Annexin-1 is an endogenous gastroprotective factor against indomethacin-induced damage

Renata C. O. Zanardo, Mauro Perretti, and John L. Wallace

Annexin-1 is an endogenous gastroprotective factor against indomethacin-induced damage. Am J Physiol Gastrointest Liver Physiol 288: G481–G486, 2005. First published October 7, 2004; doi: 10.1152/ajpgi.00299.2004.—Adherence of neutrophils to the vascular endothelium is an early and critical event in the pathogenesis of gastric injury induced by NSAIDs. Pretreatment with glucocorticoids has been shown to prevent NSAID-induced neutrophil adherence and, in turn, to protect the stomach from injury. Some of the anti-inflammatory effects of glucocorticoids, including inhibition of neutrophil adherence, are mediated via the release of annexin-1. In this study, we assessed the contribution of annexin-1 to the protective actions of a glucocorticoid (dexamethasone) against indomethacin-induced gastric damage. Dexamethasone pretreatment markedly reduced the extent of indomethacin-induced gastric damage in rats. Immune-neutralization of annexin-1 resulted in a reversal of the gastroprotective actions of dexamethasone. Similarly, pretreatment with either of two antagonists of the formyl peptide receptor family, to which annexin-1 binds, reversed the gastroprotective effects of dexamethasone. The inhibitory effects of dexamethasone on indomethacin-induced leukocyte adherence in the mesenteric microcirculation were abolished by pretreatment with an antibody directed against annexin-1 or with an antagonist of the formyl peptide receptors. These results demonstrate that annexin-1 mediates the gastroprotective effects of a glucocorticoid against NSAID-induced damage. We propose that in some circumstances, annexin-1 plays an important role as an endogenous mediator of mucosal defense.

NSAIDs are among the most widely prescribed drugs, but their use can lead to stomach ulcers and other serious gastrointestinal complications such as bleeding and perforation (30). The mechanism through which NSAIDs cause gastric damage is related to the ability of these agents to inhibit prostaglandin synthesis (28). Many studies have also demonstrated that neutrophil adherence to the vascular endothelium is a critical early event in the pathogenesis of gastric mucosal injury induced by NSAIDs. The extent of gastric injury induced by NSAIDs is markedly reduced in neutropenic rats (32), and prevention of neutrophil adherence to the vascular endothelium through treatment with antibodies against the CD18, ICAM-1, or P-selectin resulted in near-complete protection of the gastric mucosa against the damage induced by indomethacin (31, 33). McCafferty et al. (14) demonstrated that administration of dexamethasone to rats before indomethacin greatly reduced the leukocyte adherence in mesenteric postcapillary venules and the extent of gastric damage produced by this NSAID in healthy and arthritic rats. The protection afforded by dexamethasone against indomethacin-induced gastric damage was argued to be due to the ability of these compounds to inhibit ICAM-1 expression (14).

The 37-kDa protein annexin-1, previously referred as lipocortin 1, has long been thought to mediate some of the anti-inflammatory actions of glucocorticoids, including anti-adhesive and antimigratory effects (13). Within peripheral blood cells, annexin-1 is predominantly expressed by neutrophils, eosinophils, and monocytes, with lower amounts expressed in specific subsets of lymphocytes (9, 15). In human neutrophils, annexin-1 is present in large amounts in the cytosol of human neutrophils (7, 26). Within peripheral blood cells, annexin-1 is predominantly expressed by neutrophils, eosinophils, and monocytes, with lower amounts expressed in specific subsets of lymphocytes (9, 15). Glucocorticoid treatment has been shown to increase annexin-1 content in circulating neutrophils in humans (9) and in rodents (13, 23). As glucocorticoids augment cellular annexin-1 content, large amounts of the protein are externalized on the cell surface once the leukocytes adhere to the inflamed vascular endothelium (22) promoting leukocyte detachment and consequently inhibiting cell extravasation. The mechanism of action responsible for the anti-adhesive action of annexin-1 and its peptide mimetics has long remained elusive. The “formyl peptide receptor” (FPR) has been suggested to mediate at least some of the actions of annexin-1 (34). Further studies provided support for this hypothesis through the use of FPR antagonists (8, 11) and FPR-deficient mice (24). The study of Gavins et al. (8) demonstrated protective properties of annexin-1 against ischemia-reperfusion-induced leukocyte adhesion and emigration in the mesenteric microcirculation and showed a partial functional involvement of FPR and the structurally related lipoxin A4 receptor (ALX).

In the present study, we have tested the hypothesis that annexin-1 mediates the protective effects afforded by dexamethasone against indomethacin-induced gastric injury in rats. We have further examined the possibility that endogenous annexin-1 mediates inhibitory effects of dexamethasone on indomethacin-induced neutrophil adhesion in postcapillary mesenteric venules.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 175–200 g were obtained from Charles River Laboratories (Montreal, QC, Canada) and were housed in the Animal Care Facility at the University of Calgary. The rats were

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fed standard laboratory chow and tap water. All experimental protocols were approved by the Animal Care Committee of the University of Calgary, and the experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care. Unless otherwise stated, the sample size per group was at least five.

**Dexamethasone-induced gastroprotection.** Before an experiment, the rats were deprived of food, but not water, for 18–20 h. Indomethacin was dissolved in a vehicle of 5% sodium bicarbonate solution. Rats were pretreated with dexamethasone (0.1 mg/kg) or vehicle (0.9% saline) intraperitoneally, and 2 h later, they were given indomethacin orally (20 mg/kg). Three hours later, the rats were killed by cervical dislocation and the stomach was excised and opened by an incision along the greater curvature. Macroscopically visible gastric damage was then scored by an observer unaware of the treatment. The lengths of the lesions (in mm) were summed to give a gastric damage score for each stomach. After the gastric tissue was scored, it was fixed in neutral-buffered formalin and processed by routine techniques for light microscopy (hematoxylin and eosin staining). The degree of leukocyte margination within the gastric microcirculation was scored in a blind manner on a scale of 0 to 3, with the following criteria: 0 = no leukocyte margination; 1 = occasional leukocyte margination; 2 = moderate leukocyte margination; 3 = marked leukocyte margination.

Additional studies were performed in which dexamethasone was administered concurrently with indomethacin, or 1 h after indomethacin administration, to determine the effect on the extent of gastric damage (n = 5/group). These experiments were otherwise carried out as described above.

**Role of annexin-1 in dexamethasone-induced gastroprotection.** The role of endogenous annexin-1 in the gastroprotective effects of dexamethasone was first evaluated by immunoneutralization studies. Rats were treated with 0.5 ml/kg of LCPS1, a polyclonal sheep antibody raised against the N-terminal peptide (Ac2–26) of annexin-1 (4) or methasone was first evaluated by immunoneutralization studies. Rats were pretreated with dexamethasone (0.1 mg/kg) or vehicle (0.9% saline) intraperitoneally, and 2 h later, they were given indomethacin orally (20 mg/kg). Three hours later, the rats were killed by cervical dislocation and the stomach was excised and opened by an incision along the greater curvature. Macroscopically visible gastric damage was then scored by an observer unaware of the treatment. The lengths of the lesions (in mm) were summed to give a gastric damage score for each stomach. After the gastric tissue was scored, it was fixed in neutral-buffered formalin and processed by routine techniques for light microscopy (hematoxylin and eosin staining). The degree of leukocyte margination within the gastric microcirculation was scored in a blind manner on a scale of 0 to 3, with the following criteria: 0 = no leukocyte margination; 1 = occasional leukocyte margination; 2 = moderate leukocyte margination; 3 = marked leukocyte margination.

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**Annexin-1 expression.** Gastric annexin-1 expression was examined by Western blot analysis using samples from rats treated with dexamethasone and/or indomethacin, as above. Samples of gastric tissue were homogenized in lysis buffer (0.1% Triton X-100, 50 mM peptatin-A, 0.2 mM leupeptin, 1 μg/ml aprotonin, 10 mg/ml phenylmethyl sulfonyl fluoride, 50 mM Tris, and 10 mM EDTA). After centrifugation, the protein concentration of the supernatant was determined by colorimetric assay (Bio-Rad, Hercules, CA). Protein (30 μg/lane) was separated on a 7.5% polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was incubated for 1 h with blocking buffer (20 mM Tris, 100 mM NaCl, 0.5% Tween 20, and 5% nonfat dried milk) and then probed overnight with a polyclonal sheep antibody raised against full-length human annexin-1 (LCS3, 1:10,000) (21). This antibody was chosen to monitor expression of both intact and NH₂-terminally cleaved annexin-1 (11). The membrane was washed with blocking buffer in 2% nonfat milk five times for 10 min each. The membrane was then incubated with donkey anti-sheep IgG secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Again, the membrane was washed as described above. A chemiluminescence reagent (Amer sham) was added to visualize the labeling according to the manufacturer’s instructions. Densitometry was done by using a calibrated imaging densitometer (model GS-710, Bio-Rad) and analyzed with Quantity One software (Bio-Rad).

Effects of dexamethasone on neutrophil annexin-1 expression were also examined. Blood was drawn from anesthetized (Halothane) rats by cardiac puncture 2, 3, or 5 h after intraperitoneal administration of dexamethasone (0.1 mg/kg) or saline. Neutrophils were isolated using the procedure described by Boyum (3), and Western blot analyses for annexin-1 were carried out as described above.

**Intravital microscopy.** Rats were anesthetized with pentobarbital sodium (60 mg/kg ip), and cauterity incisions were made along the abdominal midline. A tracheotomy was performed to facilitate breathing. The rats were placed in a supine position, and a segment of the mesentery was exteriorized through the abdominal incision. The mesentery was carefully placed over an optically clear viewing ped estal that allowed for transillumination of a 2-cm² segment of tissue. All exposed tissue was covered with saline-soaked gauze to minimize dehydration. The temperature of the pedestal was kept at 37°C, and the mesentery was superfused with warmed bicarbonate-buffered saline (pH 7.4). An intravital microscope (Nikon L250/0.35) and a ×10 eyepiece were used to observe the mesenteric microcirculation. Post-capillary venules with diameters ranging from 20 to 40 μm were selected for the study. A video camera mounted on the microscope (Panasonic digital 5000) projected the image onto a monitor, and the images were recorded for playback analysis using a video cassette recorder. Images of the mesenteric microcirculation were recorded 5 min before indomethacin administration (baseline), at the time of indomethacin administration (time 0), and every 15 min for 60 min. Leukocyte rolling, adherence, and white blood cell velocity (V_{WBC}) were quantified from videotaped images of the vessels made over 5-min periods. Rolling leukocytes were defined as those leukocytes that rolled at a velocity slower than that of red blood cells. Leukocyte rolling velocity (V_{WBC}) was measured for the first 10–20 leukocytes entering the field of view at the time of recording and was determined as the time required for a leukocyte to traverse a given length of venule (100 μm). Leukocyte adhesion was blindly quantified as the number of leukocytes that adhered to the vessel wall for 30 s or more along a 100-μm venule length.

Effects of dexamethasone on circulating leukocyte numbers. Groups of 15 rats each were treated intraperitoneally with saline or dexamethasone (0.1 mg/kg). A subgroup of five rats from each treatment group was anesthetized with halothane 1, 2, or 3 h later, and blood was drawn by cardiac puncture for determination of circulating leukocyte numbers using a Sysmex KX-21N hematology analyzer.

**Statistical analysis.** All data are expressed as means ± SE. Groups of data were compared by using ANOVA followed by Tukey’s test. A P value of <0.05 was considered significant.

**Materials.** Indomethacin and EDTA were obtained from Sigma (St. Louis, MO). Dexamethasone was obtained from Vétoquinol (Lavaltrie, Quebec, Canada). Boc1 and Boc2 were obtained from ICN Biomedicals (Aurora, OH). Donkey anti-sheep IgG conjugated to horseradish peroxidase was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Normal sheep serum was provided by Animal Care Services, University of Calgary.

**RESULTS**

Role of annexin-1 in dexamethasone-induced gastroprotection. Indomethacin administration resulted in the formation of extensive hemorrhagic lesions in the stomach, largely confined...
to the corpus region and occurring along the crests of rugal folds. The mean damage score in rats treated with saline before indomethacin was ~50 (Fig. 1). Pretreatment with dexamethasone resulted in a significant reduction (>80%) in the extent of gastric damage following indomethacin administration. The protective effect of dexamethasone was not affected by prior administration of normal serum but was significantly attenuated when the rats were pretreated with an antibody directed against annexin-1 (Fig. 1). Histological examination of the gastric microcirculation in rats treated with indomethacin revealed clear evidence of leukocyte margination, albeit focal in nature. In rats treated with indomethacin, there was significant margination (mean score of 2.0 ± 0.2 in vehicle-treated rats; P < 0.05). Pretreatment with dexamethasone reduced the levels of indomethacin-induced leukocyte margination to control levels (mean score of 0.8 ± 0.4; P < 0.05 vs. indomethacin alone).

If dexamethasone was administered simultaneously with indomethacin (gastric damage score of 39 ± 6) or 1 h after indomethacin (gastric damage score of 41 ± 7), there was no detectable protective effect compared with a group treated with vehicle plus indomethacin (gastric damage score of 49 ± 7).

The protective effects of dexamethasone against indomethacin-induced gastric damage were also significantly diminished by pretreatment with the annexin-1 receptor antagonists (Fig. 2). When rats were pretreated with either Boc1 or Boc2, gastroprotective effects of dexamethasone were no longer evident. These agents given before indomethacin (without dexamethasone) did not significantly affect the extent of gastric damage relative to that seen with indomethacin alone (Fig. 2).

Administration of an annexin-1 antagonist (Boc2) 1 and 2 h after indomethacin resulted in a significant increase in the gastric damage score (70 ± 9) compared with rats treated with indomethacin and vehicle (gastric damage score of 39 ± 8; P < 0.05). These findings suggest an important role for annexin-1, other than that induced by a glucocorticoid, in gastric mucosal defence.

**Annexin-1 expression.** Annexin-1 was detected in the stomach of rats treated with vehicle (Fig. 3). Administration of dexamethasone did not significantly affect gastric annexin-1 expression. Indomethacin administration caused a ~45% increase in gastric annexin-1 expression, which was significantly reduced by prior administration of dexamethasone (Fig. 3).

Annexin-1 expression was also detected in neutrophils isolated from all rats studied. Dexamethasone did not significantly affect neutrophil annexin-1 expression 2 h after its administration (552 ± 28 densitometry units) compared with a group of rats not receiving dexamethasone (583 ± 14 densitometry units). At 3 h after administration of dexamethasone, however,
there was a significant increase in annexin-1 expression in neutrophils (734 ± 21 densitometry units; P < 0.05), which was no longer apparent by 5 h postdexamethasone (526 ± 60 densitometry units).

**Intravital microscopy.** Administration of indomethacin did not significantly affect the magnitude of flux of rolling leukocytes or \( V_{\text{WBC}} \) in mesenteric postcapillary venules (Fig. 4, A and B). However, indomethacin did cause a time-dependent increase in leukocyte adherence, with the earliest significant increase observed within 15 min (Fig. 4C). Pretreatment with dexamethasone caused a complete blockade of indomethacin-induced leukocyte adherence while not affecting leukocyte flux or velocity. Pretreatment with the anti-annexin-1 antibody abolished the inhibitory effect of dexamethasone on indomethacin-induced leukocyte adherence (Fig. 4C).

The role of annexin-1 in the inhibition of leukocyte adherence by dexamethasone was further examined using one of the antagonists, Boc2. Administration of Boc2 before dexamethasone and indomethacin resulted in a reversal in the effect of the glucocorticoid; that is, levels of leukocyte adherence were similar to those observed with indomethacin alone (Fig. 5C). There were no significant differences among the treatment groups in terms of the flux of rolling leukocytes (Fig. 5A) and leukocyte velocity (Fig. 5B).

**Circulating leukocyte numbers.** Dexamethasone administration resulted in significant decrease in circulating neutrophil numbers at 3 h after its administration (4.1 ± 0.3 × 10⁷/µl; P < 0.01) compared with control levels (7.6 ± 0.3 × 10⁷/µl). However, no significant changes in circulating leukocyte numbers were detected at 1 h (6.5 ± 0.3 × 10⁷/µl) or 2 h (6.1 ± 0.4 × 10⁷/µl) after dexamethasone administration. Dexamethasone administration did not significantly affect the numbers of erythrocytes or platelets in blood at any of the three time points that were examined (data not shown).

**DISCUSSION**

There is substantial evidence that the damage produced in the stomach of animals after administration of NSAIDs is mediated, at least in part, by circulating neutrophils. NSAIDs trigger adherence of neutrophils to the vascular endothelium within the gastric and mesenteric microcirculation (2, 31, 33). Immuno depletion of circulating neutrophils or prevention of adherence to the endothelium with monoclonal antibodies directed against adhesion molecules results in protection of the stomach from NSAID-induced injury (30–32). Pretreatment with dexamethasone can also significantly reduce the extent of NSAID-induced gastric injury, and this effect has been attributed to the ability of this corticosteroid to suppress NSAID-induced neutrophil adherence (14). Corticosteroids are known to downregulate expression of adhesion molecules such as ICAM-1 (16).

It has long been recognized that annexin-1, a 37-kDa member of the annexin superfamily of proteins (25), mediates many of the anti-inflammatory effects of the glucocorticoids (6). In the present study, we have provided evidence that annexin-1 mediates the protective effects of dexamethasone against NSAID-induced gastric damage, and this may occur via inhibition of NSAID-induced leukocyte adherence. Dexamethasone prevented both the gastric damage and neutrophil adherence to mesenteric venules induced by indomethacin, a result in accordance with previously published results (14). Passive immunoneutralization of rats with an antibody raised against the NH₂-terminal peptide of annexin-1 (4) before the administration of dexamethasone and indomethacin abrogated the protective effects afforded by the glucocorticoid on the gastric damage and neutrophil adherence. This same antibody, LCPS1, has been shown to be very effective in abrogating the antimigratory effect of dexamethasone in the air pouch inflamed with zymosan (20). However, as expected, administration of anti-annexin-1 to rats before indomethacin had no effect.
on neutrophil adherence or gastric damage. Second, the protective effects of dexamethasone were abolished by pretreatment with either of two antagonists of the formyl peptide receptor (FPR), Boc1 and Boc2. Walther et al. (34) suggested that annexin-1 peptides, and possibly also the full-length protein, bind to and activate FPR on neutrophils to inhibit their transendothelial migration. Consistent with this, Boc2 also significantly inhibited the preventive effect of dexamethasone on indomethacin-induced leukocyte adherence. This latter observation is consistent with the substantial evidence that annexin-1 plays a key role in modulating leukocyte-endothelial adhesive interactions (1, 12, 21, 35).

Annexin-1 is one of several ligands for the FPR family of receptors. Others include formyl-Met-Leu-Phe, lipoxin A4, and 15-(R)-epi-lipoxin A4, which is also called “aspirin-triggered lipoxin.” The lipoxins and annexin-1 share the ability to reduce the severity of NSAID-induced gastric injury (5, 27), and in both cases, the effects are blocked by antagonists to these receptors. Moreover, annexin-1 and the lipoxins share the ability to suppress leukocyte adherence to the vascular endothelium, which may explain the protective effects against NSAID-induced injury in the stomach (5, 27). FPR antagonists (Boc1 and/or Boc2) have been shown to block the inhibitory effects of annexin-1 peptides on neutrophil transmigration (24) and to blunt the protective effects of recombinant annexin-1 and its NH2-terminal peptides in a model of ischemia/reperfusion (8, 11).

The effects of indomethacin on annexin-1 expression in the stomach have not been previously reported. We observed a substantial increase in annexin-1 expression 3 h after indomethacin administration, and this upregulation was abolished by prior administration of dexamethasone. The observation that administration of Boc2 following indomethacin administration resulted in a significant exacerbation is consistent with the notion that the annexin-1 that is induced by indomethacin acts to diminish gastric injury. The fact that dexamethasone, alone, did not cause a significant change in gastric annexin-1 expression might be attributable to the fact that infiltrated neutrophils are known to be a primary cellular source of annexin-1 in damaged tissues (17, 29), and dexamethasone is known to reduce circulating neutrophil numbers (10) and neutrophil accumulation in the stomach (14). Indeed, we observed a significant decrease in circulating leukocyte numbers 3 h after administration of dexamethasone, which is the same time point at which we observed a significant induction of annexin-1 within neutrophils. The timing of the increase in neutrophil annexin-1 expression (between the 2nd and 3rd hour after administration) is consistent with the notion that annexin-1 mediates the gastroprotective effects of dexamethasone. When dexamethasone was administered simultaneously with indomethacin, or 1 h after indomethacin administration, no significant protective effect was observed.

It is worth emphasizing that we have used dexamethasone in the present study as a tool to gain a better understanding of the potential role of an endogenous anti-inflammatory substance (annexin-1) in gastric mucosal defence. Glucocorticoids have a wide range of effects, not all of which are beneficial. Indeed, glucocorticoid use can exacerbate NSAID-induced gastric ul-
cers, most likely by interfering with wound-healing processes. Whereas glucocorticoids would therefore not be considered as a viable option for preventing NSAID-induced gastric damage, analogs of annexin-1 or other agents that stimulate annexin-1 production could be attractive candidates as gastroprotective drugs.

In conclusion, we have demonstrated that annexin-1 plays an important role in mediating the gastroprotective effects of dexamethasone in a model of indomethacin-induced gastric damage. Annexin-1 was also induced in the stomach by indomethacin and acted to diminish the damage caused by that agent. These effects of annexin-1 are likely to be related, at least in part, to suppression of indomethacin-induced leukocyte adherence to the vascular endothelium. Annexin-1, similar to lipoxins that act through the same receptor, appears to be an important mediator of gastric mucosal defence.

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