Protection from diclofenac-induced small intestinal injury by the JNK inhibitor SP600125 in a mouse model of NSAID-associated enteropathy

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Abstract

Small intestinal ulceration, bleeding, and inflammation are major adverse effects associated with the use of diclofenac (DCF) or other nonsteroidal anti-inflammatory drugs (NSAIDs). The underlying mechanisms of DCF enteropathy are poorly understood, but there is increasing evidence that topical effects are involved. The aim of this study was to explore the role of c-Jun-N-terminal kinase (JNK) in DCF-induced enterocyte death because JNK not only regulates mitochondria-mediated apoptosis but also is a key node where many of the proximal stress signals converge. Male C57BL/6 mice were injected intraperitoneally with DCF or vehicle (solutol HS-15), and the extent of small intestinal ulceration was determined. A single dose of DCF (60 mg/kg) produced numerous ulcers in the third and fourth quartiles of the jejunum/ileum, with maximal effects after 18 h and extensive recovery after 48 h. To study the molecular pathways leading to enterocyte injury, we isolated villi-enriched mucosal fractions from DCF-treated mice. Immunoblot studies with a phosphospecific JNK antibody revealed that JNK1/2 (p46) was activated at 6 h, leading to phosphorylation of the downstream target c-Jun. The levels of the JNK-regulated proapoptotic transcription factor CHOP were also increased after DCF. The selective JNK inhibitor, SP600125 (30 mg/kg, ip), given both 1 h before and 1 h after DCF, blocked JNK kinase activity and afforded significant protection against DCF enteropathy. In conclusion, these data demonstrate that the JNK pathway is critically involved in the pathogenesis of DCF-induced enteropathy and suggest a potential application of JNK inhibitors in the prevention of NSAID-induced enteropathy.

Key words: NSAIDs; mitochondria; JNK; SP600125; enteropathy; small intestinal ulceration
Abbreviations:

ALP, alkaline phosphatase;
AP-1, activator protein-1;
ASK1, apoptosis signal-regulating kinase 1;
CHOP, C/EBP homologous protein;
COX, cyclooxygenase;
DCF, diclofenac;
JNK, c-Jun N-terminal kinase;
LPS, lipopolysaccharide (endotoxin);
NSAID, nonsteroidal anti-inflammatory drug;
OXPHOS, oxidative phosphorylation;
ROS, reactive oxygen species;
TNF, tumor necrosis factor-α
One of the major adverse effects of nonsteroidal anti-inflammatory drug (NSAID) therapy is gastrointestinal injury. This results in ulceration and bleeding of the mucosa, inflammation, and, in rare cases, perforation (1, 5, 46). Due to its high morbidity and mortality, NSAID-associated gastrointestinal injury is a serious clinical challenge causing a major burden on the health care system. Specifically, in the U.S. the annual number of hospitalizations for serious NSAID-associated gastrointestinal complications is estimated to be higher than 100,000 patients, and approximately 16,500 deaths occur each year; the impact of these adverse drug reactions has therefore been termed a “silent epidemic” (46).

It has become increasingly clear that, besides the well-known lesions in the stomach, the small intestine is also a major target organ of NSAID-associated toxicity (9, 20). With the advent of novel imaging techniques including capsule endoscopy it has become possible to fully appreciate the incidence and extent of small bowel injury. Unexpectedly, recent clinical studies revealed that as many as approximately two-thirds of both long-term (>3 months) and short-term (>1 week) NSAID users exhibited drug-induced lesions in the jejunum and ileum (13, 25). Unfortunately, to date there is no mechanism-based therapy or preventive treatment for drug-induced enteropathy available (17).

The underlying mechanisms of NSAID-induced enteropathy are not fully understood, but a number of modes of action have been recognized (21), (17). For example, systemic inhibition by NSAIDs of both cyclooxygenase (COX)-1 and COX-2 (the therapeutic target) may be involved, similar to the situation in the stomach (38, 41). However, off-target effects have also been implicated in the pathogenesis of small intestinal injury, including direct topical effects of the drugs on enterocytes facing the lumen (39). This is primarily due to the relatively high (millimolar) concentrations of conjugated NSAIDs in the hepatobiliary tree following ATP-dependent transport of NSAID glucuronides into the bile canaliculi, from where the metabolites are transported into the small intestine where they are cleaved and the free parent drug reabsorbed (36, 42). In cell culture studies, NSAIDs have been shown to target mitochondria and to induce uncoupling of
oxidative phosphorylation (OXPHOS) in a concentration-dependent manner (39). Furthermore, in gastric cells or hepatocytes, certain NSAIDs have been demonstrated to induce an endoplasmic reticulum (ER) stress response (43), causing increases in cytosolic (free) Ca\textsuperscript{2+} concentrations (24) and/or producing increased oxidant stress (24, 32). It is not clear to what extent reactive metabolites are involved in initiating or aggravating the injury (3, 36). Apart from these direct toxic effects of NSAIDs, indirect effects may also play a role in enterocytes. For example, due to the increased permeability of the gut, intestinal bacterial lipopolysaccharide (LPS) may activate toll-like receptor-4 (TLR4) on macrophages, which in turn can lead to tumor necrosis factor-α (TNF)-mediated cell injury and activation and recruitment of other cells of the innate immune system (45).

In view of these apparently multiple modes of action involved in NSAID enteropathy, it becomes extremely difficult to point to a specific putative mechanism that could be targeted in search of a potential therapeutic intervention strategy. However, one common pathway that links most of these initial cellular stress signals with downstream events and with ensuing cell death is the activation of c-Jun-N-terminal kinase (JNK). For example, JNK is phosphorylated by apoptosis signal-regulating kinase 1 (ASK1) or other upstream mediators, which in turn are activated by multiple stressors including oxidative stress (from, e.g., drug-induced intracellular reactive oxygen species (ROS) production, LPS signaling, TNF release), increased cytosolic [Ca\textsuperscript{2+}], or ER stress response signals (28), all converging at this pivotal signaling node. Activation of JNK, which regulates a number of cellular functions including cell proliferation and immune reactions, is particularly important in the pathogenesis of tissue injury because it can result in mitochondria-mediated lethal cell injury through regulating pro- and anti-apoptotic Bcl-2 family proteins, leading to mitochondrial permeabilization (15, 37). Currently little is known about a possible causal role of this pathway in mucosal injury of the small intestine induced by NSAIDs, despite the urgent need for preventive or therapeutic intervention. Therefore, the aim of this study was to explore the mechanistic role of JNK activation in mediating mucosal injury in a mouse model of diclofenac (DCF) enteropathy, and to ascertain whether inhibition of JNK activation afforded protection against diclofenac-induced small intestinal ulceration.
MATERIALS AND METHODS

Chemicals

Diclofenac sodium, nitrotetrazolium blue chloride (NBT), serine/threonine/tyrosine protein phosphatase inhibitors, acid and alkaline phosphatase inhibitors, proteases inhibitors and all other chemicals (unless indicated) were obtained from Sigma (St. Louis, MO). SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) was obtained from Biomol International (Plymouth Meeting, PA). Anti-JNK and anti-phospho-JNK antibodies were purchased from Cell Signaling (Danvers, MA); anti-phospho-c-Jun and anti-CHOP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-c-Jun antibody was obtained from Calbiochem EMD Chemicals (Gibbstown, NJ). Solutol HS-15 was obtained from BASF Fine Chemical Division (Ludwigshafen, Germany). Phosphate Buffered Saline (PBS) and 10% zinc formalin were obtained from Fisher Scientific (Kalamazoo, MI).

Animals

The study design and all protocols for animal care and handling were approved by the Institutional Animal Care and Use Committee of the University of Connecticut. Male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and acclimatized for at least one week before the experiments. The mice were kept on a 14/10-h light/dark cycle. Animals were 11-17 weeks old at the time of experimentation. They received mouse chow (Teklad Global Rodent Diet, Harlan Laboratories, Boston, MA) and water ad libitum.

Drug administration

Diclofenac (30, 60, or 90 mg/kg) was dissolved in a 7% (in PBS) Solutol HS-15 solution and administered intraperitoneally in a volume of 10 μL/g body weight. All animals were treated at 5 h before start of the dark cycle. SP600125, a potent and selective inhibitor of JNK, was dissolved in a 7% (in PBS) Solutol HS-15 solution and administered intraperitoneally (30 mg/kg) both 1 h prior to and 1 h after DCF administration.
Assessment of Small Intestinal Ulceration

The treated mice were euthanized by CO₂ inhalation. The abdomen and chest were rapidly opened by a midline incision, and blood was obtained by cardiac puncture. Blood was transferred to a separation tube containing clot activator and separating gel at the bottom, and serum was prepared by centrifugation and frozen at -80º C until use for chemistry analysis. A portion of the gastrointestinal tract (from stomach to cecum) was removed. The stomach was opened along the small curvature to continue on the anti-mesenteric side longitudinally along the small intestine. The small intestinal tissue was rinsed with ice-cold PBS, then incubated for 15 min in 1 mM NBT solution containing 16 mM HEPES-125 mM NaCl buffer (pH 7.4), 3.5 mM KCl, and 10 mM glucose (38). This procedure facilitates the detection of intestinal mucosal damage due to blue staining of the villi surrounding the ulcers. Stained tissue was fixed in 10% buffered formalin for 24 h and transferred to 70% ethanol. The NBT-stained small intestine was metrically divided into four segments of approximately equal length with the segment next to the stomach considered the first quartile and the segment before the cecum the fourth quartile. The luminal side of the jejunum and ileum was inspected under a 10x magnification stereo microscope (Premiere Stereoscope, Model SMP-13; Manassas, VA) to evaluate small erosions and ulcer size, number, and distribution.

Clinical Chemistry

Serum levels of total protein was determined by the bicinchoninin acid (BCA) assay (Pierce Biotechnology, Rockford, IL). Serum activity of alkaline phosphatase (ALP) was measured with a kinetic colorimetric test kit (BioAssay Systems, Hayward, CA).

Isolation of Villi-enriched Small Intestinal Mucosa

Because we found that ulcers were mostly distributed throughout the third and fourth quartiles, we used only those segments for the following steps. Isolation of the small intestinal mucosa was performed as previously described (8), including minor modifications. Briefly, to eliminate the luminal content, the longitudinally opened intestine was cleaned by vortexing the tissue (mixing speed 2800 rpm) for 30 sec in 35 mL ice-cold 1x PBS with freshly added protease inhibitors. All steps were performed at
$4^\circ$C. The first epithelial fraction (F1) was isolated by vortexing the cleaned segment for 2 min in 35 mL ice-cold 30 mM EDTA in 0.8x PBS containing proteases inhibitors. The second epithelial fraction (F2) was isolated by subsequent incubation of the segment for 15 min in 35 mL ice-cold 30 mM EDTA solution, followed by vortexing for 2 min. A third epithelial fraction (F3) was isolated by incubating the segment for 25 min in 35 mL of ice-cold 30 mM EDTA solution, followed by vortexing for 3 min. The suspensions of the isolated mucosal fractions were centrifuged and resuspended in 1x PBS solution. Samples were analyzed with differential interference contrast (DIC) microscopy. To identify the villi-enriched fractions of the small intestinal mucosa, we determined the specific activity of ALP in homogenates of the different fractions. Alkaline phosphatase is localized at the enterocyte brush border membrane and has been used as a marker for villi (14). The isolated fractions were homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Ipegal, 0.25% sodium deoxycholate, 0.2% Triton X-100, 0.1% SDS, with freshly added 1 mM PMSF and protease inhibitors). Protein content of the homogenates was determined with the BCA assay. Tissue ALP activity was determined by measuring the degradation of $p$-nitrophenol phosphate under linear conditions of protein and time.

**Western Blotting**

Because we found highest ALP activity in F2, we used this villi-enriched fraction to analyze the expression of proteins after DCF treatment. We used phosphatase inhibitors both in the EDTA solutions and lysis buffer as our study involved the analysis of phosphoproteins. The lysates were centrifuged at 20,000xg for 5 min at $4^\circ$C to remove debris. Protein content was determined by the BCA assay. Equal amounts of protein from each experimental group were reduced, denatured, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred to a nitrocellulose or polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were probed with anti-JNK (1:1000 dilution), anti-phospho-JNK (1:1000), anti-c-Jun (1:1000), anti phospho-c-Jun (1:2000), or anti-CHOP (1:1000) antibodies. The bound antigen-antibody complexes were visualized after incubation with horseradish peroxidase-conjugated secondary antibody using enhanced
chemiluminescence detection system (Millipore Corporation, Billerica, MA). Equivalent protein loading among samples was tested by detection of β-actin protein.

Statistical analysis
Ulcer numbers and serum biochemistry values were expressed as mean ± standard deviation (SD) with a $P$ value of $\leq 0.05$ to indicate statistical significance. When normality of distribution failed, a Kruskal-Wallis One Way Analysis of Variance (ANOVA) on Ranks was used followed by Dunn’s test for multiple comparison versus the respective control group. When normality test passed, a standard ANOVA was used followed by Dunnett’s test for multiple comparison versus control group. For Western blot studies, samples from at least four animals per experimental group were evaluated. Data on densitometric analysis were expressed as the means ± SD of fold changes compared with vehicle control. Statistical analysis was performed using the Kruskal-Wallis one way ANOVA on ranks, using Dunn’s test for multiple comparison versus the respective control group. To evaluate the effects of DCF with or without JNK inhibitor, the $t$-test was applied (comparing two means only). A $P$ value of $\leq 0.05$ was considered statistically significant.

RESULTS
Development of a Mouse Model of Diclofenac Enteropathy
Our first aim was to develop a mouse model of DCF enteropathy as, in contrast to rat models, detailed information on the toxic response to NSAIDs in the small intestine is limited in this species. Following administration of a single dose (30-90 mg/kg), DCF induced multiple ulcers and smaller erosions in the mouse small intestinal mucosa within less than 24 h. These ulcers were readily identified and quantitated by NBT-positive staining of the villi surrounding the focal mucosal lesions (Fig. 1). Gross pathological examination of the small intestine following an ulcerogenic dose of DCF typically revealed severe damage featuring multiple round or elongated ulcers localized primarily along the mesenteric side (Fig. 1A). Histopathological examination confirmed that the ulcers involved the entire mucosa, compromising the integrity of the submucosa and
muscularis mucosae. The lesions were characterized by loss of villi and infiltration of inflammatory cells in the necrotic part of the ulcer. The villi adjacent to the ulcer were contracted, and necrosis of the epithelium extended to the base of the crypts. Similar to the tissue injury in rats (3), the lesions were most abundant in the third and fourth quartile of the small intestine, while the first quartile was free of any apparent ulceration and the second quartile exhibited few lesions only (Fig. 1B). The reason for this differential distribution along the jejunum/ileum has not been determined but could result from differential pH in these segments and/or different bacterial populations, or it could be determined by variations in the toxicodynamic response across the length of the small intestine. Because the mouse is a relatively new model for NSAID enteropathy, we first ran a time course study with a single ulcerogenic dose of DCF. Following intraperitoneal administration of 60 mg/kg DCF, mice developed multiple ulcerations in the small intestinal mucosa that became apparent as early as at 6 h post-dose (Fig. 1C). The maximal effects were observed after 18 h, when the damage was severe with ulcers becoming confluent. Although the number of ulcers at 18 h was not significantly higher than that at 6 h, the average size of the ulcers was much greater. At 48 h post-dose, all the lesions had been repaired, and the small intestinal mucosa appeared indistinguishable from vehicle control animals, except for small scars in the mucosa, indicating that extensive tissue regeneration had occurred after a single dose of DCF (not shown). The development of small intestinal ulceration was dose-dependent (Fig. 1D). We chose a standard dose of 60 mg/kg for all subsequent experiments because higher doses did not significantly increase the number and extent of lesions, but did increase the risk of lethality. This standardized dose, which is approximately 2-3x higher than the therapeutic dose when based on body surface (rather than on mg/kg-based comparison between mice and humans), is high but necessary in order to study protective mechanisms.

Similar to the rat model (34), a single dose of DCF significantly decreased the plasma activity of ALP in a time- and dose-dependent manner (Fig.2). The degree of ALP activity changes paralleled the pathological manifestation of ulceration, making ALP a valuable biomarker of small intestinal injury in the DCF mouse model. The mechanisms underlying the decrease in serum ALP activity has not been elucidated; however, it has
recently been reported that ALP is involved in the metabolism and detoxication of bacterial lipopolysaccharide (LPS) (14), where it prevents leakage of LPS into the bloodstream. In line with this, it has been demonstrated that following administration of an ulcerogenic dose of indomethacin to rats there was an approximately 50% reduction in ALP activity in the brush border as compared to vehicle controls (39). The primary function of intestinal ALP has been suggested to be the detoxication of LPS, which is a substrate for ALP (14). Thus, the attenuated serum activity could be due to inactivation of ALP by LPS during the NSAID-induced increases in intestinal permeabilization. Furthermore, total serum protein levels (another marker of small intestinal injury) were also consistently decreased following DCF exposure (Fig. 2). After an ulcerogenic dose (60 mg/kg, ip), the total protein levels were decreased by 12% at 6 h post-dose and the loss of serum protein further progressed, attaining 21% decrease at 18 h. The underlying mechanism likely involves loss of protein due to increased intestinal permeability and bleeding. At 48 h post-dose, both serum ALP activity and total protein levels had returned to normal control values (not shown), confirming the morphological data and indicating that these serum markers of injury are reversible and paralleling the pathologic changes.

Characterization of Isolated Small Intestinal Villi

Enterocytes lining the villi are the primary target of NSAIDs in the small intestine (3). Therefore, to study the signaling pathways leading to cell injury, we isolated the small intestinal mucosa and prepared a fraction enriched in villi from the third and fourth quartile. The original method produced “footprint”-like structures (8) (Fig. 3A) which we characterized by high specific expression of both aminopeptidase N (villi) and defensin (crypts, not shown). Here, using a modified method, subsequent fractionation yielded suspensions of tissue highly enriched in villi or crypts (Fig. 3B, C). The F2 fraction (see Materials and Methods) exhibited a 47% higher specific activity of ALP activity (villus brush border biomarker) than F3 and was used for all subsequent experiments.
To explore a possible role of JNK signaling in the intestinal ulcerative damage induced by DCF, we first determined the levels of immunoreactive phospho-JNK (p-JNK) in lysates of isolated small intestinal mucosa in Western blots at various time points following administration of DCF. Both JNK1 and JNK2 can exist either as the full-length 54kDa protein (p54) or as a C-terminally truncated form of 46kDa (p46) (10). We found that the truncated form (p46) of p-JNK became increased at 3 h post-DCF in 2/4 mice, and that it was further increased at 18 h in all treated mice (Fig. 4A). Densitometric analysis (mean of 4 mice per group) revealed that p-JNK (p46) was significantly increased at 6 h, exhibiting even higher significance levels at 18 h post-dose (3.4-fold increase over basal levels) (Fig. 4B). To ascertain that the increased levels of p-JNK (p46) were not simply a consequence of a higher abundance of inactive JNK, we determined the levels of non-phosphorylated p46. We did not find any significant alterations as compared to the apparent JNK expression levels in vehicle control mice at the corresponding time points. Collectively, these findings suggest that JNK (p46) is activated prior to the appearance of any pathological changes and could therefore be involved in the signaling pathways leading to DCF-induced enteropathy.

To determine whether activation of JNK caused the phosphorylation of downstream protein targets, we assessed by immunoblotting the steady state levels of phospho-c-Jun (p-c-Jun) at an early time point (6 h post-DCF). c-Jun is part of the transcription factor AP-1 (activator protein-1) and involved in the regulation of early response genes. We found indeed a strong p-c-Jun signal (Fig. 4C), confirming that DCF enhanced the kinase activity of p-JNK.

Administration of the JNK Inhibitor, SP600125 Protects from Diclofenac Enteropathy

Diclofenac-induced stimulation of JNK phosphorylation and activation of its downstream target c-Jun suggested a role for JNK in the pathogenesis of ulcers. To further explore a possible causal role of JNK in DCF enteropathy, we used the cell-permeable, ATP-competitive small-molecule JNK inhibitor, SP600125 (4). The inhibitor or its vehicle
(Solutol HS-15, 7% in PBS) was administered intraperitoneally at a dose of 30 mg/kg both 1 h before and 1 h after DCF (60 mg/kg).

To ascertain that the JNK inhibitor was effective in blocking the downstream signaling, we first determined the immunoreactive levels of p-c-Jun. We found that SP600125 greatly attenuated the levels of immunoreactive p-c-Jun (Fig 4C). This is in line with the concept that SP600125 decreases JNK activity, while it does not interfere with JNK phosphorylation itself (26). Next, we analyzed the effects of the JNK inhibitor on DCF-induced ulcer formation. We found that SP600125 afforded significant protection from ulceration induced by DCF; both the number (Fig. 5) and size (Fig. 6) of ulcers and erosions were greatly reduced as compared to DCF alone controls. Specifically, the number of ulcers was reduced by 93% (Fig. 5A) with minor damage observed only in the third quartile (Fig. 5B). Gross examination of the small intestine stained with NBT revealed that the mucosa after combined DCF/SP600125 treatment had a control-like appearance except for a few shallow, small ulcers (Fig. 6A-C) and a low number of erosions that did not exhibit loss of villi (not included in the ulcer count). Histopathologic evaluation of the few small ulcers confirmed that the degree of severity was mild, featuring minor loss of villi and without compromising the integrity of the submucosa and muscularis mucosae and a reduced number of infiltrated neutrophils (Fig. 6D). The erosions in the DCF/SP600125-treated mice were only superficial changes in the mucosa without affecting crypts, submucosa, or muscularis mucosae, and no inflammation was observed (Fig. 6E). Taken together, the data clearly indicate that the JNK inhibitor afforded full protection from DCF enteropathy, and they suggest that JNK may play a key role in the cellular signaling leading to NSAID-induced small intestinal injury.

Role of CHOP and mitochondria-mediated cell death

Recent studies have indicated that ER stress can activate JNK, which in turn leads to CHOP upregulation and apoptosis (23, 47). CHOP is a transcription factor that has been associated with mitochondria-mediated cell death. To ascertain whether CHOP may be involved in DCF enteropathy, we measured the time-dependent changes of CHOP protein in the isolated villi-enriched fraction of the small intestine following an ulcerogenic dose (60 mg/kg) of DCF (Fig. 7). We found a time-dependent increase in immunoreactive
CHOP attaining a 2.1-fold increase at 18 h as compared to the basal levels, but not at earlier time points. Because CHOP is positively regulated by JNK, these data could provide a link between DCF-induced JNK activation and enterocyte apoptosis. However, because CHOP activation is not an early event, it is more likely that CHOP-mediated pathways could aggravate the injury at an advanced stage rather than being the primary event.

DISCUSSION

The aim of this study was to evaluate the mechanistic role of JNK in DCF-induced small intestinal injury in the mouse. We found that JNK plays a pivotal role in the pathogenesis of DCF-induced small intestinal ulceration and that targeting JNK could become a potentially novel way for therapeutic intervention. These conclusions were based on a number of observations. First, a single ulcerogenic dose of DCF selectively increased the levels of one activated (phosphorylated) JNK form (p46) prior to the development of ulcers. Furthermore, and importantly, we found that co-administration of the selective JNK inhibitor, SP600125, fully protected from DCF-induced small intestinal injury, indicating that JNK and its downstream signaling pathways are critical in mediating DCF-induced lethal cell injury in enterocytes.

JNK are a protein family of serine/threonine kinases, encoded by three genes, JNK1, JNK2, and JNK3. The gene products can be alternatively spliced to give α and β forms of proteins (p54 and p46) (10). JNK1 and JNK2 are ubiquitously expressed (including enterocytes), while JNK3 is present in the nervous system and cardiomyocytes and not expressed in the small intestine. The functions of the forms differ, but they are all activated by different forms of cellular stress, among which oxidative stress including ROS generated in mitochondria and other cellular sources is a major activator (29, 37). Further downstream, JNK can phosphorylate a number of targets; one pathway is activation of c-Jun, a protein that is also expressed in murine enterocytes (18). Activation of c-Jun enables activation of AP-1 and plays a role in the regulation of the expression of
a number of proinflammatory cytokines including TNF and IL-1β and the activation of
the innate immune system (2, 12). On the other hand, JNK also activates other proteins,
including Bcl-2 family members, and is therefore involved in the regulation of
mitochondria-mediated cell death (11, 35). For example, JNK can phosphorylate
(activate) Bax and other pro-apoptotic proteins, while at the same time it phosphorylates
and thereby inactivates Bcl-XL and other anti-apoptotic proteins (19). Because both
mitochondria (39, 40) and the innate immune system (45) have been implicated in
mediating NSAID-associated enteropathy, inhibition of JNK by a chemical inhibitor
likely blocks multiple important downstream pathways.

We found indeed that the specific JNK inhibitor, SP600125 almost completely prevented
ulcer formation following administration of a normally ulcerogenic dose (60 mg/kg) in
mice. SP600125 is a small-molecule, ATP-competitive inhibitor with low selectivity for a
specific JNK form (it inhibits both JNK1 and JNK2), but it is highly selective and has a
>300-fold selectivity over the related MAPKs, ERK and p38 (4). SP600125 is an
established tool to evaluate JNK-dependent processes in vivo or in vitro (7), including
chemically induced injury to the gastrointestinal tract. For example, SP600125 protected
from dextran sulfate sodium-induced colitis in mice (2), or the inhibitor prevented JNK-
dependent gastric lesions induced by ethanol in rats (27). At the high dose (30 mg/kg, ip)
that we used, SP600125 has been shown to decrease JNK-regulated TNF plasma levels in
mice treated with LPS (4). While it is not yet clear to what extent TNF is causally
involved in DCF enteropathy, it is, however, unlikely that the observed protection by
SP600125 from DCF ulceration is solely due to an inhibitory effect on TNF, as JNK
inhibition also prevents the activation of other pathways including mitochondrial
permeabilization (19, 22, 33). Another downstream target that may be indirectly inhibited
by SP600125 is COX-2; it has been shown that COX-2 is regulated by JNK and that the
enzyme was downregulated by SP600125 following induction of COX-2 by LPS in
murine macrophages (30). In enteropathy, loss of COX-2 activity, however, would
promote rather than protect from damage; therefore, downregulation of COX-2 is
unlikely to be the sole cause for the protection provided by SP600125. Another JNK-
regulated pathway is the activation of the pro-apoptotic transcription factor CHOP. It has
been shown that JNK activation during ER stress leads to increases in CHOP levels (23) and that inhibition of JNK by SP600125 attenuates CHOP expression (47), which is commensurate with our findings.

While these downstream effects (mitochondria-mediated and/or innate immune system-mediated enterocyte cell demise and its protection by SP600125) can be explained by the key role of JNK, the proximal pathways leading to JNK activation in the first place are much less clear. Although DCF has been shown to cause early increases in [Ca^{2+}]_{i} and to enhance oxidant stress in other cell types such as hepatocytes (24), it is not clear whether similar changes are involved in enterocytes. Both increased [Ca^{2+}]_{i} and oxidant stress could lead to activation of Ask1-dependent signaling and JNK phosphorylation (31). Alternatively, the high concentrations of the electrophilic and protein-reactive DCF acyl glucuronide and \textit{iso}-glucuronides present in the gut, which have been shown to cause DCF adduct formation to enterocyte plasma membrane proteins (3, 6, 36, 44), could initiate some signaling events; however, a causal role of these protein adducts has never been proven. Once activated, JNK can in turn either phosphorylate Bcl-2 proteins that regulate mitochondria-mediated apoptosis, or, alternatively, translocate to mitochondria where JNK can directly trigger mitochondrial permeabilization (16).

In conclusion, this study demonstrates that specific JNK forms (p46) are activated in the small intestinal mucosa early following administration of an ulcerogenic dose of DCF in mice and that the highly selective JNK inhibitor, SP600125 completely prevents the injury, implicating a mechanistic role of JNK in the pathogenesis of NSAID ulceration. These findings could lead to a potential therapeutic application of novel JNK inhibitors in drug-induced intestinal ulceration, as currently there is no mechanism-based treatment available to treat or prevent NSAID-induced enteropathy. Furthermore, the use of the generally intestine-safer selective COX-2 inhibitors (as opposed to the mixed COX-1/2 inhibitors like DCF, which have higher gastrointestinal liability) has been restricted in recent years after it was detected that some COX-2 inhibitors have a higher risk for cardiovascular disease; therefore the number of prescriptions for the traditional NSAIDs will likely be increasing. In light of the severity and the high incidence of these drug-
induced intestinal adverse effects, further mechanistic studies on how to prevent NSAID enteropathy seems therefore both timely and topical.

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REFERENCES


41. Tanaka A, Hase S, Miyazawa T, and Takeuchi K. Up-regulation of cyclooxygenase-2 by inhibition of cyclooxygenase-1: A key to nonsteroidal anti-


Legend to Figures

**Fig. 1.** Characterization of diclofenac (DCF)-induced small intestinal ulcers in C57BL/6 mice. (A) Mice were administered a single dose of DCF (60 mg/kg, ip) and sacrificed 18 h later. An opened segment of quartile 3 of the small intestine revealed ulcers on the mesenteric side of the mucosa. Villi surrounding the ulcers appeared dark blue after NBT staining, while the necrotic tissue remained in the center of the ulcer. The lower panel is a histologic section (H&E staining) from the ulcer in the upper panel (arrow). The mucosa overlying the mesentery appears severely damaged with focal destruction and necrosis of villi, extending to the submucosa and muscularis mucosae. Note the abundant infiltration of inflammatory cells. (B) Quantitative analysis and distribution of ulcers across quartiles 1 through 4 of the mouse small intestine. Mice were treated with a single dose of DCF (60 mg/kg, ip), and the mucosa was analyzed at the indicated time points. Each point represents the mean ± SD for 4-7 animals/group. (C) Time course of small intestinal ulceration after treatment of mice with a single dose of DCF (60 mg/kg, ip). Each point represents mean ± SD for 4-7 animals. (D) Dose-dependent development of ulcerative damage in the small intestine. Mice were treated with a single ip dose of DCF and sacrificed after 18 h. Each column represents mean ± SD for 5-8 animals/group. *, $P < 0.05$ versus vehicle control.

**Fig. 2.** Clinical-chemical biomarkers of small intestinal ulceration induced by DCF (60 mg/kg, ip). Mice were sacrificed at various time points, and serum levels of total protein and alkaline phosphatase (ALP) activity were determined. Each point represents the mean ± SD for 4-7 animals. *, $P < 0.05$ versus 0 h (protein); #, $P < 0.05$ versus 0 h (ALP).

**Fig. 3.** Morphologic characterization of villi-enriched isolated mucosa. (A) Footprint-like structures isolated from mouse small intestine (quartiles 3 and 4) consisting of both villi and crypts. (B) Representative micrograph showing a villus from the F2 fraction, and (C) a crypt from the F3 fraction. Original magnification was 200x (B) or 400x (C).

**Fig. 4.** Time-dependent effects of DCF on JNK activation (phosphorylation). Mice were treated with a single dose of DCF (60 mg/kg, ip) and sacrificed at the indicated time
points. Villi-enriched fractions were prepared from the small intestinal mucosa quartiles 3 and 4. Proteins from tissue lysates were resolved by SDS-PAGE and analyzed by immunoblotting with specific antibodies against phosphorylated JNK (p-JNK) or JNK.

(A) Representative blots demonstrating an increase in the expression of p-JNK (p46 form) during the progression of ulcerative damage. (B) Densitometric analysis of JNK and p-JNK bands at various time points. Beta-actin was used as loading control. Each column represents the mean ± SD for 4 animals/time point; *, P < 0.05 versus 0 h. (C) Western blot showing the effects of DCF and SP600125 on c-Jun phosphorylation. In a separate experiment, groups of mice were treated with DCF (60 mg/kg, ip) or vehicle in the presence or absence of the JNK inhibitor, SP600125 (30 mg/kg, ip, given both 1 h before and 1 h after DCF) or vehicle. The mice were sacrificed 6 h post-DCF. Phospho-c-Jun was probed by immunoblot techniques. The figure is representative of four different mice per group.

**Fig. 5.** Effects of the JNK inhibitor SP600125 on the extent and distribution of small intestinal ulcers induced by DCF. Mice were administered a single dose of DCF (60 mg/kg, ip) with or without SP600125 (30 mg/kg, ip, given both 1 h before and 1 h after DCF). The mice were sacrificed at 18 h post-DCF. Note that in the presence of SP600125 the ulcerative damage was almost completely abolished. (A) Total ulcer number. Each column represents the mean ± SD for 4-6 animals/group; *, P < 0.05 versus DCF alone; (B) Distribution of ulcers across quartiles 1 through 4 of the small intestine. Each point represents the mean ± SD for 4-6 animals/group.

**Fig. 6.** Macroscopic and histopathologic characterization of the cytoprotective effects of SP600125 against DCF-induced ulceration. (A) Close-up view of one of the few small intestinal ulcers induced by DCF (60 mg/kg, ip) in presence of SP600125. Note the small size of the circular shaped ulcer; (B) Opened segment of quartile 3 of the small intestine showing the mesenteric side of the mucosa from the same experimental group as in panel A. Note the “vehicle control-like” macroscopic appearance of the mucosa; (C) Close-up view of an erosion. Note that the central villi are retained in the minor lesion; (D) Histopathologic analysis of the ulcer displayed in panel A, confirming the moderate
mucosal damage on the mesenteric side; (E) Microscopic view of the erosion displayed in panel C showing the superficial injury confined to the mucosa; no evidence of inflammation was observed. H&E stain, original magnifications 10x (B), 30x (A, C), and 100x (D, E).

Fig. 7. Immunoblot analysis of DCF-induced CHOP expression in villi-enriched fractions of the small intestinal mucosa. Mice treated with a single dose of DCF (60 mg/kg, ip) and sacrificed at the indicated time points. Proteins from the mucosal tissue lysates were resolved by SDS-PAGE and analyzed by immunoblotting with a specific antibody against CHOP. (A) Representative blot showing an increase in the expression of CHOP during the progression of ulcerative damage. (B) Densitometric analysis of CHOP protein expression at different time points relative to 0 h. Beta-actin was used as loading control. The blots are representative of 4 blots with different animals/time point. Each column represents the mean ± SD for 4 animals/time point; *, $P < 0.05$ versus 0 h.
A

B

C

D

Fig. 1
Ramirez et al.
Fig. 2
Ramirez et al.
Fig. 3
Ramirez et al.

A

Villus

Crypts

B

C
Fig. 4
Ramirez et al.

A

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C

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Figures and data showing the expression of JNK and p-JNK after DCF treatment.
Fig. 5
Ramirez et al.
Fig. 7
Ramirez et al.

A

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Relative density - Fold change

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