylamide gels were used to separate lysates and subsequently transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Prior to probing with calpain 1 or 2-specific antibodies, membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (Bio-Rad) supplemented with 0.1% Tween-20 (Sigma-Aldrich). Primary antibodies directed to calpain 1 and 2 (1:1000, Abcam, Cambridge, MA, USA) and actin (1:2000, Sigma-Aldrich) as well as secondary antibodies conjugated to horse radish peroxidase (Santa Cruz Biotechnology) were obtained commercially. Enhanced chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific) was used to detect protein-specific immunocomplexes, and equal loading was evaluated by stripping and re-probing membranes with anti-actin antibody. Bands underwent densitometry analysis using imaging software (AlphaEaseFc, AlphaInnotech, San Leandro, CA), and calpain 1 or 2 expression was first normalized to actin, and then compared to control. The calpain 1 antibody specifically detects 2 bands for calpain 1 at 60 and 80 kDa (both are shown in the calpain 1 protein expression figures). The 80 kDa band is the complete, functional calpain 1 protein and was used to quantify calpain 1 protein expression for comparison between treatments, whereas the 60 kDa band represents a partially cleaved calpain 1 protein. Immunoblotting experiments were replicated at least four times, and summary results of densitometry measurements in immunoblotting experiments represent analysis of lysates from four to seven individuals.

2.6 Cell culture and siRNA experiments

A crypt-like intestinal cell line, IEC-6 (Quaroni et al. 1979), derived from rat duodenal epithelia was grown as previously described (Freeman et al. 2007; McCormack and Johnson 2001; Raveendran et al. 2008; Silver et al. 2012; Silver et al. 2010; Silver et al. 2015) on culture-treated plastic in DMEM (supplemented with 5% fetal bovine serum, 10 μg/mL insulin, and 50 μg/mL gentamicin) at 37 °C with 5% CO_2. Only passages 15–20 were used in these experiments.

For silencing of gene expression experiments, pools of four siRNA sequences specific to calpain 1 or calpain 2 as well as a pool of non-targeting sequences were purchased commercially (Dharmacon GE Biosciences, Lafayette, CO). IEC-6 cells (1.5×10^6 cells per reaction) were nucleofected with 40 pmol of siRNA using the Cell Line Nucleofector Kit V and program U-031 on a Nucleofector 2b Device (Lonza, Walkersville, MD) and seeded onto 60 mm collagen I-coated plates. In dual siRNA experiments, cells were treated with 80 pmol (40 pmol of each of sicalpain 1 or 2 or 80 pmol of non-targeting siRNA). Control

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experiments indicated that nucleofection efficiency was 75% at 48 h or 90% at 24 h. Cells were incubated for 48 h at 37 °C following nucleofection, after which migration assays were performed and protein expression was evaluated. Additionally, cells were monitored visually for signs of cell death or toxicity, and no differences were observed between treatments with non-targeting or calpain specific siRNAs in this regard.

2.7 Migration Assays

Migration assays were performed as previously described (Freeman et al. 2007; Raveendran et al. 2008; Silver et al. 2010; Silver et al. 2015). Cells were seeded onto 60 mm collagen I-coated plates, and then treated for 48 h with indomethacin (100 μM) or 0.1% DMSO as vehicle control, or for 24 h with ALLM (100 μM, calpain protease inhibitor II) or 0.1% ethanol (EtOH) as vehicle control. These concentrations of indomethacin or NS-398 are consistent with previous *in vitro* studies examining the effects of NSAIDs on cell migration of cultured epithelial cells (Freeman et al. 2007; Pai et al. 2001; Raveendran et al. 2008; Silver et al. 2012; Silver et al. 2010; Silver et al. 2015). Additionally, 100 μM NS-398 has high inhibitory activity on COX 2, but little to no effect on COX 1 activity at this concentration (Futaki et al. 1994; Gierse et al. 1995). Cells treated with siRNA were incubated 48 h on 60 mm or 35 mm collagen I-coated plates prior to assay as stated above. Monolayers were then wounded by scraping a razor blade across the surface of the plate creating a scratch line and removing roughly one-quarter of the monolayer. Immediately after wounding, plates were photographed (time = 0 h) to ensure the quality of the scratch lines. Subsequently, photographs of migrating cells were also taken at 6 h post wounding. Photographs were then evaluated to quantify the percentage of a standardized region of interest (600 μm^2) placed on the scratch line occupied by migrating cells. Results were then normalized to the appropriate control (0.1% DMSO for indomethacin, 0.1% EtOH for ALLM, or non-targeting siRNA for calpain siRNAs). Three separate fields were photographed per plate and the experiment was repeated four times.

2.8 Calpain Activity Assays

Evaluation of calpain activity was performed in live IEC-6 cells using the fluorescent 7-amino-4-chloromethylcoumarin, tert-butoxycarbonyl-L-leucyl-L-methionine amide substrate (BOC-LM-CMAC; Rosser et al. 1993) as has been previously published (Glading et al. 2000; Silver et al. 2010). Briefly, cells were cultured in optical quality, collagen-coated 35 mm plates (Matek, Ashland, MA) for 48 h after nucleofection or for 48 h during treatment with NSAIDs (100 μM indomethacin or NS-398). Cells were washed twice with 1X phosphate buffered saline (PBS), then treated with 25 μM BOC-LM-CMAC in 1X PBS for 20 min. Cells were then washed two more times with PBS and imaged on a Zeiss 700 Confocal microscope (Carl Zeiss AG, Oberkochen, Germany) using the 40X objective under oil. The Zen software (v2.3 Black, Carl Zeiss AG) and ImageJ (National Institutes of Health) were used to evaluate images and process them for publication. Six to eight images were taken per plate and experiments were repeated three times.

2.9 Statistical Analysis

Significant differences between treatment groups were determined using a one-way analysis of variance (ANOVA) with a *post hoc* least significant difference test when there were two

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or more treatment groups to compare to control. Alternatively, a Student's *t*-test was used when there were only two groups to compare (e.g. 0.1% DMSO versus 100 μM indomethacin migration).

3. Results

3.1 Silencing of calpain expression inhibits cell migration

Monolayers of cultured IEC-6 cells were treated with non-targeting, calpain 1, or calpain 2 siRNA for 48 h, or indomethacin (100 μM, 48 h) or ALLM (calpain inhibitor II, 100 μM, 24 h), then wounded with a razor blade, and cell migration was measured 6 h later. Our results clearly show that treatment with indomethacin, ALLM, or calpain 1 or 2 siRNA are sufficient to significantly inhibit IEC-6 cell migration (Fig. 1). Interestingly, indomethacin and ALLM, non-specific inhibitors of calpain expression or activity (Raveendran et al. 2008; Silver et al. 2010), reduced cell migration slightly more than specifically inhibiting protein expression of either calpain 1 or calpain 2. Subsequent analysis of whole cell lysates from these migration experiments showed that siRNA silencing was specific, with siCapn1 silencing calpain 1 protein expression by nearly 60% without affecting calpain 2 expression, and siCapn2 reducing calpain 2 protein expression also by 60% without affecting calpain 1 expression (Figs. 2A and B). ALLM had no effect on calpain 1 or calpain 2 protein expression (data not shown).

Measurement of calpain protease activity following treatment with siRNA revealed some surprising results, however. Indomethacin and NS-398 both caused significant reductions in fluorescence associated with cleavage of the BOC-LM-CMAC substrate by calpain proteases (Figs. 3A–C), as has previously been shown (Silver et al. 2010). However, treatment with siRNA complementary to calpain 1 or 2 had no significant effect on BOC-LM-CMAC fluorescence (Figs. 3D–F), suggesting that this method is not sensitive enough to detect the changes in calpain activity associated with inhibition of only 1 protease at a time, even though it is sufficient to disrupt cell migration.

Subsequent experimentation was performed also evaluate the effects of inhibition of both calpain 1 and 2, a situation more similar to treatment with NSAIDs, using siRNA. The dual silencing experiments resulted in 60% suppression of calpain 1 and 2 protein expression (data not shown), similar to the individual silencing experiments. Further, treatment with sicalpain 1 and 2 resulted in significant (40%) suppression of IEC-6 cell migration after wounding (Figs. 4A–C); nearly exactly the sum of inhibition of migration observed when calpain 1 and 2 were silenced individually (Figs. 1G and 4C). Interestingly though, suppression of calpain 1 and 2 protease expression did not significantly reduce BOC-LM-CMAC fluorescence (Figs. 4C and E).

3.2 Pathological effects on duodenal villi

Gross examination of small intestines from rats treated with NSAIDs for 72 h revealed multiple erosions visible on the mucosal surface of the duodenum with indomethacin or NS-398 treatment that were absent in control tissues (data not shown). In addition, sections of duodenum obtained from these rats were examined for morphometric and pathological

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effects resulting from NSAID treatment. This examination revealed that indomethacin, but not NS-398, slightly, yet significantly decreased both crypt (Figs. 5A and B) and villus (Figs. 6A and B) length following 72 h of treatment.

3.3 NSAIDs inhibit duodenal cell migration

During the course of NSAID treatment, rats were also given a single pulse of BrdU at specific time points (6, 12, 18, and 24 h) prior to euthanasia in an effort to understand the effects of treatment with these NSAIDs on cell migration in rat duodenum *in vivo*. Sections of duodenum from these rats were then fixed in formalin, embedded in paraffin, and later sectioned for immunohistochemistry (two slides per rat; three to five rats per time point per treatment group). Subsequent staining with an anti-BrdU antibody allowed us to compare the extent of migration of labeled cells as a fraction of the crypt-villus axis length as a function of time during treatment with either indomethacin or NS-398 (Figs. 7A and B). Labeling with BrdU at 6 h (data not shown) and 12 h prior to euthanasia revealed no significant difference in migration between control and NSAID treatment groups (Fig. 7C); however, at 18 and 24 h, our analysis revealed a significant reduction (20–25%) in cell migration in indomethacin and NS-398-treated rats (Fig. 7C).

3.4 NSAIDs suppress calpain protease expression in rat duodenum

Following measurement of migration rates of enterocytes up the villus axis, muosal scrapings from rat duodenum were subject to gene and protein expression analysis. NSAID treatment had no significant effect on gene expression of calpain 1 (Fig. 8A) or 2 (Fig. 8B), but indomethacin or NS-398 reduced protein expression of calpain 1 (Fig. 9A) by 30 and 40%, repectively, and calpain 2 (Fig. 9B) by 20 and 50%, respectively.

4. Discussion

This is the first report to document inhibition of cell migration of epithelial cells from crypt to villus tip in intact rat duodenum by oral NSAID treatment. Previous studies have established that NSAIDs inhibit wound healing of chemically-induced ulcers without affecting cell proliferation *in vivo* in gastric epithelia (Penney et al. 1995; Schmassmann et al. 1995), though these experiments did not examine rates of cell migration. Additionally, other studies focused on effects of NSAIDs on cell migration *in vitro*, demonstrating that wound healing and cell migration are inhibited by NSAID treatment in cultured gastric or intestinal epithelial cells (Freeman et al. 2007; Giap et al. 2002; Raveendran et al. 2008; Silver et al. 2012; Silver et al. 2010; Silver et al. 2015). Our results extend these prior findings to show that cell migration is affected under normal conditions by oral NSAID treatment in rat duodenum (previous studies focused on gastric epithelia), an area of the small intestine highly sensitive to NSAID-induced toxicity. Furthermore, our results provide a foundation on which to evaluate the importance of inhibition of cell migration in clinical GI toxicity using a wider array of NSAIDs as well as examining migration in more distal portions of the GI tract including jejunum and ileum. Recent evidence shows that jejunum and ileum are far more susceptible to NSAID-induced toxicity than previously appreciated (Kanbayashi and Konishi 2015; McCarthy 2009; Satoh et al. 2012, 2014; Wallace 2013).

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Histological analysis of intestinal sections revealed slight, yet significant shortening of villi and crypts following oral treatment with indomethacin (Figs. 5 and 6). However, the modification of the villi and crypts was slight (shortening of crypts and villi by 9% and 13%, respectively), and it is unlikely that this shortening can account for the observed reduced rates of cell migration, particularly since the values measured for cell migration were expressed as a function of crypt-villus length.

In addition to revealing the effects of NSAIDs on cell migration in rat duodenum, our results also potentially identify a crucial mechanism through which this inhibition occurs: specifically inhibition of calpain 1 and 2 protein expression. Calpain 1 and 2 are the most well-studied calpains and are the members of this family that are consistently associated with cell migration (Glading et al. 2002; Leloup and Wells 2011; Perrin and Huttenlocher 2002), yet few studies have evaluated the effects of NSAID treatment on calpain protease expression in the GI. These have been limited to examining calpain 9 expression in the colon and its relevance to the ability of NSAIDs to inhibit neoplasia (Saini and Sanyal 2014; Vaish and Sanyal 2012), or to various *in vitro* studies with cultured intestinal epithelial cells (Raveendran et al. 2008; Silver et al. 2010; Silver et al. 2015). In these *in vitro* studies, treatment of IEC-6 cells with indomethacin or NS-398 inhibited calpain protease expression and activity (Raveendran et al. 2008; Silver et al. 2010), and treatment with indomethacin, NS-398, or ALLM caused dose-dependent inhibition of cell migration (Raveendran et al. 2008). Interestingly, co-treatment of IEC-6 cells with lower doses of indomethacin or NS-398 with ALLM had an additive effect on inhibition of cell migration following wounding, a result that is consistent with inhibition of calpain activity through the combination of reductions in calpain expression (NSAIDs) and direct suppression of calpain activity (ALLM; (Raveendran et al. 2008). Our results are consistent with these previous studies in that treatment with calpain siRNA inhibited calpain protease expression in IEC-6 cells and that these treatments, as well as ALLM, inhibited IEC-6 cell migration. This is the first time, however, that NSAIDs have been shown to inhibit the expression of calpains 1 and 2 in the small intestine.

Interestingly, the data collected on the effects of NSAIDs or calpain siRNA on calpain activity as measured using the BOC-LM-CMAC assay yielded some unexpected results. Whereas previous (Silver et al. 2010) and current experiments showed that treatment with indomethacin or NS-398 clearly reduced calpain activity, reductions in BOC-LM-CMAC fluorescence were not apparent following inhibition of the expression of either calpain 1 or 2 or both (Figs. 1 and 4). Treatment with calpain siRNA suppressed protein expression of calpain 1, or 2, or both by 60% (Fig. 2) in individual or combination experiments, whereas previously treatment with 100 μM indomethacin or NS-398 reduced calpain 1 expression by 30 – 40% or calpain 2 expression by 40 – 60% in IEC-6 cells (Raveendran et al. 2008; Silver et al. 2010). These results suggest that the BOC-LM-CMAC assay may not be sensitive enough to detect the changes in calpain activity that occur with the very specific inhibition of calpain expression with siRNA or that cleavage of BOC-LM-CMAC may be occurring through calpain-independent pathways. Alternatively, our data may suggest, unsurprisingly, that NSAIDs with ulcerogenic potential are affecting calpain activity in multiple ways beyond simply suppressing protein expression, and other mechanisms, such as

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depolarization of membrane potential (Silver et al. 2015) or changes in Ca^{2+} utilization are likely involved.

Calcium utilization and intracellular calcium levels are highly relevant in our discussion given that calpains are Ca^{2+}-dependent proteases. NSAID treatment has been shown to cause increases in intracellular Ca^{2+} and activation of the ER stress pathway in cultured hepatocytes (Fredriksson et al. 2011; Maiuri et al. 2016), and previous experiments show that wounding IEC-6 monolayers to stimulate migration (as in this study) results in increases in intracellular Ca^{2+} (Chung et al. 2015). Judging by the dependence of calpain activity on Ca^{2+} concentration (Franco and Huttenlocher 2005) and an expected rise in Ca^{2+} levels as a result of wounding IEC-6 monolayers, we might expect NSAID treatment of wounded monolayers to enhance calpain protease activity. However, we observed a significant reduction in cleavage of BOC-LM-CMAC as a result of treatment with indomethacin or NS-398 (Fig. 3A–C; Silver et al. 2010). Our findings of reduced calpain activity are consistent with previous reports in IEC-6 cells where treatment with indomethacin had no effect on intracellular Ca^{2+} (Yamawaki et al. 2014) and with the reduction in calpain protease expression (Fig. 9; Raveendran et al. 2008; Silver et al. 2010). In the future, it would be valuable to investigate the combined effects of NSAID treatment and wounding to determine the exact effects on Ca^{2+} availability in IEC-6 cells.

Our results are particularly relevant to present research initiatives in light of the interest in calpain proteases as they relate to cancer and the apparent ability of NSAIDs, specifically COX 2-selective NSAIDs, to inhibit development and spread of colorectal cancers (Vaish et al. 2013; Vaish and Sanyal 2012). Ubiquitous calpains, such as 1 and 2, regulate cell migration by mediating adhesion and de-adhesion steps (the two calpain isoforms are selectively compartmentalized), and dysregulation of the activity of these calpains can result in decreased apoptosis, increased proliferation, and stimulation of cell migration (reviewed in Leloup and Wells 2011). In contrast, NSAIDs inhibit the expression and activity of calpains 1 and 2, previously shown *in vitro* and now *in vivo*, and have been suggested to have chemopreventative effects on colorectal cancer (Bertagnolli et al. 2006; Cooper et al. 2010; Rao and Reddy 2004; Smalley et al. 1999). Inhibition of calpain 1 and 2 expression in rat duodenal epithelia and cultured epithelial cells is consistent with the ability of NSAIDs to inhibit metastasis and invasion by colorectal cancer cells and enhances the clinical relevance of our results, though more research is necessary to explore the interaction between NSAIDs, calpains, and colorectal cancer cells.

Interestingly, our analysis revealed that whereas gene expression for calpain 1 was unaffected and was apparently, though not significantly, increased for calpain 2 in rat duodenal mucosa, protein expression was significantly reduced for both calpain 1 and 2. These results are not necessarily contradictory in light of the fact that there are numerous regulatory steps between mRNA production and protein translation, including post-transcriptional regulation, RNA processing, alternate or differential splicing, RNA editing, or inclusion or exclusion of regulatory units, which can change gene or protein expression levels. In fact, efforts at correlating changes in gene expression identified using transcriptomic analyses with those observed in proteomic analyses suggest that message abundance for a particular gene does not necessarily predict the relative occurrence of the

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corresponding protein (Vogel and Marcotte 2012). Our results may, in the case of calpain 2, reflect calls for increased mRNA production to replace lost calpain 2 protein or, in the case of calpain 1, temporal discrepancies between loss of protein, possibly through an increase in protein catabolism, and the lack of a resulting demand for more protein.

NSAIDs are a structurally diverse group of compounds that collectively can reduce pain and inflammation presumably through inhibition of COX enzymes. As noted above, NSAIDs are typically categorized as selective for one COX isoform or another or non-selective. The drugs and drug regimens used in this study were selected to reflect the diversity of the NSAID class of drugs and standard practices for the experimental use of these drugs. Indomethacin is a non-selective inhibitor of COX enzymes that has a high ulcerogenic potential and causes ulcers in drug-treated animals at the doses used in this study (Kato et al. 2001; Peskar et al. 2001; Tavares 2000), whereas NS-398 is a COX 2-selective inhibitor (coxib) that does not cause ulceration in drug treated animals, but inhibits ulcer healing once they are induced (Brzozowski et al. 2001; Kato et al. 2001; Peskar et al. 2001; Tavares 2000; Tomisato et al. 2004). Animals were treated with indomethacin and NS-398 at a dose of 10 mg/kg/day in this study, which is consistent with the experimental model of indomethacin-induced GI damage that is commonly used (Ishida et al. 2013; Kato et al. 2009; Lim et al. 2012; Rohde et al. 2015; Satoh and Urushidani 2016) as well as their experimental use by other research groups in studying the anti-nociceptive effects of NSAIDs (Brzozowski et al. 2001; Euchenhofer et al. 1998; Lichtenberger et al. 2015; Peskar et al. 2001; Petchi et al. 2015; Pozzoli et al. 2007). It will be interesting to evaluate the effects of a greater range of NSAIDs for their effects on cell migration in duodenal epithelia and in the more distant epithelia of the jejunum and ileum in future experiments to determine if there is a correlation between inhibition of calpain protease expression and inhibition of migration following NSAID treatment there.

As with our *in vivo* experiments, similar consideration was given to the selection of drug treatment regimens for our *in vitro* experiments treating IEC-6 cells with indomethacin, NS-398, and ALLM at concentrations of 100 μM. These concentrations of indomethacin or NS-398 are consistent with previous *in vitro* studies examining the effects of NSAIDs on cell migration of cultured epithelial cells (Freeman et al. 2007; Pai et al. 2001; Raveendran et al. 2008; Silver et al. 2012; Silver et al. 2010; Silver et al. 2015), and 100 μM NS-398 has little to no effect on the activity of hetrologously expressed human or isolated sheep COX-1 enzymes (Futaki et al. 1994; Gierse et al. 1995). ALLM is an effective inhibitor of calpain activity and has been shown to dose-dependently inhibit IEC-6 cell migration in standard scratch assays (Raveendran et al. 2008).

In conclusion, our results showed for the first time that short-term oral NSAID treatment with indomethacin or NS-398 inhibited cell migration in duodenal epithelial cells. We also demonstrated that inhibition of total protein expression of calpain 1 and 2 proteins by NSAIDs or calpain siRNA or inhibition of calpain activity with ALLM, is sufficient for inhibition of cell migration in cultured epithelial cells. Additionally, inhibition of expression of calpain 1 and 2 proteases occurred *in vivo* in duodenal mucosa and may represent a mechanism through which NSAIDs cause GI toxicity.

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Abbreviations

NSAID	nonsteroidal anti-inflammatory drug
GI	gastrointestinal
COX	cyclooxygenase
PPI	proton pump inhibitor
PG	prostaglandin
IEC	intestinal epithelial cell
ALLM	N-acetyl-L-leucyl-N-[(1S)-1-formyl-3-(methylthio)propyl]-L-leucinamide
BrdU	bromodeoxyuridine
DMEM	Dulbecco's Modified Eagle's Medium
NS-398	N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide
DMSO	dimethyl sulfoxide
KSUVDL	Kansas State University Veterinary Diagnostic Laboratory
EtOH	ethanol
BOC-LM-CMAC	7-amino-4-chloromethylcoumarin, tert-butoxycarbonyl-L-leucyl-L-methionine amide

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Highlights

- Suppression of calpain protein expression with siRNA inhibited IEC-6 cell migration
- NSAIDs, but not calpain siRNA, suppressed calpain activity in IEC-6 cells
- NSAIDs inhibited calpain protein expression and crypt cell migration in rat duodenal mucosa

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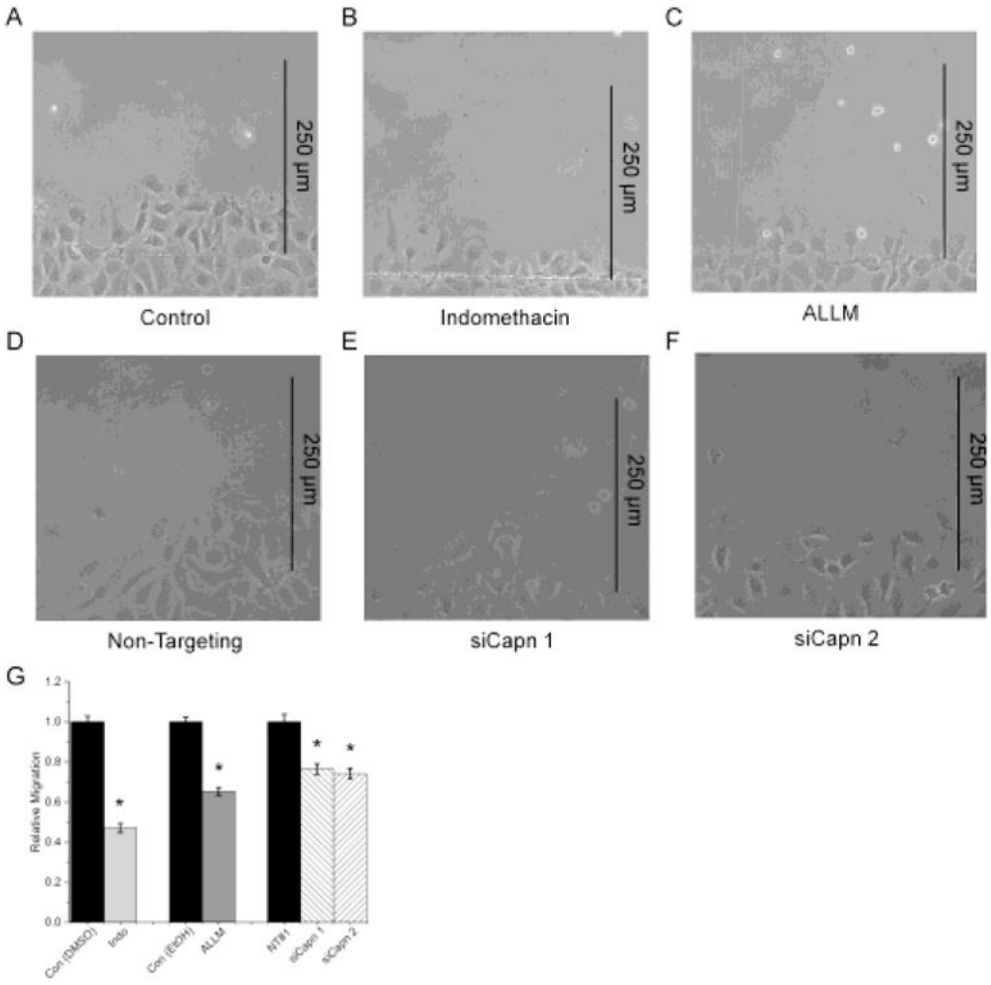

Figure 1.

Inhibition of migration of IEC-6 cells by NSAIDs or inhibition of calpain expression or activity. A–F. Photographs, 6 h post-wounding, of migrating IEC-6 cells treated with control (A, 0.1% DMSO for 72 h), 100 μM indomethacin (72 h, B), 0.1% ethanol (EtOH for 24 h – not shown), or 100 μM ALLM (24 h, C), or nucleofected (48 h prior to wounding) with non-targeting siRNA (D), calpain 1 siRNA (E), or calpain 2 siRNA (F). Photographs of cells treated with 0.1% EtOH are not shown since they appeared identical to cells treated with 0.1% DMSO. G. Summary results for migration experiments showing average coverage of a standardized area of interest (600 μm^2) covered by migrating cells for each treatment (n = 9 per treatment). Significant differences from the appropriate control are indicated by *.

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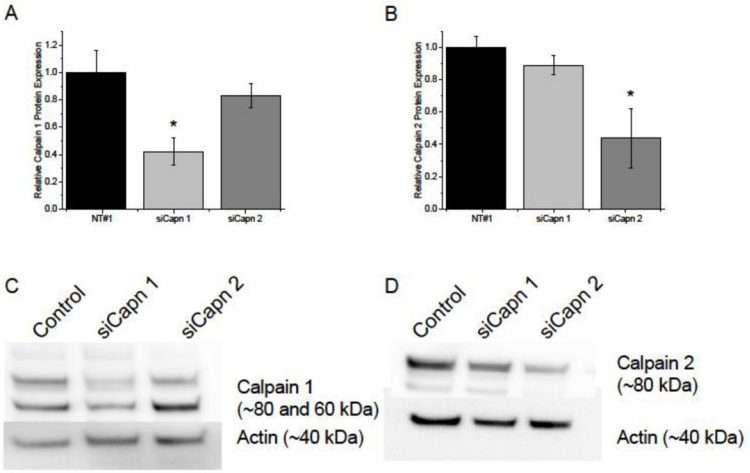

Figure 2.

Western blotting results for protein expression of calpain 1 (A) or 2 (B) in IEC-6 cells nucleofected with non-targeting siRNA or siRNA specific to calpain 1 or 2 ($n = 4$ per treatment group) for 48 h. Exemplar blots for calpain 1 (C top panel, 60 or 80 kDa bands), calpain 2 (D top panel, 80 kDa band), or actin (40 kDa band, used as the loading control, C and D bottom panel). In this experiment, blots were first probed for calpains 1 or 2, and subsequently stripped and reprobed for actin. Completeness of stripping was tested for by applying secondary antibody and checking for chemiluminesence following stripping. For quantification of calpain 1 protein expression, the 80 kDa band was used for all treatments since this is the complete, active form of calpain 1. * indicates a significant difference from control ($p < 0.05$).

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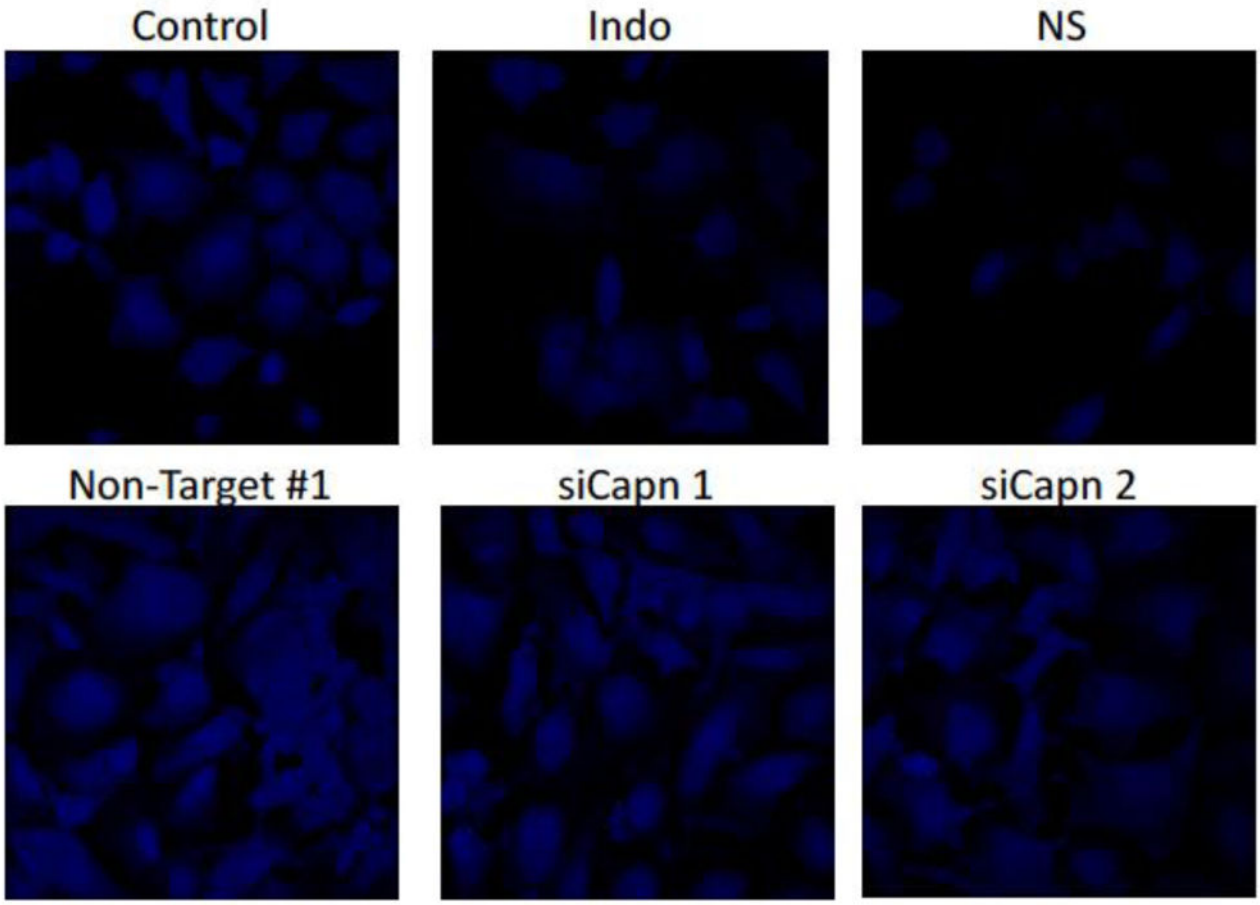

Figure 3.

Effects of loss of calpain protease expression on calpain activity. Cells were nucleofected with non-targeting siRNA (A) or siRNA specific to calpain 1 (B) or calpain 2 (C) 48 h prior to measurement of BOC-LM-CMAC fluorescence using confocal microscopy. D. Measurement of BOC-LM-CMAC fluorescence in IEC-6 cells as measured on a 96-well plate (n = 12 for each experiment) using a fluorescence plate reader 48 h after nucleofection with siRNA.* indicates a significant difference in fluorescence between treatment groups.

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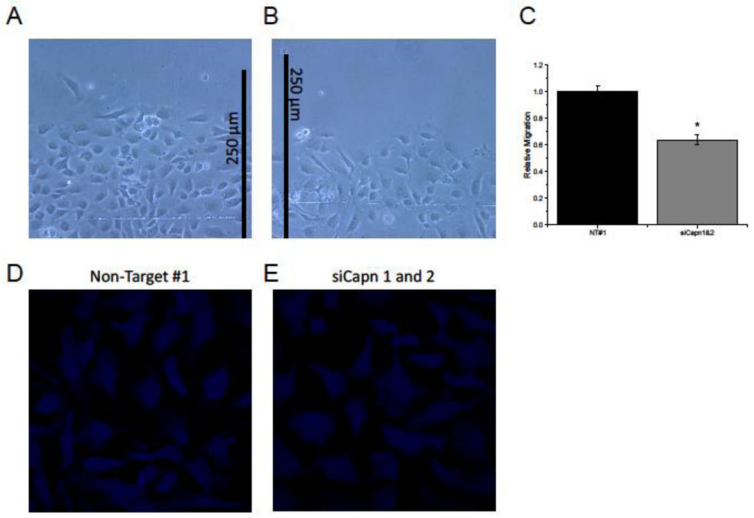

Figure 4.

Effects of treatment with siRNA to both calpains 1 and 2 on IEC-6 migration and calpain activity. Cells were nucleofected with non-targeting siRNA (A) or calpain 1 and 2 siRNA (B) and photographs were taken of cell migration 6 h after wounding in a scratch assay. C. Summary of results showing inhibition of cell migration by nucleofection with calpain siRNA. The effect on calpain activity was also measured in cells previously nucleofected with non-targeting siRNA (D) or calpain 1 and 2 siRNA (E) using the BOC-LM-CMAC fluorescence assay. Significant difference from control ($p < 0.05$) is indicated by * (Student's t-test).

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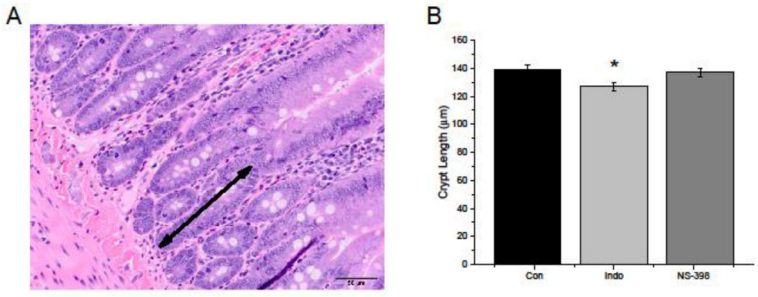

Figure 5.

Effects of NSAID treatment on duodenal crypt length. A. Photograph of a section of duodenum stained with hematoxylin and eosin with an arrow showing how crypt length was measured. B. Average crypt length in duodenum of rats treated with indomethacin ($n = 16$) or NS-398 ($n = 19$) for 72 h versus control ($n = 19$). * indicates a significant difference from control ($p < 0.05$).

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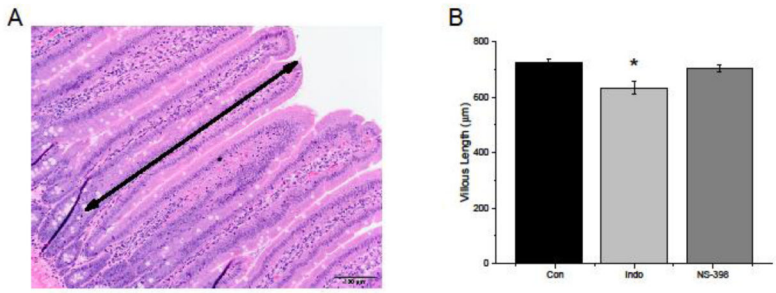

Figure 6.

Effects of NSAID treatment on duodenal villus length. A. Photograph of a section of duodenum stained with hematoxylin and eosin with an arrow demonstrating how villus length was measured. B. Average villus length in duodenum of rats treated with indomethacin (n = 16 rats) or NS-398 (n = 19 rats) for 72 h versus control (n = 19 rats). * indicates a significant difference from control ($p < 0.05$).

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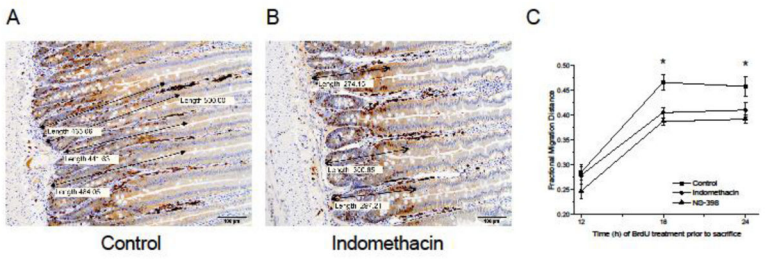

Figure 7.

Effects of NSAID treatment (72 h) on cell migration in rat duodenum. A and B. Photographs of sections of duodenum from rats treated with control (A) or indomethacin (B) 24 h after pulsing with BrdU and stained with an anti-BrdU antibody and marked with arrows showing how migration distance was measured. C. Average fractional migration distance of BrdU-labeled epithelial cells as a ratio of the migration distance to the average length of the crypt to villus axis in the duodenum of rats treated with indomethacin or NS-398 versus control at 12, 18, or 24 h after pulsing rats with BrdU ($n = 3 – 5$ rats per time point per treatment group). The average length of the crypt to villus axis for each treatment group was calculated by adding together their respective average crypt and villus lengths from Figs. 1B and 2B. * indicates a significant difference from control ($p < 0.05$).

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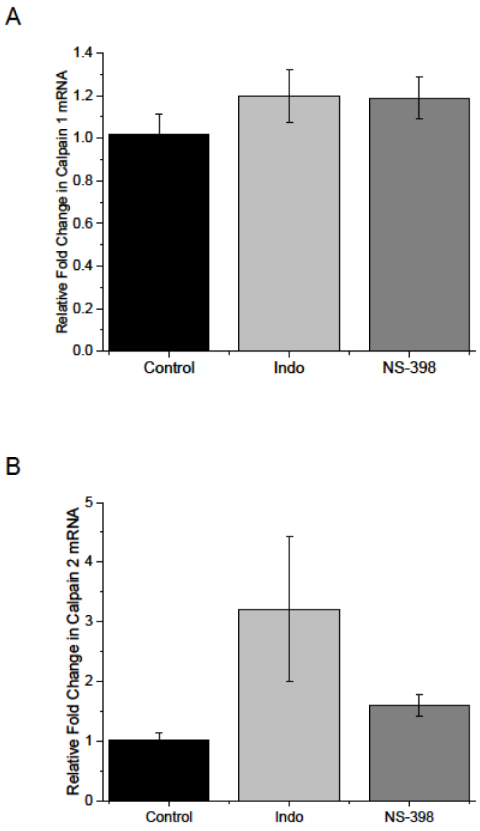

Figure 8.

Results of qRT-PCR experiments showing relative fold change in gene expression of calpain 1 (A) or 2 (B) in duodenal mucosa of rats treated with indomethacin ($n = 4$) or NS-398 ($n = 5$) for 72 h relative to control ($n = 5$). Relative fold change was calculated using the ΔΔ Ct method (Livak and Schmittgen 2001) using GAPDH and 18S as internal controls.

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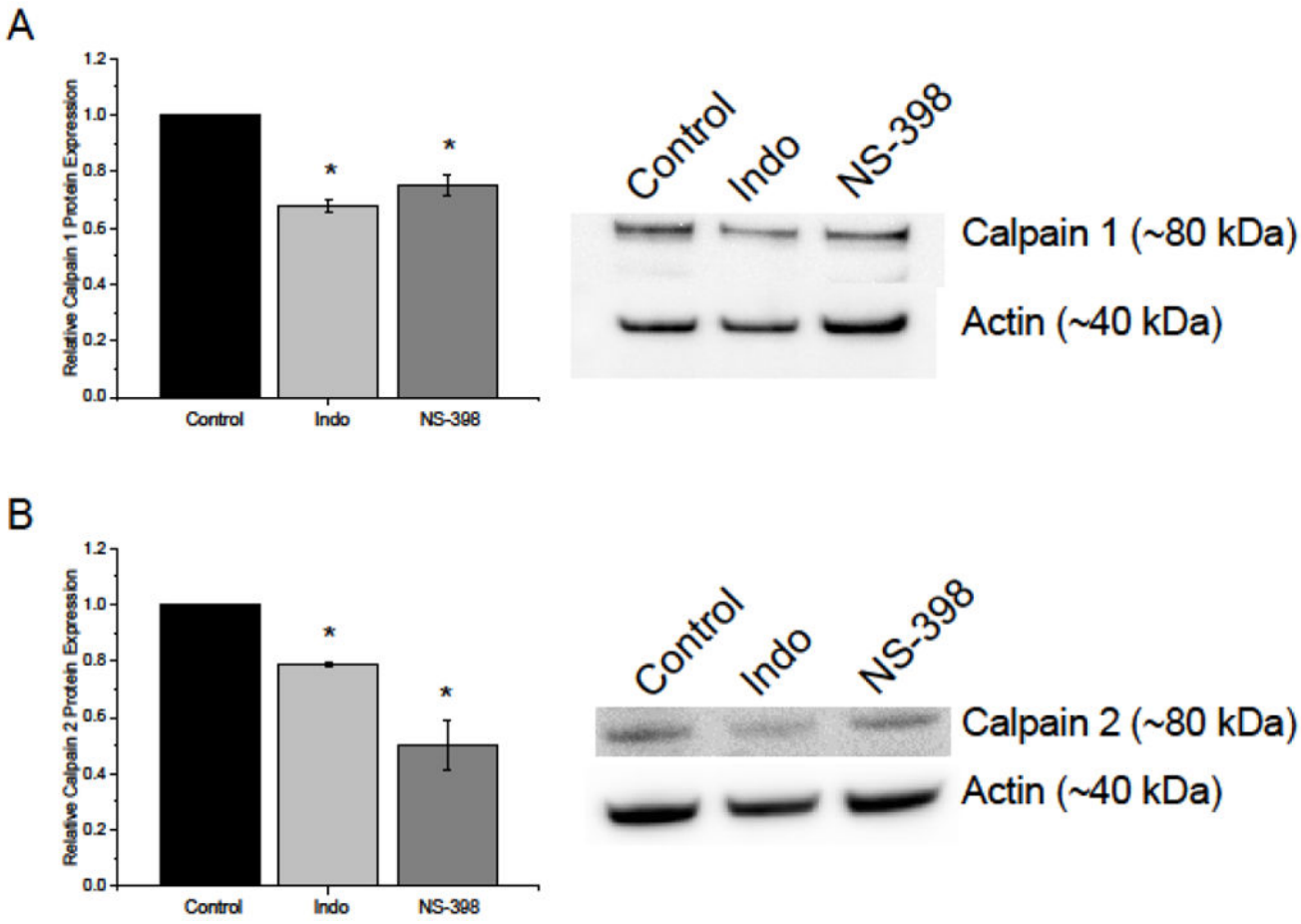

Figure 9.

Western blotting results evaluating protein expression of calpain 1 (A) or 2 (B) in duodenal mucosa of rats treated with indomethacin or NS-398 for 72 h relative to control ($n = 4$ per treatment group). Left panels: summary results for calpain 1 (A) or 2 (B) expression. Right panels: Exemplar blots for calpain 1 (2 bands at 80 or 60 kDa) or 2 (80 kDa) as well as for actin (40 kDa), which was used to normalize calpain expression prior to statistical comparison. * indicates a significant difference from control ($p < 0.05$).