Macrophage inflammatory protein-2 mediates the bowel injury induced by platelet-activating factor

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Han, Xin-Bing, Xueli Liu, Wei Hsueh, and Isabelle G. De Plaen. Macrophage inflammatory protein-2 mediates the bowel injury induced by platelet-activating factor. Am J Physiol Gastrointest Liver Physiol 287: G1220–G1226, 2004. First published August 19, 2004; doi:10.1152/ajpgi.00231.2004.—Platelet-activating factor (PAF) is a potent endogenous mediator of bowel inflammation. It activates neutrophils that are needed to initiate the inflammatory response. Macrophage inflammatory protein-2 (MIP-2), a critical C-X-C chemokine secreted by macrophages and epithelial cells, is a potent chemotactant for neutrophils. Whereas MIP-2 has been previously shown to mediate the injury in various organs, its role in acute intestinal injury has never been assessed. In this study, we first investigated the effect of PAF on MIP-2 expression in the intestine. Anesthetized young adult male Sprague-Dawley rats were injected intravenously with either PAF (1.5 µg/kg) or saline. Sixty minutes later, ileal MIP-2 gene expression was determined by semiquantitative RT-PCR, and plasma and ileal MIP-2 protein was determined by ELISA. In a second step, we assessed the role of MIP-2 in PAF-induced bowel injury. Rats were pretreated with rabbit anti-rat MIP-2 antibodies or control IgG for 90 min and then injected intravenously with PAF (2.5 µg/kg) for 90 min. We found that, in the rat intestine, 1) MIP-2 mRNA was only minimally expressed constitutively in sham-operated animals; 2) MIP-2 mRNA was significantly upregulated in response to PAF; 3) MIP-2 protein plasma levels and local production of MIP-2 in the ileum were markedly induced by PAF; 4) the administration of anti-rat MIP-2 IgG, but not control rabbit IgG, markedly reduced PAF-induced bowel injury (injury scores of 0.19 ± 0.09 vs. 1.12 ± 0.43, P < 0.05), hypotension, and leukopenia but did not reduce PAF-induced hemoconcentration. Thus we conclude that MIP-2 mediates PAF-induced intestinal injury.

chemokines; polynuclear neutrophils

PLATELET-ACTIVATING FACTOR (PAF), a proinflammatory phospholipid endogenously produced in the intestine during acute bowel injury (37), is thought to play a role in the pathogenesis of necrotizing enterocolitis (12) and bowel injury associated with endotoxin shock (14) and ischemia/reperfusion (4). In a rat model, systemic injection of PAF produces a dose-dependent intestinal necrosis (13) that predominates in the small intestine, especially in the ileum. Neutrophils are probably the key effector cells for PAF-induced bowel injury, because 1) PAF activates neutrophils (35) and promotes neutrophil-endothelial adhesion (35), an initial event of the inflammatory response, and 2) neutropenic rats are protected from PAF-induced intestinal injury and systemic inflammation (39).

The recruitment of neutrophils to sites of inflammation is driven by locally produced chemokines. There are four families of chemokines in which CC and CXC (classified based on the position of the first 2 cysteins) are the major two families (11). In rats, the CXC chemokines include MIP-2 and growth-regulated oncogene α/cytokine-induced neutrophil chemoattractant-1 (Gro/CINC-1), which are potent chemotactants for neutrophils. The CC chemokines, such as monocyte chemoattractant protein-1 (MCP-1), are main chemotactants for monocytes/macrophages and lymphocytes.

In this study, we investigated the effect of PAF on the gene expression and protein production of a major chemokine target. Neutrophils, macrophage inflammatory protein (MIP)-2, in the ileum and compared it with other chemokines. We then examined the role of MIP-2 in PAF-induced systemic inflammatory changes including hypotension, hemoconcentration, leukopenia, neutrophil recruitment, and acute bowel injury.

MATERIALS AND METHODS

Rabbit polyclonal anti-rat MIP-2 IgG and normal rabbit IgG were purchased from Biosource International (Camarillo, CA). An enhanced chemical luminescence kit was obtained from Amersham (Arlington Heights, IL). PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine; Biomol Research Lab, Plymouth Meeting, PA) was prepared in 5 mg/ml bovine serum albumin as previously described (41).

Animal experiment. Male Sprague-Dawley rats (80–120 g) were anesthetized with Nembutal (65 mg/kg ip) and tracheotomized as previously described (7). The carotid artery and jugular vein were cannulated for continuous blood pressure (BP) monitoring, blood sampling, and drug administration. In the first part of the experiments, the effect of PAF on chemokine induction was examined by using a low dose of PAF (1.5 µg/kg; low dose was used to avoid severe tissue necrosis, which may interfere with RNA extraction). The animals were either sham operated or injected with PAF (1.5 µg/kg iv). After 1 h, rats were killed, their small intestine was processed for mRNA extraction, and chemokine gene expression was determined by RT-PCR.

The second part of the experiment was aimed at determining the role of MIP-2 on PAF-induced bowel injury. Rats were injected intraperitoneally with either saline, a rabbit polyclonal antibody directed specifically against MIP-2 (1 mg/kg; Biosource International), or control rabbit IgG (1 mg/kg; Biosource International). The same anti-MIP-2 antibody had been successfully used to block MIP-2 in a rat model of intestinal toxin A enteritis (5) and in an experimental autoimmune neuritis (45). Ninety minutes later, the animals were injected with either PAF (2.5 µg/kg iv) or an equal volume of normal saline (NS) for 90 min (the dose of PAF was chosen so it would induce intestinal injury without causing mortality within the experimental period). Mean arterial BP was monitored continuously throughout the experimental period. Blood samples were collected 90

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was carried out on the serum and intestine homogenates using a commercially available rat MIP-2 ELISA kit (Biosource International). All standards, controls, and samples were run in duplicate. The absorbance of each well was read at 450 nm with an MR600 microplate reader (Thermomax-Molecular Devices; Fisher Scientific Instrument, Nepean, ON, Canada), and background absorbency values of blank wells were subtracted from the obtained values. MIP-2 measurements were expressed as picograms of MIP-2 per milligram of proteins for intestine homogenates and picogram of MIP-2 per 50 µl plasma for sera samples. Five animals per treatment group were analyzed.

**Determination of intestinal myeloperoxidase activity.** Intestinal MPO activity was measured according to a published method (36). Briefly, intestinal tissues were homogenized in 0.5% hexadecyltri-methyl-ammonium bromide in 0.05 M potassium phosphate buffer and EDTA (5 mM), pH 6.0, and sonicated and then frozen and thawed three times to release MPO from the neutrophil granules. Each sample aliquot of 50 µl was then mixed with 450 µl of substrate (0.167 mg/ml O-dianisidine HCl plus 0.0005% hydrogen peroxide in 50 mM potassium phosphate buffer at pH 6.0). After 20 min of reaction, the change in absorbance at 460 nm was measured with a spectrophotometer (24) (Beckman Instruments, Fullerton, CA).

**Statistical analysis.** Two-sided Student’s t-test was used for the comparison of any two single groups. Data are presented as means ± SE. The difference between groups was considered significant when \( P < 0.05. \)

**RESULTS**

PAF markedly induces the gene expression of MIP-2 but only moderately upregulates gene expression of CINC-1 and MCP-1. We first examined whether MIP-2 and Gro/CINC-1, two major chemokines controlling neutrophil infiltration in the rat, and MCP-1 were expressed constitutively in the rat ileum and whether they were upregulated by PAF. We found that MIP-2 mRNA was almost undetectable constitutively in the ileum of rats not surgically prepared (not shown). However, both Gro/CINC-1 and MCP-1 mRNA were present. In sham-operated rats, a very low amount of MIP-2 became detectable in some animals (Fig. 1A). Sixty minutes after PAF injection, MIP-2 gene expression was very strongly upregulated \([8.13 ± 1.51\text{-fold increase } (P < 0.001, n = 7); \text{Fig. 1A}],\) whereas Gro/CINC-1 and MCP-1 were moderately increased \((4.92 ± 1.02-3.48 ± 0.35\text{-fold, respectively } (P < 0.001, t-test, n = 7); \text{Fig. 1A})\).

PAF markedly increases the plasma and ileal MIP-2 protein levels. To determine whether this strong increase in MIP-2 gene expression was concomitant with the upregulation of MIP-2 protein, we examined the level of MIP-2 released in the circulation and locally produced in the intestine before and after PAF challenge. MIP-2 protein was barely detectable in the ileal tissue (133.6 ± 45.2 pg/mg of protein; Fig. 2A) and in the plasma \((18.2 ± 18.2\text{ pg/ml of plasma}; \text{Fig. 2B})\) collected from sham-operated animals. PAF treatment induced a strong increase in MIP-2 protein levels in both the ileum \((1,487.8 ± 551.4\text{ pg/mg of protein}; \text{Fig. 2A})\) and the plasma \((2,610 ± 554\text{ pg/ml of plasma}; \text{Fig. 2B})\) corresponding to an 11- and 143-fold increase, respectively \((P < 0.05, t\text{-test})\).

Pretreatment with anti-MIP-2 antibodies reduces PAF-induced neutrophil sequestration in the ileum. As previously reported (27), PAF caused a significant increase in MPO activity in the rat ileum (Fig. 3). Pretreatment with anti-MIP-2 antibodies significantly decreased the PAF-induced increase in MPO activity \((0.066 ± 0.009\text{ for IgG + PAF vs. 0.026} ± P < 0.05). \)
0.006 for anti-MIP-2 + PAF, P < 0.01, t-test), suggesting that MIP-2 plays a critical role in mediating PAF-induced neutrophil infiltration in the gut (Fig. 3).

Pretreatment with anti-MIP-2 prevents PAF-induced intestinal necrosis. The administration of PAF induces severe gross bowel injury, scored at 1.64 ± 0.56 (Table 1). To investigate the role of MIP-2 in PAF-induced bowel injury, a specific rat anti-MIP-2 antibody or control IgG was administered 90 min before PAF injection. Animals pretreated with control IgG showed a trend toward a lesser degree of bowel injury than when pretreated with NS (gross injury score of 1.12 ± 0.43 in IgG-PAF vs. 1.64 ± 0.56, in NS-PAF treatment) (Table 1). However, this difference was not statistically significant. Animals pretreated with anti-MIP-2 antibodies were almost totally protected from the gross intestinal injury induced by PAF [gross injury score of 0.19 ± 0.09 in anti-MIP-2-PAF vs. 1.12 ± 0.43 in control IgG-PAF treatment (P < 0.01, t-test; Table 1)].

The histological examination confirmed the gross assessment (Table 1). PAF alone or in combination with IgG (Fig. 4) caused microscopic injury (score of 1.67 ± 0.21) varying from the loss of epithelial cells at the villus tips (Fig. 4C) to extensive mucosal necrosis with loss of villi (Fig. 4, A and B). In contrast, pretreatment with anti-MIP-2 antibodies significantly prevented the bowel injury induced by PAF (score of 0.67 ± 0.28; Fig. 4, D–F).

Anti-MIP-2 pretreatment markedly attenuated PAF-induced systemic inflammation, including hypotension and leukopenia, but showed no effect on PAF-induced hemococoncentration. Pretreatment with control IgG, intraperitoneally caused a transient decrease in BP (71.78 ± 6.36% of baseline value) with a return to normal within 15 min (Fig. 5). Pretreatment with
anti-MIP-2 antibodies intraperitoneally caused a marked but transient hypotension (31 ± 6.69% of baseline value) with a slower return to the normal range over 90 min (Fig. 5). Injection of vehicle (saline) itself showed no effect on systemic BP (data not shown). Both groups were then treated with saline or PAF for 90 min. In those treated with saline, the mean BP remained stable during the next 90 min (Fig. 5) and comparable with sham-treated animals. In those animals treated with PAF, all developed hypotension. Animals that had been pretreated with either NS or control IgG developed a rapid and severe hypotension (6.68 ± 4.09 and 15.74 ± 5.9% of baseline BP values, respectively), which persisted during the experimental period (end BP of 51.27 ± 18.5 and 60 ± 15.37% of baseline values, respectively). In animals pretreated with anti-MIP-2 antibodies, the hypotension following PAF injection was less severe (30.26 ± 6.86% of baseline BP value) and more transient with return to nearly baseline value within 90 min (94 ± 3.11% of baseline value; Fig. 5). This difference was statistically significant ($P < 0.05$, $t$-test).

PAF induced a marked leucopenia [with a decrease in peripheral WBC count to 49.33 ± 10.27% of baseline value ($P < 0.01$, $t$-test; Fig. 6)], whereas the peripheral WBC count did not change over time in the sham-treated group (Fig. 6). In the group treated with anti-MIP-2 antibodies only, there was a slight decrease in the WBC count that was not statistically significant (95.12 ± 1.72% of baseline value; Fig. 6). In animals pretreated with control IgG, there was no significant improvement in PAF-induced leucopenia (Fig. 6). However, in animals pretreated with anti-MIP-2 antibodies, PAF-induced leucopenia was markedly and significantly attenuated (peripheral WBC count of 85.76 ± 10.3 vs. 54.58 ± 8.99% of baseline)

### Table 1. Anti-MIP-2 antibodies block PAF-induced bowel injury in rat small intestine

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<tr>
<th>Treatment</th>
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<th>Histological Results</th>
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<tr>
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<td>Means ± SE</td>
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<td>Means ± SE</td>
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<tr>
<td>PAF</td>
<td>1.64±0.56</td>
<td>1.7 (0.3–2.85)</td>
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<tr>
<td>IgG + PAF</td>
<td>1.12±0.43</td>
<td>0.8 (0.4–2.8)</td>
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<tr>
<td>Anti-MIP-2</td>
<td>0.19±0.09*</td>
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Values are means ± SE. Rats were pretreated intraperitoneally with either rabbit anti-macrophage inflammatory protein-2 (MIP-2) antibodies (1 mg/kg, rabbit origin), control rabbit IgG (1 mg/kg), or saline for 90 min. They were then injected intravenously with either platelet-activating factor (PAF 2.5 μg/kg) or saline. After 90 min, rats were killed, and gross and microscopic intestinal injury severity scores were attributed as explained in MATERIALS AND METHODS. Pretreatment with anti-MIP-2 IgG markedly inhibited PAF-induced intestinal injury ($P < 0.05$, $t$-test), whereas control IgG had no significant protective effect. *$P < 0.05$ and †$P < 0.01$ vs. IgG + PAF.

![Fig. 4](https://example.com/figure4.png)

Fig. 4. Microscopic changes of the rat small intestine following PAF treatment. Various degrees of mucosal necrosis were present in the rats treated with PAF following IgG pretreatment (A–C), whereas rats pretreated with anti-MIP-2 antibodies (Ab) and then treated with PAF did not show evidence of mucosal injury (D–F).
values in rats pretreated with anti-MIP-2 antibodies vs. control IgG, respectively; \( P < 0.05 \), t-test).

PAF induced a marked hemoconcentration (with an increase in hematocrit by 31.28 ± 5.35%; Fig. 6), whereas the hematocrit remained stable over time in the sham-treated group or in the animals treated with anti-MIP-2 alone (Fig. 6). Pretreatment with anti-MIP-2 antibodies or control IgG did not significantly improve PAF-induced hemoconcentration.

**DISCUSSION**

PAF is thought to be a central mediator involved in the bowel injury associated with necrotizing enterocolitis (NEC) and endotoxic shock (12). It is a potent endogenous phospholipid that mediates the intestinal injury induced by hypoxia-reperfusion (4), TNF (37), and LPS (14). PAF-receptor antagonists have been shown to prevent experimental NEC induced by hypoxia-cold stress-formula feeding in newborn rats (3). When injected intravenously, PAF induces hypotension and a dose-dependent isolated bowel necrosis predominantly in the small intestine, as seen in NEC (10). It activates NF-κB, a major transcription factor involved in inflammation, very rapidly (6) and induces the gene expression of PLA2 (41), TNF (15), NF-κB p50 precursor p105 (40), and PAF-receptor (43). PAF has been shown to activate platelets, and many types of inflammatory cells, especially neutrophils (1), stimulate leukocytes adhesion to endothelial cells (17, 18), increase microvascular permeability (18), and cause neutrophil infiltration during sepsis in the gastrointestinal (GI) tract (2). We previously found that neutrophils were involved in PAF-induced NF-κB activation (6). Furthermore, several previous studies suggest that PAF-induced injury requires leukocyte-endothelial cell adhesion (38, 39). Antibodies against the \( \beta \)-integrin CD 11b or CD 18 markedly attenuated PAF-induced shock, bowel injury, and mortality (39). These observations indicate that neutrophils are main effector cells that mediate PAF-induced bowel injury (39) and mortality (38).

The traffic of neutrophils toward sites of inflammation is regulated by chemokines, a superfamily of low-molecular-weight, secreted small peptides (21). There are more than 50 chemokines identified to date. The major chemokine families are termed CC and CXC chemokines. CXC chemokines such as IL-8 (in humans) and GRO (Gro/CINC-1 and MIP-2 in rats) primarily activate and attract neutrophils. The C-C chemokines target mainly monocytes and T cells but are also active on eosinophils, basophils, dendritic cells, natural killer cells, and, to a much lesser degree, neutrophils. The small intestine is known to produce various chemokines on stimulation and following infection (34). Besides various resident immune cells, which are likely sources of chemokines, the intestinal epithelium has also been shown to secrete chemokines such as IL-8, Gro, MCP-1, regulated on activation,
normal T cell-expressed, and presumably secreted, MIP-1α, MIP-1β, and MIP-2 (26).

There is evidence that MIP-2 is one of the major inducible chemokines that lead to neutrophil infiltration and subsequent tissue injury in several animal models of inflammation and injury. MIP-2 has been shown to mediate the lung injury induced by IgG immune complex (33), LPS (31), aspiration pneumonia (32), influenza virus (30), and fecal peritonitis [the cecal ligation and puncture (CLP) model] (42). MIP-2 mediates the liver injury induced by adenovirus infection (25), the kidney injury induced by ischemia/reperfusion (23), and the neutrophil influx in antibody-induced glomerulonephritis (9). The role of MIP-2 in promoting neutrophil infiltration is organ specific: MIP-2 is necessary for polymorphonuclear neutrophil (PMN) migration into the lung and peritoneum but not to the liver in the fecal peritonitis model induced by CLP (22). MIP-2 has also been shown to act on extravascular leukocyte recruitment but not on rolling or adhesion: immunoneutralization of MIP-2 did not reduce LPS-induced leukocyte rolling and adhesion in postsinusoidal venules but abolished the extravascular recruitment of leukocytes in the livers of endotoxemic mice (19). Besides mediating organ injury in several models of inflammation affecting the lung (30, 42), kidney (9), and liver (25), MIP-2 has also been shown to mediate reperfusion-induced firm leukocyte-endothelial adhesion in the colon (29). These observations lead us to hypothesize that MIP-2 may play a central role in mediating the acute intestinal injury induced by PAF.

In this study, we found that PAF strongly upregulates MIP-2, whereas it is almost undetectable constitutively. In contrast, Groα/CINC-1 and MCP-1 are constitutively expressed in the intestine and moderately upregulated by PAF. PAF also caused the local production of MIP-2 in the ileum and its release into the plasma. Furthermore, we found that MIP-2 plays an essential role in the bowel injury induced by PAF, because MIP-2 blocking antibodies nearly totally protect against PAF-induced intestinal injury but not control IgG.

Anti-MIP-2 antibodies significantly attenuated PAF-induced systemic inflammation including hypotension induced by PAF. Indeed, at 90 min following PAF injection, rats pretreated with control IgG or NS alone were still severely hypotensive, whereas rats pretreated with anti-MIP-2 antibodies had already recovered a near-baseline BP (the recovery was rapid, despite a more profound hypotension during the pretreatment period). The effect of PAF on the cardiovascular system is complex (24). PAF causes dilation of resistance vessels (independently of prostaglandin production) (44), reduction of venous return, venodilation (mediated by prostaglandin), and has a direct negative inotropic effect. Furthermore, PAF induces vascular leakage, leading to hypovolemia, and increases pulmonary vascular resistance, both decreasing the left atrial filling (24). At doses sufficient to induce marked bowel injury, the hypotension is usually irreversible. However, similar doses of PAF induce only transient hypotension in leukocyte-depleted rats (39), suggesting that the late hypotensive effect is not a direct action of PAF on the cardiovascular system but is leukocyte dependent. Blocking leukocyte-endothelial adhesion with anti-β2 integrin has a protective effect on BP similar to that of leukocyte depletion (39). In the present study, we showed that inhibiting neutrophil recruitment and chemotaxis by anti-MIP-2 did not significantly affect the initial drop in BP induced by PAF but effectively blocked the second phase of hypotension, and the BP rapidly recovered. It is possible that PMN recruitment, activation, and adhesion to the endothelial cells lead to the release of reactive oxygen radicals, resulting in endothelial injury, vascular leakage, and subsequent tissue damage. Interestingly, anti-MIP-2 antibodies failed to protect against PAF-induced hemoconcentration. The mechanism of PAF-induced hemoconcentration may also be complex. Previous investigations (28) showed that plasma atrial natriuretic peptide (ANP), a hormone involved in plasma volume regulation, was elevated following LPS administration, which was significantly blocked by PAF receptor antagonists. Furthermore, LPS-induced hemoconcentration was largely inhibited by PAF receptor antagonists or splenectomy, suggesting a complex interaction between PAF-induced ANP and the spleen in causing hemoconcentration. It is possible that anti-MIP2 blocks the effect on vascular leakage by inhibiting PMN recruitment but has little or no effect on other mechanisms such as ANP secretion. As a result, the increase in HCT is incompletely inhibited.

PAF induces a profound leukopenia and causes neutrophil infiltration in the GI tract within 30 min (2). Anti-MIP-2 antibodies significantly reduced PAF-induced leukopenia and PAF-induced neutrophil infiltration of intestinal tissues. Although several mechanisms involving complex interactions of different cell types may contribute to the inflammatory response induced by PAF in vivo, the present study further supports the central role played by neutrophils in PAF-induced bowel injury through the upregulation of MIP-2. The cell source of MIP-2 is unclear. Candidates include several cell types of the intestinal mucosa capable of expressing MIP-2, such as fibroblasts (8), endothelial cells (20), neutrophils (16), macrophages (16), and epithelial cells (8, 26).

In summary, we found that PAF, even at a low dose (which does not cause gross bowel injury) induces both C-C and C-C chemokines, especially MIP-2, in the intestine. MIP-2, in turn, may cause neutrophil sequestration into the intestinal tissue and mediates PAF-induced intestinal injury. Indeed, pretreatment of rats with an anti-MIP-2 antisera before PAF exposure markedly attenuated PAF-induced bowel injury as well as the hypotension and leukopenia induced by PAF. The inhibition of MIP-2 might provide a novel therapeutic approach to acute bowel injury, such as NEC, for which no specific pharmaceutical agents are currently available.

GRANTS

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REFERENCES


