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Review

Mechanisms underlying intestinal injury induced by anti-inflammatory COX inhibitors

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Abstract

By far the most attention has been paid to the deleterious actions of nonsteroidal anti-inflammatory drugs (NSAIDs), including isoform selective agents that inhibit cyclooxygenase (COX), on the upper gastrointestinal tract, particularly the gastric and duodenal mucosa. However, recent studies confirm a relatively high incidence of serious clinical events, especially with the more-established drugs of this class, involving the small intestine. Pathogenic factors that have been proposed from early studies in such enteropathy have included the enterohepatic circulation of the nonsteroidal anti-inflammatory drugs, inhibition of cyclooxygenase, surface epithelial changes and focal microvascular events. More recent work has concerned the role of infiltrating inflammatory cells, the relative roles of cyclooxygenase isoforms, COX-1 and COX-2 and the key involvement of inducible nitric oxide (NO) synthase and its product in combination with superoxide, peroxynitrite. In the present review, evidence for the underlying involvement of each these processes, and their sequential integration in the development of the intestinal injury and ulceration promoted by nonsteroidal anti-inflammatory drugs has been considered.

Keywords: NSAID; COX (Cyclo-oxygenase); COX-2 inhibitor; Coxib; Enteropathy; Intestinal permeability; Enteral bacteria; Neutrophil; Nitric oxide; iNOS; Peroxynitrite

Contents

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1. Introduction

When considering the side-effect profile of anti-inflammatory drugs, particularly the nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenase (COX) and more recently, the isoform-selective COX-2 inhibitors, it is clear that by far the most attention has been paid to the deleterious actions on the upper gastrointestinal tract, particularly the gastric and duodenal mucosa. Thus, apart from experimental investigations and a limited number of clinical studies on the lower gut over the years, gastroduodenal injury have been the main focus of clinical and indeed commercial emphasis in the development of newer agents. This may be primarily because of the ease and accuracy of direct determination of acute and more chronic gastroduodenal injury and because any such peptic symptoms are the most rapid of onset and probably most prevalently noted by the patients themselves when taking a course of NSAIDs.

Clinical pharmacology studies from a number of groups, dating from the 1980s, have documented the effects of NSAIDs on the small intestine, usually in healthy volunteers, using a range of techniques including direct endoscopic observation, radiolabelled inflammatory cell localisation and faecal excretion of markers and the use epithelial permeability probes (Bjarnason et al., 1987a,b, 1993; Davies et al., 1992; Morris et al., 1991; Sigthorsson et al., 1998b, 2000; Smecuol et al., 2001; Smale and Bjarnason, 2003). Prior to this work, acute administration of aspirin suspension to humans has been shown to cause a rapid response of erythrocyte extrusion and focal erosion of the jejunal villi within 5 min of exposure, as determined in biopsies by electron microscopy (Ivey et al., 1979), an action that now might be considered to be primarily an immediate topical irritant action.

Epidemiological and smaller clinical surveys have also indicated that the chronic ingestion of NSAIDs is a risk factor for a number of small intestinal adverse events, including bleeding, perforation, ulceration, strictures and obstruction (Langman et al., 1985; Allison et al., 1992; Lanas et al., 1997; Wilcox et al., 1997). More recently, data from a prospective study in over 8000 rheumatoid patients confirms that incidents of serious lower gut clinical events account for some 40% of all serious gastrointestinal events (Laine et al., 2003). This rate of incidence for small intestinal complication with NSAIDs is thus far greater than has been previously suspected by most clinical gastroenterologists and rheumatologists, and possibly the pharmaceutical industry, although this is what could have been anticipated by the previous more-limited case and cohort studies. Moreover, it comes as little surprise to the pharmacologists who have evaluated the profound actions of such agents on the lower gut in experimental models.

The mechanistic basis for the intestinal damage has only slowly become more clear, although significant and novel information has been gathered over the past 10 years. The early studies, commencing some 35 years ago, have indicated that NSAIDs, particularly indomethacin, can induce the chronic development of lesions in the jejunal-ileal region of the rat gastrointestinal tract after 1 to 3 days of acute or repeated administration (Kent et al., 1969; Somogyi et al., 1969; Thomas et al., 1969; Brodie et al., 1970; Robert, 1975; Fang et al., 1977; Whittle, 1981). This slowly developing intestinal injury, in contrast to the acute gastric mucosal defects and superficial lesions caused by NSAIDs that usually undergo relatively rapid repair with epithelial restitution, can result in a fulminant and lethal local inflammatory response, ultimately involving large sections of the small intestine. This extreme injurious response, seen particularly in rodents, is accompanied by the development of adhesions and eventual intestinal perforation, even after a single anti-inflammatory dose. Although clearly, the side-effect profile in the therapeutic use of these agents is not at all so severe, the processes leading the intestinal ulceration in the experimental models warrant careful consideration in the evaluation of the pathological basis of the intestinal injury from NSAIDs and the newer anti-inflammatory agents in patients.

Pathogenic factors that have been proposed from early studies as initiating events for the damage have included the enterohepatic circulation of NSAIDs, interference with oxidative processes, surface epithelial changes and local microvascular events (Whittle, 1992). Inhibition of protective prostaglandins in the intestine has been a common theme of many experimental studies (Robert, 1975; Whittle, 1992), although the precise role of cyclooxygenase (COX)-inhibition in such injury had not been clear from these early studies (Whittle, 1981). The effectiveness of antibiotics in reducing NSAID-induced intestinal lesions, and the resistance of germ-free rats to indomethacin-induced intestinal injury, suggested that bacteria and bacterial products was an inflammatory stimulus in the NSAID-induced enteropathy (Kent et al., 1969; Robert and Asano, 1977).

In the present review, the older literature and the more recent studies over the past decade that have aided in our understanding of this previously relatively ignored iatrogenic disease promoted by NSAIDs have been considered. An attempt has been made to reconcile the findings from all

these various reports in the formulation of a scheme describing the sequential pathological processes involved in intestinal injury and ulceration induced by NSAIDs.

2. Mechanisms involved with early phases of intestinal injury

2.1. Enterohepatic recirculation

A number of NSAIDs, including indomethacin, diclofenac and flurbiprofen, have been shown to undergo enterohepatic circulation in the rat and this process has been implicated in the resulting intestinal injury and ulceration (Yesair et al., 1970; Wax et al., 1970; Duggan et al., 1975; Reuter et al., 1997; Eeckhoudt et al., 1997). Such recirculation and excretion of these agents into the gut lumen via the bile thus produces a more prolonged exposure of the jejunal mucosa to high concentrations of the agents and their active metabolites. Following indomethacin administration for example, the upper intestinal lumen concentrations of indomethacin and its metabolites have been shown to be extremely high in comparison with the caecum, colon and even plasma levels in rats (Nygard et al., 1995). It is thus likely that these processes would increase the propensity of these NSAIDs to produce local epithelial injury and barrier dysfunction in the small intestine. Moreover, concurrent exposure to the surface-active properties of the secreted bile could promote this local luminal irritation.

The relative extent of this recirculation process may account for some of the differences between the oral and parenteral potency of these agents in causing injury in experimental studies. Thus, in experimental studies, indomethacin exhibited comparable activity in provoking rat jejunal macroscopic and microvascular injury over a 24-h period when given either orally or subcutaneously. However, the degree of this injury over 24 h by both diclofenac and flurbiprofen was somewhat greater when these agents were administered orally than when given by subcutaneous administration (Evans and Whittle, 2001).

A diclofenac derivative, nitrofenac that unlike the parent, does not undergo such enterohepatic circulation, causes far less macroscopic injury to the rat small intestine (Reuter et al., 1997), an effect also attributed in part to its nitric oxide releasing capacity (Reuter et al., 1994). It is not known whether this derivative forms an acyl glucuronide, the major biliary metabolite that is associated with enteropathy of the parent compound (Seitz and Boelsterli, 1998). Diclofenac given orally has been shown to cause small-intestinal enteropathy and inflammation in humans (Gut et al., 1996; Sigthorsson et al., 1998b). The atypical agents, aspirin or nabumetone that are not excreted in the bile, do not cause extensive rat intestinal macroscopic injury (Whittle, 1981; Reuter et al., 1997), although some macroscopic damage to the rat intestine following high doses of aspirin has been recorded in an early study (Brodie et al., 1970). Moreover, these latter two agents did not cause detectable changes in intestinal permeability or inflammation, as reflected by faecal excretion of radiolabelled white cells, in patients on long-term therapy with either of these drugs (Sigthorsson et al., 1998b). However, a causal relationship between the potential for intestinal injury and their enterohepatic recirculation in humans with a range of NSAIDs has yet to be established in the clinic.

2.2. Epithelial permeability changes

Administration of NSAIDs can cause significant changes in the permeability of the rat small intestine, as determined by the use of a range of markers (Zuccato et al., 1992, Davies et al., 1994, 1996; Reuter et al., 1997; Tibble et al., 2000). Such changes in permeability are considered to be a reflection of the change in epithelial integrity, as a prelude to gross ulceration. These permeability markers have also proved of substantial use in the clinical evaluation of small intestinal injury (Smale and Bjarnason, 2003).

Early clinical work had demonstrated that oral ingestion of aspirin, ibuprofen or indomethacin by healthy volunteers or rheumatoid patients increased the permeability of the small intestine to radiolabelled markers (Bjarnason et al., 1984, 1987a,b). This effect was also observed after rectal administration of these agents, indicating that this was a systemic action and not just a reflection of any local irritation. In these studies, over 60% of the subjects exhibited blood and protein loss with demonstrable inflammation of the small intestine (Bjarnason et al., 1987a,b). The extent of this mucosal injury was considered milder than that observed in patients with Crohn's ileitis. However, radiologically detected abnormalities in the ileum suggested that ulceration and strictures could ultimately develop on chronic administration of these agents. Other workers have shown that treatment with the typical NSAIDs, indomethacin, flurbiprofen or naproxen increased epithelial permeability of the human small intestine to labelled markers (Davies et al., 1996; Smecuol et al., 2001). In studies on intestinal permeability and inflammation in 68 patients receiving NSAIDs on a chronic basis, there appeared to be no difference between the propensity for such actions between a wide range of standard NSAIDs evaluated, excepting aspirin and nabumetone, nor were these events correlated with the duration of treatment (Sigthorsson et al., 1998b).

The local processes involved with these changes in epithelial cell permeability have been suggested to be comparable to those previously proposed to be involved with the surface irritancy of salicylates and NSAIDs on the gastric mucosa (Whittle, 1992, 2003a). One such concept on the nature of the topical effect is based on the very early findings that NSAIDs can uncouple oxidative phosphorylation and affect mitochondrial function (Whitehouse and Haslam, 1962; Mahmud et al., 1996; Sigthorsson et al.,

1998a; Somasundaram et al., 2000). Thus, it is suggested that in high concentrations such as those that may be achieved in the lumen after repeated dosage or enterohepatic recirculation, these agents cause uncoupling of this oxidative process. This leads to changes in mitochondrial energy production and hence a reduction in cellular integrity, promoting the observed changes paracellular permeability (Somasundaram et al., 2000).

Other work has also extended consideration of the changes in the known gastric mucosal protective properties of the overlying mucosal hydrophobic layer by NSAIDs, suggesting that similar actions operate in the intestine (Lichtenberger et al., 1995). Thus, it has been demonstrated that physically associating NSAIDs with zwitterionic phospholipids reduced their ability to provoke intestinal injury, an affect attributed to the reduction in local barrier disruption by the NSAIDs (Lichtenberger et al., 1995; Bertrand et al., 1999).

Experimental studies with known agents that uncouple or inhibit the oxidative processes suggest that the observed epithelial changes in integrity alone are insufficient to promote the extensive lesions seen with typical NSAIDs (Somasundaram et al., 2000). Others have likewise concluded from studies with diclofenac and nitrofenac that the initial epithelial injury as seen by permeability changes with both agents does not itself lead to subsequent frank intestinal ulceration (Reuter et al., 1997). Thus, it can be concluded that such epithelial injury with a concomitant change in permeability of the surface barrier is an early, and probably necessary, event in the pathogenic process, leading eventually to the characteristic intestinal lesions and ulceration induced by NSAIDs, but only if the subsequent stages in the overall process are initiated.

2.3. Early microcirculatory events

Changes in the local microcirculation have long been associated with gastric injury provoked by NSAIDs (Whittle, 1992, 2003a; Wallace, 1997) and it would appear highly probably that microvascular events would contribute to the pathogenesis of intestinal injury and ulceration by these agents. Thus, poorly vascularised sites along the mesenteric margin have been identified as being highly susceptible to the injury promoted by indomethacin (Anthony et al., 1997). Much of the experimental work has centred on the very early microvascular events in the superficial capillaries that may be the initiating step for the subsequent evolution of the ulceration and inflammation.

In a series of detailed studies using a number of elegant techniques, the very early microvascular events that follow indomethacin challenge have been evaluated in the rat jejunum. These studies have focused on the villus region, since vascular changes at this site would be expected to compromise the integrity of the epithelial layer lying immediately above. This work identified focal slowing of blood flow leading to localised stasis in the capillaries at the villus tip, events that preceded the loss of the epithelium at the villus tip (Nygard et al., 1994; Anthony et al., 1995; Kelly et al., 1998). In a very detailed study, the initial microvascular process occurring within 10 min of exposure to indomethacin involved the development of endothelial projections at the tip of the villus vascular arcade, without any change in villous blood flow or epithelial structure (Kelly et al., 2000). This was followed by a stepwise reduction in villous blood flow as the endothelial projections within the capillaries increased and occluded the vascular lumen, accompanied by endothelial vacuolisation, until stasis of blood flow in the villus tip was achieved by 45 min. Only at this time was degeneration of the overlying epithelial layer and its detachment from the lamina propria noted (Kelly et al., 2000).

Such studies thus clearly suggest that the initial changes in the microcirculation with endothelial projections and subsequent reduced capillary perfusion brought about by indomethacin reduce the functional integrity of the overlying epithelial cells, leading to their subsequent death and detachment, events that would reflect the local ischemia and cellular anoxia so produced. These latter events causing dysfunction of the epithelial cells may also be associated with the proposed concurrent action of the NSAIDs on the oxidative processes of these cells. Thus, both the reduction in villous blood flow and a more-direct local epithelial metabolic injury may interact, perhaps synergistically, to promote the focal cellular damage and progressive disruption to the epithelial barrier.

The biochemical basis and the mediator systems involved in these initial events at the level of the microvascular endothelium, and whether they are shared by other NSAIDs, are not known. Effects on the oxidative process of the villus endothelium, as with the epithelium, cannot be entirely excluded. Direct vasoconstrictor or other effects on vessel diameter would appear unlikely in this capillary network, although effects on the submucosal microcirculation having secondary actions on villous blood flow cannot be excluded. The effects of NSAIDs on the gastric microcirculation are well documented, which are considered to reflect the reduction of locally produced vasoactive prostanoids such as prostacyclin (Whittle, 1992) through inhibition of the COX-1 isoform (Wallace et al., 2000). It is not known if such a role for COX, particularly COX-1, products also operate in the intestinal microcirculation, and how they would interact with other vasoactive mediators such as nitric oxide (NO) in the regulation of the supporting blood flow essential for the maintenance of the epithelial barrier integrity.

2.4. Enteral bacteria and endotoxins

Disruption of the epithelium may not itself be a dramatically injurious action to the intestinal mucosa, as these cells may well undergo rapid repair with restitution, as in other regions of the gut, and hence restore the continuity

of the epithelial barrier. However, the progressive loss of structural integrity of the epithelial barrier and the consequent increased intestinal permeability would allow the underlying mucosal tissues to be exposed to the gut-luminal contents including antigens and microorganisms. Early work with antibacterial agents and with germ-free rats led to the suggestion that indomethacin-induced enteropathy involves luminal bacteria (Robert, 1975, 1977; Fang et al., 1977). It has also been shown that NSAIDs give rise to the overgrowth of Gram-negative bacteria (Kent et al., 1969; Yamada et al., 1993), and increased luminal numbers of enteric bacteria (Reuter et al., 1997) although the mechanisms underlying this latter response are not fully clear.

All these findings suggest that indigenous bacteria, unrestricted by the epithelial barrier, will migrate into the mucosal tissue. Indeed, high power magnification of the jejunum from rats, 24 h after indomethacin administration showed predominantly Gram-negative bacteria adhering and invading to the mucosa (Evans and Whittle, 2001), while others have also noted an enterobacterial invasion into the jejunum 24 h after indomethacin (Tanaka et al., 2002b). It has also been noted that alterations in intestinal motility may be associated with this bacterial translocation from the gut lumen and eventual intestinal injury (Tanaka et al., 2002b).

In an early study where bacterial overgrowth of *Escherichia coli*, *Bacteroides* and *Clostridium* was observed, reduction in lesion severity was achieved using a mixture of neomycin, polymyxin B and bacitracin (Kent et al., 1969). In other work, pretreatment with the broad-spectrum antibiotic ampicillin, or the anti-anaerobic metronidazole, prevented the development of intestinal mucosa injury and microvascular permeability (Whittle et al., 1995; Evans and Whittle, 2001).

These findings can also be extended to the clinical setting. Studies in human volunteers have thus demonstrated that metronidazole can reduce the small-intestinal injury from NSAIDs, as determined by changes in the excretion of radiotagged erythrocytes and white cells and by intestinal permeability probes (Bjarnason et al., 1992; Davies et al., 1992).

Polymyxin B, which binds to, and hence inactivates, lipopolysaccharide also prevents the onset of tissue injury in the small intestine (Whittle et al., 1995; Evans and Whittle, 2001). It is possible that the local release of the lipopolysaccharide from the translocated bacteria in the intestinal mucosa is the stimulus for the observed local production of the cytokines, including tumour necrosis factor-α, in the tissue following NSAIDs (Bertrand et al., 1998). Moreover, an inhibitor of cytokine production has been shown to reduce indomethacin-induced intestinal injury (Konaka et al., 1999a). The production of these pro-inflammatory cytokines can provoke a cascade of events that would bring about the subsequent protracted phase of the development of intestinal tissue injury, including the expression and up-regulation of a number of inflammatory genes. Thus, the translocation of enteral bacteria and the subsequent tissue and cellular responses to this intramucosal challenge appears to be an essential phase towards the development of the gross intestinal enteropathy and inflammatory response.

2.5. COX inhibition

There has been much discussion of the role of COX inhibition in the pathogenesis on gut damage, both in the stomach and intestine (Whittle, 2003b). It has been long known that aspirin, in doses sufficient to bring about a substantial inhibition of COX, does not cause a marked enteropathy by comparison with the other classical NSAIDs such as indomethacin (Whittle, 1981). More recent work has indicated that parental aspirin does not affect rat intestinal mitochondrial function nor cause changes in intestinal permeability (Somasundaram et al., 2000). However, when combined with the uncoupler of oxidative phosphorylation, dinitrophenol, that alone causes epithelial disruption but not macroscopic ulceration, the typical NSAID-provoked intestinal damage and inflammation was observed following aspirin administration (Somasundaram et al., 2000). Such findings of an interaction between these two classes of effects in the development of intestinal ulcers echo those in the gastric mucosa (Whittle, 1992, 2003a), indicating that both a topical irritant action affecting epithelial integrity and COX inhibition is involved in the subsequent extensive gut injury.

The exploration of the involvement of the inhibition of both the COX isoforms, COX-1 and COX-2 in intestinal injury has also followed on from observations in the rat gastric mucosa (Wallace et al., 2000), where inhibition of both isoforms has been shown to be required for the gastric damage. Thus, recent detailed studies in rats using COX-1 and COX-2 inhibitors have demonstrated that inhibition of both isoforms is required for the development of the characteristic jejunal lesions seen over 48 h with NSAIDs (Tanaka et al., 2002a,b). Studies with an isoform selective COX-1 inhibitor suggested that it fails to provoke gross intestinal inflammation, as there is up-regulation of COX-2 with the production of prostanoids. This perhaps reflects a compensatory response to COX-1 inhibition provoked by an unknown mechanism, but which appears to attenuate the processes leading to ulceration. Thus, concurrent administration of a selective COX-2 inhibitor, which itself did not cause ulceration but which attenuated the subsequent production of the prostanoids by the COX-1 inhibitor, did lead to the typical intestinal injury and ulceration seen with NSAIDs (Tanaka et al., 2002a,b).

These studies are complemented by other elegant work using isoform-selective COX inhibitors in selective COX gene-deleted mice (Sigthorsson et al., 2002). Like rats, mice develop intestinal lesions following administration of NSAIDs. Moreover, indomethacin caused similar injury in either COX-1 or COX-2 gene-deleted mice, while in wild-

type mice, neither a selective COX-1 or COX-2 inhibitor caused any significant intestinal ulceration. However, administration of a COX-1 selective inhibitor in COX-2 knockout mice, or a COX-2 inhibitor in COX-1 knockout mice, did cause a similar degree of intestinal ulceration as did indomethacin (Sigthorsson et al., 2002). Thus, the use of both gene-deletion and pharmacological probes in rodents indicates the obligatory requirement for the activity of both COX isoforms to be attenuated, for the development of intestinal ulceration. Further studies with such isoform-selective inhibitors on the full time course of events leading to ulceration are needed to clarify whether both COX isoforms must be simultaneously inhibited, which may help identify a temporal relationship between COX-dependent processes and intestinal damage.

2.6. Role of COX products

Since the inhibition of the two COX isoforms appear to be a key stage in the early to mid-term events in the pathology of the intestinal ulceration caused by NSAIDs, it is important to understand the role the endogenous products made by these isoform. It is likely that they are involved in the maintenance of the integrity of the intestinal mucosa, as well as limiting the various detrimental events during these phases. Both COX isoforms are generally capable of synthesising a similar range of products, particularly the prostanoids, which can have a wide profile of protective actions on gut function. Such beneficial effects include enhancing epithelial continuity, increasing mucus and phospholipid secretion, stimulating electrolyte transport as well as effects on the microcirculation (Whittle, 1992). All of these actions may influence the functional integrity of the intestinal mucosa in its secretory and barrier roles, interference of which could be envisaged to lead to the changes in epithelial permeability and microvasculature observed in the early stages of the NSAID-induced intestinal ulceration.

Identification of the relative roles of the products made by the COX-1 and COX-2 informs in the small intestine will require use of effective pharmacological and gene probes. Studies with an experimental selective COX-1 inhibitor have so far suggested that COX-1 inhibition can promote bacterial invasion into the intestinal mucosa, an effect not shared with a selective COX-2 inhibitor alone (Tanaka et al., 2002b). Thus such events may well reflect a predominant role for the products of COX-1, compared with those from COX-2 produced constitutively or in response to challenge, in the maintenance of the epithelial barrier integrity.

Regarding the vascular events, it could be considered that as in the gastric mucosa (Wallace et al., 2000) the microvascular blood flow in the intestinal mucosa is meditated or modulated by the vasoactive prostanoids such as prostaglandin E_2 and prostacyclin formed by COX-1, while the products of COX-2, assumed to be prostanoids such as prostacyclin, could be involved in the regulation of neutrophil adherence to the microvascular epithelium. However, the use of COX isoform selective inhibitors in studies on intestinal mucosal and submucosal blood flow and intravascular cell adherence are required to define these relative roles and help explain the requirement for concurrent COX-1 and COX-2 inhibition in the development of intestinal enteropathy.

2.7. Involvement of inflammatory cell infiltration

The involvement of neutrophils in the pathogenesis of the intestinal lesions produced by NSAIDs, as with the injury in the gastric mucosa (Wallace, 1997; Wallace et al., 1990), has been studied using a number of different approaches. Thus, significant infiltration of neutrophils was observed by morphological evaluation in the rat intestine, some 6 h after administration and this was associated with an increase in the leukotriene production (Nygard et al., 1994). Using radiolabelled neutrophils, a more recent study have shown that infiltration of these cells into the intestinal mucosa and lumen could be detected by 6 h after indomethacin challenge, and continued to accumulate for 48 h (Stadnyk et al., 2002).

In a study on the site-selective actions of NSAIDs, myeloperoxidase activity as an index of neutrophil infiltration, was increased in the jejunum 24 to 72 h following subcutaneous indomethacin administration, but remained unchanged in the ileum and colon over this 72-h period (Evans et al., 2000). Earlier studies had showed increased myeloperoxidase activity in intestinal mucosa at 6, 12 and 24 h after indomethacin (Miura et al., 1991; Yamada et al., 1993), although in some animals, there were changes in mucosal permeability at 24 h with no elevation of myeloperoxidase activity (Yamada et al., 1993).

In functional studies, local perfusion of the rat jejunum with indomethacin caused an increase in clearance of a radiotagged probe, and an effect that was reversed by depletion of neutrophils with an antisera (Chmaisse et al., 1994). Not all studies with neutrophil-depleting antibodies, however, have shown effectiveness in preventing intestinal injury by NSAIDs. Thus depletion of circulating neutrophils to less than 7% by an antisera did not affect the gross intestinal mucosal injury induced by multiple dose of indomethacin, although it could suggest that neutrophil recruitment in that model was a consequence of the lesion formation rather than the cause (Yamada et al., 1993). Similarly in other work on injury induced in the gut by NSAIDs, a marked infiltration of neutrophils was only seen in the ileal region, while depletion of neutrophils with an antiserum failed to affect gut injury (Melarange et al., 1995). Other studies have, however, demonstrated that a reduction in neutrophil count by an antisera does limit the extent of intestinal injury (Konaka et al., 1999a).

More recent work also supports the role of neutrophils in the intestinal injury and inflammation provoked by

NSAIDs. Thus, in a study in mice, indomethacin-induced intestinal injury was shown to be dependent on leukocyte recruitment and activation, while T cells and B cells were not critical for the injury (Beck et al., 2000). In another study to investigate the role of cell adhesion factors in the intestinal injury, the expression of the endothelial intracellular adhesion molecule-1 (ICAM-1) in intestinal tissue was elevated 6 and 24 h after administration of a single dose of indomethacin to rats, while an early increased expression of ICAM-1 was noted in the intestinal mucosa but not muscle. A more complex picture of ICAM-1 expression emerged following multiple doses, although immunoneutralization of ICAM-1 reduced the overall intestinal injury (Krieglstein et al., 2001). The expression of mRNA for a number of factors involved with chemoattraction, interleukin 1β, tumour necrosis factor-α and monocyte inflammatory peptide 2 was also detected (Stadnyk et al., 2002).

Expression of the ICAM-1 ligand CD11b/CD18 on leukocytes was also shown to be enhanced, when determined after 48 h after indomethacin challenge, while an antibody to CD11b attenuated the intestinal injury (Krieglstein et al., 2001). In another study, the accumulation of radiotagged neutrophils was attenuated by antibodies to both CD11a and CD 11b (Stadnyk et al., 2002).

It is probable that the infiltration of neutrophils in the intestinal mucosa following NSAID administration is involved in the later stage development of microvascular injury associated with the inflammatory response, and is involved in the release of cytotoxic moieties and reactive oxygen species, causing damage to the vascular endothelium and underlying tissue, while interacting with other local inflammatory mediators.

2.8. Late changes in microvascular permeability

Although early endothelial changes have been observed involving the intestinal villus tip following acute indomethacin challenge (Kelly et al., 2000), detectable microvascular leakage of radiolabelled albumin in the small intestine, as an index of endothelial injury, was not detected until some 18 h after indomethacin administration (Whittle et al., 1995). Indomethacin, diclofenac and flurbiprofen, when administered orally or subcutaneously likewise increased the microvascular leakage in the rat jejunum determined 24 h after challenge. The onset and time-dependent increase in macroscopic tissue damage, observed in the jejunum, correlated with the onset of the microvascular leakage of radiolabelled albumin (Evans and Whittle, 2001).

In a study on the site-selectivity of the injury to the intestine, the time-dependent increase in vascular leakage and myeloperoxidase activity was closely associated in the jejunum (Evans et al., 2000). Moreover, the lack of increased microvascular leakage in the rat ileum, colon and caecum following indomethacin, accords with the lack of both macroscopic tissue damage and the increase in myeloperoxidase activity these latter tissues.

2.9. Critical role of NO in lesion development

2.9.1. Modulator role of constitutive NO synthase

Nitric oxide (NO) is a potent vasodilator and is a key physiological modulator of vascular tone produced by vascular endothelial cells through activity of a constitutive NO synthase, eNOS (Alderton et al., 2001). NO also modulates the permeability and integrity of the vascular endothelium, being a potent inhibitor of white cell adhesion (Arndt et al., 1993). It is assumed that this latter action of NO on microvascular integrity reflects a physiological function although this role may be more apparent under pathological conditions including low-grade trauma or acute inflammation. Indeed, concurrent inhibition of constitutive NO synthase leads to an acute substantial increase in microvascular injury within 1 h following indomethacin, suggesting that early events following challenge by NSAIDs are down-regulated by NO formed by a constitutive NO synthase, probably the eNOS isoform (Laszlo and Whittle, 1998).

2.9.2. Role of iNOS

By contrast, the excessive production of NO in the gut by the inducible isoform, iNOS, following challenge with endotoxin is involved in elevated microvascular permeability in the gastrointestinal mucosa and in epithelial cytotoxicity (Boughton-Smith et al., 1993; Tepperman et al., 1993; Lamarque et al., 2000). Unlike the constitutively expressed isoforms, iNOS (also termed NOS II or NOS-2) is induced by cytokines and bacterial lipopolysaccharides, and once expressed, has the ability to produce sustained and substantial amounts of NO (Whittle, 1994, 1997).

Although it is a highly labile moiety and rapidly decomposes to relatively inert products such as nitrite and nitrate, NO can also interact with the reactive oxygen moiety, superoxide, to form peroxynitrite which can also decompose to the reactive hydroxyl radical (Beckman et al., 1990). Peroxynitrite is a highly cytotoxic species that oxidises a number of key molecular species including ascorbate, sulphydryls and thiols, producing membrane lipid peroxidation, causing DNA injury and activating poly (ADP)-ribose synthase (Radi et al., 1991; Virag and Szabo, 2002). Thus, a number of cytotoxic moieties can be potentially generated from NO in the inflammatory environment, where reactive oxygen species are concurrently produced by both inflammatory cells and the involved tissue (Moilanen et al., 1999).

In experimental studies, a time-dependent expression of iNOS activity was observed in the jejunum, following subcutaneous administration of indomethacin in the rat, which preceded the elevation of jejunal microvascular leakage (Whittle et al., 1995). The expression of iNOS observed in the jejunum was not seen in the ileum, colon or

caecum of the rat, correlating with the site of detectable macroscopic jejunal damage and microvascular leakage (Evans et al., 2000). The iNOS activity, the microvascular leakage and the macroscopic damage was reduced by the antibacterial agents, ampicillin, metronidazole and polymyxin B, none of these agents themselves directly inhibiting the iNOS enzyme in vitro (Whittle et al., 1995; Evans and Whittle, 2001). This microvascular injury was also attenuated by an isoform nonselective NO synthase inhibitor, when administered at the time of iNOS expression and also in other studies, by a highly selective iNOS inhibitor (Whittle et al., 1995; Evans and Whittle, 2001). Other groups have confirmed these findings that subcutaneous indomethacin administration leads to iNOS expression and increased iNOS activity in the rat small intestine associated with the development of intestinal lesions (Bertrand et al., 1998; Konaka et al., 1999a,b; Chen et al., 1999; Tanaka et al., 2002b).

2.9.3. Interaction of NO with oxygen species

Endogenous superoxide may be generated from a number of cellular sources and enzymes including local tissue as a result of activation of xanthine oxidase. Since neutrophils are also considered to be a major cellular source of superoxide generation, it is of relevance that neutrophils are considered to play a later role in the development of intestinal damage, as described above. In addition, in vivo microscopy techniques have identified changes in blood flow and neutrophil rolling in the intestinal microcirculation some 6–12 h following indomethacin administration (Miura et al., 1991).

A role for reactive oxygen metabolites has been suggested from studies using the xanthine-oxidase inhibitor, allopurinol, which reduced the intestinal injury provoked by indomethacin (Konaka et al., 1999b). Piroxicam-induced intestinal injury was also shown to be associated with an increase in tissue xanthine oxidase activity with a concomitant decrease in superoxide dismutase (SOD) activity and glutathione levels (Villegas et al., 2001). In more direct studies with the longer acting pegylated superoxide dismutase formulation, SOD-PEG, this agent substantially attenuated the intestinal microvascular and macroscopic injury following indomethacin challenge (Evans and Whittle, 2001). Such findings, along with the effectiveness of the selective iNOS inhibitors to prevent the development of such lesions, thus point these process as a final pathway, with the key role for both NO and superoxide, and hence of peroxynitrite, in the pathogenesis of NSAID-induced intestinal injury and ulceration.

3. Systemic inflammatory response following NSAIDs

Although much experimental work has probed the development of the enteropathy after a single dose of NSAIDs, studies using multiple doses show a more extreme, sometimes highly lethal progression of this damage (Yamada et al., 1993; Reuter et al., 1997). Moreover, administration of indomethacin for just 2 days provoked the expression of iNOS in the lung, liver, spleen and kidney, as well as in the jejunum, caecum, colon and ileum. Such widespread expression of iNOS was associated with an increase in microvascular leakage in these organs located both within and outside the gut, thus contrasting with the localised actions on the jejunum following a single dose of indomethacin (Evans and Whittle, 2003).

Pretreatment with the antibacterial agents, metronidazole or ampicillin as well as polymyxin B, significantly inhibited microvascular injury and the expression of iNOS activity in all the organs studied, indicating the involvement of enteral bacteria in these events (Evans and Whittle, 2003). Although the microvascular leakage in all organs was reduced by a highly selective iNOS inhibitor, it is likely that the combination of superoxide and NO to form the cytotoxic peroxynitrite radical is responsible for the tissue injury, as in the more-acute indomethacin-induced enteropathy (Evans and Whittle, 2001, 2003).

The provocation of microvascular leakage as a consequence of the expression of iNOS, in organs remote from the initial site of injury in the jejunum is thus likely to be dependent on bacteria, bacterial endotoxins or lipopolysaccharide entering the systemic circulation from the extensively damaged gut mucosa. There is a relationship between gut-derived bacteria and the development of the multiple organ dysfunction syndrome (Nieuwenhuijzen et al., 1996). Moreover, urinary nitrate levels have been considered as a marker of bacterial translocation in the gut (Oudenhoven et al., 1994). These actions of enterally derived bacterial products are likely to be augmented by the production and release into the circulation of pro-inflammatory and stimulatory mediators such as cytokines by the inflamed jejunum.

Such severe widespread disease following these agents, reminiscent of systemic inflammatory response syndrome or SIRS (Qureshi et al., 2001), is likely to be species specific as rodents being highly susceptible to the intestinal injury with NSAIDs, while these extreme effects have not been reported in humans.

It is also feasible that such referred induction of iNOS could be involved in other aspects of intestinal disease occurring at other sites. It has been reported that in COX-2 gene-deleted mice ileocaecal lesions can also be detected, as also found with a long-term treatment with NSAIDs as well as COX-2 inhibitors (Sigthorsson et al., 2002). Others have also reported that caecal lesion can occur in rats on long-term treatment with NSAIDs (Nygard et al., 1995) or after 24 h with an experimental anti-inflammatory agent (Rainsford, 1988). The pathological processes underlying these latter gut lesions, which appear to be distinct from those in the jejunum associated with NSAIDs administration, have not been identified. They appear to be independent of any

local irritant action since these actions occurs in those animals not receiving NSAIDs but made deficient in COX-2 by gene-deletion, and could reflect this process of referred induction at sites distinct from the primary site of infection and inflammation in the jejunum.

4. Sequential mechanisms involved in NSAID-enteropathy

In an attempt to reconcile the myriad of events and processes that have been described to occur in the small intestine following challenge with NSAIDs, the following sequence of events that lead to the characteristic intestinal injury can be discerned. This pathological pathway indicates a close temporal inter-relationship between all of these processes leading to the eventual intestinal inflammatory enteropathy.

The experimental studies thus suggest that the initial changes in the villous microcirculation seen within minutes of exposure to indomethacin, with progressive occlusion and blood flow stasis, lead to a reduction in the functional integrity of the overlying epithelial cells, causing their subsequent death and detachment. Whether this initial vascular response is a consequence of inhibition of the synthesis, release or activity of vasoactive products derived from the COX-1, COX-2 or both isoforms, or the involvement of other vasoactive mediators is not yet clear. These latter changes in epithelial integrity may also be associated with the concurrent action on the oxidative processes of these cells, with the reduction in blood flow enhancing or synergising with this local epithelial metabolic injury.

The development of the local topical irritant and biochemical actions of NSAIDs appear to require the prolonged exposure of the intestinal mucosa to the drug, and this may be achieved as a consequence of enterohepatic recirculation, at least in rodents. Bile, if present, also may play a role in augmenting these early irritant actions. The superficial and focal epithelial injury that is seen within an hour of exposure to a NSAID, if not attenuated, leads to the gradual dysfunction of the mucosal barrier, and this can be detected by changes in permeability to probes in both experimental and clinical studies. These early processes that occur over the initial hours following challenge appear to be modulated by endogenous mediators, such as NO formed constitutively, and also possibly constitutive COX-1 products such as prostaglandin E_2 or and prostacyclin.

The progressive change in intestinal epithelial barrier integrity subsequently allows the ingress of indigenous bacteria and their products into the mucosa. A key role for such bacteria is evident from both experimental and clinical studies, and it is considered that the bacterial products such as lipopolysaccharide, initiate the cascade release of other pro-inflammatory mediators such as the cytokines, as well as altering intestinal motility. These mediators in turn lead to the activation of tissue cytotoxic and destructive processes, a key feature being the expression of iNOS. Such events appear to be slow in onset and experimental evidence suggests that they take place from 6 to 18 h following challenge with indomethacin, the time needed for the bacterial translocation into the mucosa though the damaged epithelial barrier, mediator synthesis and release, and the consequent known slow expression of iNOS.

The concurrent infiltration of neutrophils into the intestinal mucosa during this phase of the pathogenic process further augments the progressive injury, one such important function being possibly the elaboration of reactive oxygen moieties such as superoxide, while injured endothelial cells can also be a source of these reactive species. While such mediators will themselves bring about tissue injury, superoxide in combination with the exaggerated and prolonged production of NO from iNOS will form the highly destructive species peroxynitrite. The promotion of the intestinal microvascular leakage of proteins, as an index of endothelial injury, is associated with the onset of iNOS expression and activity. The experimental studies with both superoxide dismutase and inhibitors of iNOS, especially when administered at the later phases, strongly implicates peroxynitrite as the final highly damaging moiety in the development of the intestinal microvascular and macroscopic injury that leads to the extensive inflammation and ulceration.

5. Conclusions

Studies with pharmacological tools at the various stages of the proposed pathological sequence suggest that it is feasible to alter the subsequent progression of events leading to the characteristic intestinal injury and fulminant inflammation caused by NSAIDs. Thus, interference with the proposed near-final stage of peroxynitrite production, achieved with specific pharmacological inhibitors directed at iNOS or scavenging superoxide or other reactive oxygen species including peroxy-radicals themselves, as well as the reduction in neutrophil infiltration are all effective in the experimental setting in reducing the gross tissue injury, as discussed above.

The use of antibacterial agents to limit enteral translocation has shown efficacy against the macroscopic and microvascular intestinal injury in the experimental model. Moreover, they can limit the intestinal permeability changes of markers in clinical pharmacological studies (Bjarnason et al., 1991; Davies et al., 1992). Natural and synthetic prostanoids such as prostaglandin E_2 or misoprostol can also reduce the development of intestinal ulceration in rodents and also the permeability changes in humans (Robert, 1975; Bjarnason et al., 1989).

Predicated on the role of COX-1 inhibition in the development of gastrointestinal injury by NSAIDs, the pharmacological targeting and pharmaceutical development of isoform-selective COX-2 inhibitors provided a

logical progression for novel anti-inflammatory therapeutic agents. Regarding the intestine, the experimental findings in animal models support the reduced side-effect profile of the agents that preferentially inhibit COX-2 as well as the subsequent more-highly selective coxibs, celecoxib and rofecoxib (Tibble et al., 2000; Tanaka et al., 2002a). However, experimental work also suggested that rather than this beneficial therapeutic ratio being just the consequence of retaining endogenous gut prostanoids by failing to inhibit COX-1 activity, the pathological process for characteristic intestinal ulceration required both COX isoforms to be inhibited (Sigthorsson et al., 2002; Tanaka et al., 2002a,b). This latter finding would not, however, alter the observed attenuation of the potential of such new anti-inflammatory drugs to cause injurious events on the intestine, although its basis will need mechanistic redefinition.

Importantly, the clinical pharmacology of the COX-2 inhibitors conducted so far support the reduction of undesirable action of the intestine. Studies with the atypical NSAID, nimesulide in healthy volunteers, considered more selective for inhibition of COX-2 indicated that it had less effect than did naproxen in promoting excretion of calprotectin, a maker of intestinal inflammation (Shah et al., 2001). Studies with permeability probes in healthy volunteers indicated that celecoxib had no action in promoting intestinal permeability as compared to the changes seen with naproxen or indomethacin after a 2-day treatment regimen, although meloxicam, considered to have preferential COX-2 selectivity, did cause alterations in intestinal permeability (Smecuol et al., 2001). In an investigation of the intestinal actions of a 7-day, once daily, treatment protocol in healthy volunteers, rofecoxib, evaluated at dose levels greater than the therapeutic dose, did not cause any change in intestinal permeability, whereas indomethacin, as anticipated, caused a substantial increase (Sigthorsson et al., 2000).

These clinical pharmacology findings from studies in volunteers are supported by a large study in rheumatoid patents taking either naproxen or rofecoxib for 1 year. The rates of serious gastrointestinal events with rofecoxib was 54% lower than with naproxen, while the actual rates of lower gastrointestinal serious events accounted for 39% and 43% of the total serious events in the naproxen and rofecoxib cohorts respectively (Laine et al., 2003). Thus, the coxib group that showed as substantially reduced propensity for gut injury compared to a typical NSAID, although there still remained a component of the overall serious gastrointestinal events that reflected intestinal injury with this new class of anti-inflammatory analgesic.

Whether the COX-2 inhibitors show less intestinal adverse events as a consequence of a lack of inhibitory action on the gut COX-1 isoform, or their physicochemical properties that allows them to avoid luminal local irritation, oxidative metabolic changes and barrier disruption, awaits further evaluation. The fact that they do not affect platelet COX-1 activity, thromboxane formation and platelet aggregation, and hence have no effect on the haemostatic processes involved in gut bleeding may also be a contributory factor, although this lack of platelet activity is also considered to be a risk factor in co-existing cardiovascular disease. How the second and third generation of coxibs will compare to this already reduced intestinal side-effect profile of these now-established coxibs is awaited with interest.

The past decade has brought significant findings to help understand the complex sequence of events that underlies the intestinal pathology that results from treatment with the classical NSAIDs. The temporal relationship of each phase of this sequential process of enteropathy, as outlined above, has support from a range of experimental approaches. Those aspects that have already been evaluated in the clinical setting, including changes in epithelial permeability, the involvement of inflammatory cells and enteral bacteria, as well as the involvement of COX inhibition and its isoforms, give support to the working concept. Such information should help in the design of new anti-inflammatory agents and aid in the prediction of their potential side-effect profile on the small intestine.

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