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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.

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 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

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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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, 2010, 2015; Somasundaram et al., 2000).

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, 2010, 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, 2015). Furthermore, inhibition of calpain activity by ALLM, or calpain inhibitor II (*N*-acetyl-L-leucyl-N-[(1*S*)-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 have 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 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 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, 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-[(1*S*)-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 h 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.