Indomethacin increases susceptibility to injury in human gastric cells independent of PG synthesis inhibition

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Nonsteroidal anti-inflammatory drugs (NSAIDs), because of their analgesic and anti-inflammatory actions, are the most frequently used medicines in the world and account for nearly 5% of all prescribed medications (34). However, the clinical efficacy of NSAIDs is not without adverse side effects. It is well accepted that NSAID administration is associated with gastrointestinal (GI) complications ranging from dyspepsia and abdominal pain to bleeding and perforated ulcers. The incidence of serious or life-threatening complications associated with NSAID use is 1–2% per NSAID user per year (10).

In the early 1970s, Sir John Vane proposed that aspirin (acetylsalicylic acid; ASA) and other NSAIDs inhibit PG synthesis and that this intrinsic property may account for both their anti-inflammatory properties and their ability to induce GI injury (41). After that original proposal and as a result of numerous studies, it has become widely accepted that the GI complications induced by NSAIDs are caused in large part by the systemic effects of endogenous PG inhibition (15, 25, 30, 44). Suggested mechanisms whereby PG inhibition results in GI injury include the reduction of mucus and/or bicarbonate release and decreased mucosal blood flow (26). However, several studies indicate that both the therapeutic actions and the ability of NSAIDs to promote injury may not only be related to their capacity to inhibit PG synthesis but may also involve additional mechanisms (1, 24, 42, 43).

We have recently investigated adaptive cytoprotection under in vitro conditions in a human gastric cell line (22). Adaptive cytoprotection can be defined as the process whereby administration of a low concentration of a damaging agent (not damaging by itself) is able to attenuate injury to GI mucosa on subsequent exposure to higher concentrations of the same or differing damaging agents. Our previous data suggested that adaptive cytoprotection exists under in vitro conditions independent of intact blood flow, neural innervation, or circulating humoral mediators. Furthermore, this protective response did not appear to be dependent on endogenous PG synthesis. However, our data also suggested that pretreatment of human gastric cells with indomethacin reversed protection conferred by the mild irritant and also appeared to increase cellular susceptibility to injury. These findings suggested one of two possibilities: that either the inhibition of basal PGs significantly disrupts cellular integrity (as has been suggested in the literature for the past several decades) or indomethacin may have other actions in addition to promoting mucosal injury.

It has been proposed that calcium (Ca$^{2+}$) homeostasis is critical in maintaining mucosal integrity (37) and that this cation plays a major role in promoting mucosal injury induced by a variety of noxious agents (38, 39). This relationship between Ca$^{2+}$ and cellular injury is by no means specific to GI mucosae and has been described in many other cell types initiated by diverse causes of injury including ischemia-reperfusion, chemical exposure, radiation, and infection (9). The objective of this study was to determine if indomethacin increases...
cellular susceptibility to injury through the inhibition of PGs or acts through an alternate mechanism and what role Ca\(^{2+}\) may play in this process. Although indomethacin has limited use in the clinical arena and its popularity has decreased recently with the advent of newer and safer NSAIDs, this cyclooxygenase inhibitor has been and will continue to be extensively employed in the research setting to indirectly deduce the physiological role of PGs. For this reason the current study is highly relevant.

**MATERIALS AND METHODS**

Cells. The human gastric cell line, known as AGS (CRL 1739), was obtained from American Type Culture Collection (Rockville, MD) at passage 49. We have previously characterized this cell line morphologically and have determined AGS cells to be quite similar to gastric mucous cells (PAS\(^{+}\), alcin blue\(^{+}\)) with an ability to differentiate when postconfluent (22). Cells were maintained at 37°C in an atmosphere of 5% CO\(_2\) and 100% relative humidity. Cells were split on a weekly basis at a ratio of 1:6 on reaching confluence and were detached using 0.5 g porcine trypsin and 0.2 g EDTA tetrasodium per liter Hank's balanced salt solution (HBSS), and then plated into either 24- or 48-well plates (Costar, Cambridge, MA) for experiments or into 150-cm\(^2\) flasks for propagation. Cells grown for permeability work were split at a ratio of 1:2 into 3-µM Biocoat Collagen I Cells Culture Inserts (0.3-cm\(^2\) growth area; Becton Dickinson Labware, Bedford, MA), and experiments were performed at 7 days postconfluence. All other experiments were performed at 1 day postconfluence. Cell passage was maintained between 50 and 65, and medium was changed every 2–3 days. AGS medium consisted of Ham's F-12 supplemented with 10% fetal bovine serum, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B.

Solutions. Before all experiments, the medium was aspirated and replaced with HBSS plus 10 mM HEPES (H-8264, Sigma Chemical, St. Louis, MO). Experiments involving Ca\(^{2+}\)-free buffer utilized HBSS plus 10 mM HEPES and 2 mM 1,2-bis(2-aminophenoxy)ethane N,N,N',N'-tetraacetic acid (H-6648, Sigma, 137 mM NaCl, 5.7 mM NaHCO\(_3\), and 5.3 mM KCl). All test compounds were dissolved in either HBSS or Ca\(^{2+}\)-free HBSS. N,N',N'-tetakis(2-pyridylmethyl)ethylenediamine (50 µM, Molecular Probes) was used in all solutions as a heavy metal scavenger (19). Throughout the experiment, cells were maintained at a temperature of 37°C with a heated stage.

Measurement of cellular injury. Cellular injury was quantitated using two different assays, one measuring plasma membrane integrity and the other measuring cytoplasmic enzyme leakage. We employed the fluorescent agent ethidium homodimer-1 (Et; 8 µM, Molecular Probes) to monitor plasma membrane integrity. Et enters cells through damaged membranes and exhibits enhanced fluorescence on binding to nucleic acids (18). This fluorescent probe produces a bright red fluorescence in dead cells which was measured with a fluorescent multiwell plate reader at 485 nm excitation and 620 nm emission wavelengths. Injury is reported as relative fluorescence.

Cellular injury was also assessed by release of lactate dehydrogenase (LDH) into the buffer. LDH content was determined using the CytoTox 96 assay (Promega, Madison, WI), which is based on a coupled enzymatic reaction that results in the conversion of a tetrazolium salt into a red formazan product. The amount of red formazan product is directly proportional to the amount of LDH in the buffer. After the reaction the formazan product was quantitated spectrophotometrically by measuring its absorbance at 490 nm (Bio-Rad model 3550 microplate reader; Hercules, CA) in 96-well plates (Costar). Total protein concentration was also quantitated with a bicinchoninic acid (BCA) protein assay kit (Pierce Chemicals, Rockford, IL), and data are presented as µU/mg protein.

PG synthesis. Newly synthesized PGs are not stored intracellularly but released into the extracellular space. For this reason, we used the following protocol to quantitate PG synthesis in AGS cells. After the respective experiment buffer was immediately transferred to microcentrifuge tubes and stored at −80°C, and plates containing cells were immediately frozen for protein determinations. Samples were then thawed, and PGE\(_2\) content was assayed using a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). This assay utilizes an acetylcholinesterase tracer and a specific PGE\(_2\) monodonal antibody. PGE\(_2\) concentrations were determined by spectrophotometric analysis of all samples after addition of Ellman's reagent and compared with standard curves generated under identical conditions. Levels of other eicosanoids [PGF\(_2\alpha\), leukotriene B\(_4\) (LTB\(_4\)), and thromboxane B\(_2\) (TXB\(_2\))] were measured in a similar fashion. Total protein concentration per well was measured colorimetrically.
with BCA protein assay kits (Pierce Chemicals) to determine picogram per milligram protein. To optimally evaluate the efficacy of the various cyclooxygenase inhibitors, PGE2 release in response to a positive agonist (20 µM digitonin) was quantitated.

Permeability. Permeability of the epithelial cell monolayer was quantified by measuring the apical-to-basolateral flux of Texas Red conjugated BSA (66 kDa, Molecular Probes). During the respective treatment fresh HBSS (750 µl) was pipetted into the basolateral chamber and experimental HBSS solutions (300 µl) containing BSA (50 µg/ml) were pipetted into the apical chamber. Duplicate samples of 100-µl aliquots were subsequently obtained from the apical and basolateral chambers at baseline and after the experiment and pipetted into 96-well plates (fluorescent clear bottom plate; Costar). Fluorescent signals were quantitated using a Cytofluor II fluorescent multiwell plate reader employing 530 nm excitation and 620 nm emission spectra. Clearance (Cl) was calculated according to the following equation

\[
\text{Cl (nl·h}^{-1}·\text{cm}^{-2}) = \frac{J_{ab}}{[\text{BSA}]_b \times S}
\]

where \( J_{ab} \) is apical-to-basolateral flux of BSA (light units/h), \([\text{BSA}]_b\) is the concentration at baseline (light units/ml), and \( S \) is the surface area (0.3 cm²) (40).

Experimental design. We have previously demonstrated that 100 µM indomethacin significantly decreases PGE2 synthesis and does not cause cellular injury (22). In addition, this concentration of indomethacin (100 µM) has been employed by other investigators to inhibit PG synthesis under in vitro conditions (5, 49). Furthermore, this concentration approximates plasma and tissue levels obtained with therapeutic doses (2, 7). Thus this concentration of indomethacin was utilized for initial experiments.

The first two experiments were designed to determine the effect of indomethacin pretreatment on changes in intracellular Ca\(^{2+}\) and injury in AGS cells exposed subsequently to low, nondamaging concentrations of either deoxycholate (DC) or a common Ca\(^{2+}\) ionophore (A-23187) or higher, damaging concentrations of both respective agents. In the third experiment we added exogenous PG during indomethacin pretreatment to ascertain whether exogenous PGs could reverse responses observed in the first experiment. The ability of varying concentrations of indomethacin, ibuprofen, and ASA to inhibit endogenous PGE2 release was then established in the fourth experiment. In the fifth experiment, the effect of varying concentrations of indomethacin on changes in intracellular Ca\(^{2+}\) and cellular injury in AGS cells exposed to a damaging concentration of DC was determined. The responses induced by DC exposure after indomethacin pretreatment were then compared with pretreatments with equipotent concentrations of ibuprofen and ASA in the sixth experiment. We then investigated the role of extracellular Ca\(^{2+}\) with regard to the predisposition to injury induced by indomethacin. Finally, we assessed permeability changes induced by exposure to the various cyclooxygenase inhibitors alone in the presence or absence of extracellular Ca\(^{2+}\) to determine if indomethacin alone had any effect on cellular function.

Statistics. Statistical evaluation was performed by ANOVA with a Scheffe post hoc test. Data (n = 6–13/group) are reported as means ± SE. \( P < 0.05 \) was used to determine statistical significance.

RESULTS

Indomethacin pretreatment, changes in intracellular Ca\(^{2+}\) and cellular injury. In control cells intracellular Ca\(^{2+}\) and cellular injury remained stable over the 20-min experimental period (data not shown). Indomethacin pretreatment was associated with mild elevations in [Ca\(^{2+}\)] \( (177 ± 15 \text{ vs. } 103 ± 19 \text{nM, } P < 0.05) \) but by itself was not damaging. Compared with control cells, 50 and 250 µM DC evoked an early increase in intracellular Ca\(^{2+}\) which decreased with time. This effect was more pronounced with 250 µM DC which induced cellular injury after sustained increases in intracellular Ca\(^{2+}\) (Figs. 1 and 2). Indomethacin pretreatment augmented the increases in intracellular Ca\(^{2+}\) and induced cellular injury, as measured by Et uptake and LDH release, in cells exposed to 50 µM DC and significantly accentuated both the changes in intracellular Ca\(^{2+}\) and injury in cells subsequently treated with 250 µM DC (see Figs. 1 and 2).

In contrast, cells exposed to 5 and 30 µM A-23187 demonstrated a gradual increase in intracellular Ca\(^{2+}\)
content over the 20-min time period (Fig. 3). Significant cellular injury was observed in cells exposed to 30 µM but not 5 µM A-23187. In cells exposed to 5 µM A-23187, indomethacin pretreatment augmented the increases in intracellular Ca$^{2+}$ and induced Et uptake. Indomethacin pretreatment significantly accentuated both the changes in intracellular Ca$^{2+}$ and Et uptake in cells subsequently treated with 30 µM A-23187 (see Fig. 3). These data suggest that inhibition of endogenous PGs, which by itself does not induce cellular injury, significantly disrupts Ca$^{2+}$ homeostasis and predisposes AGS cells to injury induced by DC and A-23187.

Effect of exogenous PGs. In view of the above findings, we next attempted to reverse the aforementioned effects of indomethacin pretreatment with exogenous PG treatment. Various concentrations of PGE$_2$ were added concomitantly during the indomethacin pretreatment period as well as during the entire posttreatment period. Compared with control, AGS cells exposed to 250 µM DC demonstrated significant increases in both intracellular Ca$^{2+}$ and cellular injury, an effect that was significantly augmented with indomethacin pretreatment. The addition of PGE$_2$, at concentrations ranging from 2.5 to 10 µM, did not reverse the increased susceptibility to injury induced by indomethacin pretreatment (data not shown). These data suggested that indomethacin may predispose AGS cells to injury through a mechanism which is not directly related to PG metabolism.

Inhibition of PG synthesis. Under control conditions, PGE$_2$ synthesis in AGS cells averaged 50 ± 11 pg/mg protein, levels too low to accurately measure significant decreases in response to cyclooxygenase inhibition. Thus we assessed PGE$_2$ inhibition in cells treated with a positive agonist. Cells exposed to 20 µM digitonin for 20 min demonstrated a significant and substantial increase in PGE$_2$ release (1,311 ± 172 pg/mg protein). Preliminary data demonstrated that indomethacin, ibuprofen, and ASA alone did not induce significant cellular injury until concentrations approached 500 µM, 500 µM, and 2 mM, respectively (data not shown). Thus we employed nondamaging concentrations of these three cyclooxygenase inhibitors for subsequent experimentation. In cells stimulated by digitonin, indomethacin (1-100 µM) inhibited PGE$_2$ synthesis by 98–99%. Ibuprofen (100 µM) and ASA (500 µM) pretreatment significantly inhibited PGE$_2$ release by 93 and 89%, respectively. These data are shown in Fig. 4. Although there was a trend toward increased PGE$_2$ inhibition as induced by 100 µM indomethacin, compared with cells treated with 100 µM ibuprofen or 500 µM ASA, this did not achieve statistical significance.

Separate studies demonstrated that resting levels of PGI$_2$, LTB$_4$, and TxB$_2$ were very low (<5 pg/mg protein) in AGS cells. Furthermore, we did not observe a significant increase in the aforementioned eicosanoids after exposure to 20 µM digitonin (data not shown). These data suggested, at least in the AGS cell line, that PGE$_2$ is the major prostanoid affected by NSAID treatment.
Effect of indomethacin concentration. Because indomethacin appeared to equally inhibit PGE₂ synthesis at concentrations ranging from 1 to 100 µM, the effect of varying concentrations of indomethacin on intracellular Ca²⁺ content and cellular injury was then investigated (Figs. 5 and 6). The augmentation by indomethacin pretreatment on changes in intracellular Ca²⁺ and cellular injury induced subsequently by a damaging concentration of DC was highly concentration dependent. After 20 min of exposure to 250 µM DC, there was no difference between cells pretreated with 1–10 µM indomethacin and those pretreated with buffer, whereas pretreatment with 50–100 µM indomethacin significantly augmented intracellular Ca²⁺ accumulation, Et uptake, and LDH release. These data further suggest that indomethacin, independent of effects on PG synthesis inhibition, increases cellular susceptibility to injury.

Effect of other cyclooxygenase inhibitors. As previously shown, there was no significant difference with regard to PGE₂ synthesis inhibition among 100 µM indomethacin, 100 µM ibuprofen, and 500 µM ASA (see Fig. 4). We next investigated the effects of these equipotent concentrations of ibuprofen and ASA to determine if alternate cyclooxygenase inhibitors had similar effects to indomethacin on intracellular Ca²⁺ and cellular injury (Table 1). In cells exposed to DC, indomethacin (100 µM) pretreatment enhanced the increases in intracellular Ca²⁺, induced both Et uptake and LDH release in cells exposed to 50 µM DC, and accentuated cell injury induced by 250 µM DC treatment. This predisposition to injury was not appreciated when cells were posttreated with 250 µM DC in Ca²⁺-free buffer. These data are depicted in Table 2 and further suggest that indomethacin predisposes AGS cells to injury through a Ca²⁺-mediated mechanism.

Effect of NSAIDs on cellular permeability. BSA clearance in control cells was ~1 nl·h⁻¹·cm⁻². AGS cells treated for 1 h with 50–100 µM indomethacin demonstrated significantly increased clearance to BSA compared with control cells. However, increased permeability of the epithelial cell monolayer was not observed in cells treated with 100 µM ibuprofen (1.06 ± 0.04 nl·h⁻¹·cm⁻²) or 500 µM ASA (1.08 ± 0.03 nl·h⁻¹·cm⁻²). Interestingly, the changes in BSA clearance in AGS cells exposed to 50 and 100 µM indomethacin were not evident when extracellular Ca²⁺ was removed. These data are depicted in Fig. 7 and suggest that 100 µM indomethacin predisposes AGS cells to injury through a Ca²⁺-mediated mechanism.

Role of extracellular Ca²⁺. We have previously investigated, in AGS cells, the role of extracellular Ca²⁺ in injury induced by DC (23). Our data suggested that in the absence of extracellular Ca²⁺, 250 µM DC elicited an initial Ca²⁺ surge followed by a rapid return to baseline values. Furthermore, removal of extracellular Ca²⁺ significantly attenuated injury elicited by 250 µM DC.

These previous observations were confirmed in the current study. We again noted that indomethacin pretreatment significantly increased intracellular Ca²⁺ elevations and enhanced Et uptake and LDH release induced by exposure to 250 µM DC in Ca²⁺-containing buffer. However, the predisposition to injury induced by indomethacin pretreatment was not observed when cells were posttreated with 250 µM DC in Ca²⁺-free buffer. These data are depicted in Table 2 and further suggest that indomethacin predisposes AGS cells to injury through a Ca²⁺-mediated mechanism.

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indomethacin treatment, although not directly associated with cellular injury, significantly increases cellular permeability, an effect that appears to be dependent on extracellular Ca$^{2+}$.

**DISCUSSION**

It is generally accepted that GI complications induced by NSAIDs are a direct result of the systemic effects of PG synthesis inhibition. The current study suggests that while pretreatment of human gastric cells with indomethacin is itself not damaging, it is associated with elevated [Ca$^{2+}$]$_i$ and increased cellular permeability. Furthermore, indomethacin pretreatment significantly predisposes human gastric cells to injury induced by two dissimilar agents (DC and A-23187), both of which are associated with intracellular Ca$^{2+}$ accumulation.

Our initial assumption was that the effects of indomethacin were related to its inhibitory effect on basal PG production. However, subsequent data support the premise that indomethacin initiates additional mechanisms of action that appear to be responsible for the disruption of gastric cellular integrity. In support of this contention we noted that the addition of exogenous PGs did not reverse the predisposition to cell injury induced by indomethacin pretreatment. Second, the observed effects elicited by indomethacin were directly dependent on the concentration employed and not on its ability to inhibit PG synthesis. Finally, similar effects were not duplicated by two other common cyclooxygenase inhibitors (ibuprofen and ASA) when employed at equipotent concentrations.

Several earlier studies have suggested that NSAID gastric damage correlates with inhibition of PG synthe-

Table 1. Comparison of equipotent concentrations of indomethacin, ibuprofen, and aspirin

<table>
<thead>
<tr>
<th>Pretreatment, 60 min</th>
<th>Post-treatment, 20 min</th>
<th>Intra-cellular Ca$^{2+}$, mM</th>
<th>Ethidium uptake, relative fluorescence</th>
<th>LDH release, mU/mg protein</th>
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</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>HBSS</td>
<td>355$\pm$27</td>
<td>867$\pm$78</td>
<td>100 µM indo-</td>
</tr>
<tr>
<td>HBSS</td>
<td>50 µM DC</td>
<td>587$\pm$25</td>
<td>700$\pm$47</td>
<td>methacin</td>
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<tr>
<td>100 µM indo-</td>
<td>50 µM DC</td>
<td>225$\pm$15$^*$$^†$</td>
<td>875$\pm$24$^*$$^†$</td>
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<td>methacin</td>
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<td>813$\pm$80</td>
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<tr>
<td>500 µM ASA</td>
<td>50 µM DC</td>
<td>151$\pm$21</td>
<td>768$\pm$90</td>
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<td>HBSS</td>
<td>250 µM DC</td>
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<td>975$\pm$36$^*$$^†$</td>
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<tr>
<td>100 µM indo-</td>
<td>250 µM DC</td>
<td>346$\pm$19$^*$$^†$</td>
<td>3,167$\pm$273$^*$$^†$</td>
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<td>1,892$\pm$157$^*$$^†$</td>
<td>985$\pm$30$^*$$^†$</td>
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<tr>
<td>100 µM ibuprofen</td>
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<td>1,852$\pm$137$^*$$^†$</td>
<td>1,046$\pm$44$^*$$^†$</td>
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<tr>
<td>500 µM ASA</td>
<td>250 µM DC</td>
<td></td>
<td></td>
<td>1,046$\pm$44$^*$$^†$</td>
</tr>
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</table>

Values are means $\pm$ SE; n = 6–12/group. LDH, lactic dehydrogenase; HBSS, Hanks’ balanced salt solution; DC, deoxycholate; ASA, acetylsalicylic acid. $^* P < 0.01$ vs. HBSS pre- and posttreatment; $^† P < 0.01$ vs. HBSS pretreatment and 50 µM DC posttreatment. $\ddagger P < 0.01$ vs. HBSS pretreatment and 250 µM DC posttreatment.

Table 2. Role of extracellular Ca$^{2+}$ on susceptibility to injury induced by indomethacin

<table>
<thead>
<tr>
<th>Pretreatment (Ca$^{2+}$ containing), 60 min</th>
<th>Post-treatment, 20 min</th>
<th>Intra-cellular Ca$^{2+}$, mM</th>
<th>Ethidium uptake, relative fluorescence</th>
<th>LDH release, mU/mg protein</th>
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<td>526$\pm$19</td>
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<td>100 µM indomethacin</td>
<td>250 µM DC</td>
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<td>847$\pm$25$^*$$^†$</td>
<td>1,586$\pm$31$^*$$^†$</td>
</tr>
<tr>
<td>100 µM indomethacin</td>
<td>250 µM DC (Ca$^{2+}$ free)</td>
<td>167$\pm$29</td>
<td>811$\pm$35$^*$$^†$</td>
<td>1,558$\pm$72$^*$$^†$</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE; n = 6–13/group. $^* P < 0.01$ vs. HBSS pre-and posttreatment; $^† P < 0.01$ vs. HBSS pretreatment; $\ddagger P < 0.01$ vs. HBSS pretreatment and 250 µM DC (Ca$^{2+}$ containing) posttreatment.

Fig. 6. LDH release in AGS cells pretreated with either buffered saline (HBSS) or varying concentrations of indomethacin (Indo, 60 min) and subsequently exposed to 250 µM DC. $^* P < 0.01$ vs. control, $^† P < 0.01$ vs. cells pretreated with HBSS; n = 10–12/group.

Fig. 7. Changes in cellular permeability to BSA induced by 60-min treatment with varying concentrations of indomethacin in either calcium-containing or calcium-free buffer. $^* P < 0.01$ vs. control, $^† P < 0.01$ vs. indomethacin treatment in calcium-containing buffer; n = 6/group.
sis (25, 30, 44). Although it was initially felt that NSAIDs (especially ASA) may act as topical irritants, subsequent work demonstrated that NSAID injury is similar with oral, parenteral, or rectal administration, thus further implicating a more important effect of systemic PG inhibition (16, 30).

In contrast to the aforementioned hypothesis, recent data support the existence of other mechanisms of NSAID action, with regard to both clinical efficacy and GI injury. To date, few studies have related the degree of PG synthesis inhibition with anti-inflammatory effect, and it has been shown that significantly higher doses of NSAIDs are required to suppress inflammation than to inhibit PG synthesis (1). Interestingly, in patients with arthritis, salicylate, a weak cyclooxygenase inhibitor, appears to be as effective as ASA in both controlling pain and limiting inflammation (29). Similarly, PGs alone have been shown to inhibit inflammation in other animal models of arthritis (50).

Whittle (47) investigated the temporal relationship between PG inhibition and small bowel lesion formation in rats. His data suggested that indomethacin may cause intestinal damage through a PG-independent process. Human trials have failed to demonstrate a correlation between tissue PG concentration and the gross or microscopic appearance of gastric lesions. Redfern et al. (31) administered oral doses of indomethacin (sufficient to reduce mucosal PGs) to humans and observed no gastric lesions. However, upon increasing the dosage, they documented no further decrease in PG synthesis but did detect significant gastric ulceration.

Recent observations in cyclooxygenase 1 knockout mice further support our contention that indomethacin elicits gastric injury through mechanisms independent of PG synthesis inhibition. These mice did not spontaneously develop gastric ulceration but did demonstrate significant GI injury when exposed to indomethacin (24).

Previous investigators have reported that the concentration of indomethacin utilized in the current study (100 µM) is noninjurious under in vitro conditions (8, 11). Our findings are clinically relevant because data from both human and rat experimentation suggest that serum indomethacin concentrations are frequently greater than 20 µM and may even approach 150 µM (2, 7). Furthermore, in vitro concentrations may also be significantly less than that attainable under in vivo conditions because NSAIDs, in acidic environments such as the stomach, tend to penetrate lipid membranes and accumulate within cells (27).

Our data indicate that indomethacin, while it remains noninjurious in the concentrations we employed, still predisposes cells to injury subsequently induced by bile salts. Because the gastric mucosa is continuously exposed to noxious irritants such as acid, refluxed bile salts, and ethanol, this observation has clinical relevance. Other investigators have reported similar findings. Indomethacin has been implicated in aggravating bile-induced mucosal lesions in rats and dogs (45, 46).

Yamada et al. (49) investigated indomethacin-induced enteritis in IEC-18 (rat ileal epithelial) cells. They noted that neither indomethacin (exceeding concentrations of 200 µM) nor rat bile exposure individually induced mucosal cell injury. However, when administered in combination they observed significant cytotoxicity. Whittle et al. (48) explored gastric mucosal injury in rats and reported that indomethacin pretreatment alone did not cause significant mucosal injury but did elicit enhanced damage by Nω-monomethyl-L-arginine (a nitric oxide synthase inhibitor) or capsaicin treatment.

Predisposition to mucosal injury induced by indomethacin appears to involve a profound disturbance in Ca2+ homeostasis. Our work also reinforces that of Tepperman and Soper (36), who proposed that injury induced by indomethacin in rabbit gastric mucosal cells appeared to involve extracellular Ca2+. We have recently investigated the role of extracellular Ca2+ and the relationship between intracellular Ca2+ accumulation and injury induced by both DC and A-23187 in AGS cells. Our data indicate that sustained Ca2+ accumulation resulting from the influx of extracellular Ca2+ precedes cellular injury induced by both agents. However, transient changes in intracellular Ca2+ content as observed in a Ca2+-free environment do not appear to significantly affect cellular injury (23). Proposed mechanisms whereby sustained elevations in intracellular Ca2+ cause cellular toxicity include disruption of the cytoskeleton, phospholipid hydrolysis, and protease and endonuclease activation (28).

Several mechanisms (independent of PG synthesis inhibition) have been proposed whereby NSAIDs potentially decrease inflammation and elicit GI mucosal injury. NSAIDs are lipophilic and insert into lipid bilayers, thereby uncoupling protein-protein interactions and potentially disrupting signal transduction mechanisms (1, 3). Other reported cellular effects of NSAIDs include disturbances in oxidative phosphorylation, active transport, transmembrane ion fluxes, and cell-to-cell adhesion (3, 13, 14). These alternate mechanisms may also partially account for the observed ability of indomethacin to disrupt Ca2+ homeostasis.

The current study supports the premise that indomethacin exhibits properties independent of other cyclooxygenase inhibitors (ibuprofen and ASA) that predispose human gastric cells to injury. Data from human studies suggest that indomethacin has greater ulcerogenic potential than ibuprofen and other NSAIDs (12, 17). Clinically, indomethacin is still extensively used in obstetrics to delay uterine contractions and in the neonatal unit to facilitate patent ductus arteriosus closure. However, the major significance of the current study is that indomethacin is still commonly used to indirectly deduce the physiological role of PGs in a given process being studied.

Our findings suggest that indomethacin may have additional properties that significantly affect mucosal integrity. Thus data resulting from studies (either in vitro or in vivo) employing only indomethacin as a PG synthesis inhibitor should be interpreted with caution. As an example, the majority of the early work investigating adaptive cytoprotection demonstrated that this
protective response was blocked with indomethacin pretreatment (4). It was thus proposed that adaptive cytoprotection was in large part mediated by endogenous PGs. Although many subsequent studies, including those in our laboratory, confirmed Robert’s (4) original hypothesis that indomethacin blocked adaptive cytoprotection, the role of endogenous PGs remained controversial. We and others were unable to demonstrate that mild irritant pretreatment correlated with enhanced endogenous PG synthesis (6, 21, 22, 35). The current work may provide a partial explanation for these observations in that indomethacin, by disturbing intracellular Ca\(^{2+}\) homeostasis and predisposing human gastric cells to injury through mechanisms independent of PG synthesis, may negate the protective effects conferred by the mild irritant.

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