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Platelet-activating factor modulates gastric mucosal inflammatory responses to *Helicobacter pylori* lipopolysaccharide

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Abstract

Platelet-activating factor (PAF) is a phospholipid messenger implicated in mediation of inflammatory events associated with the resolution of inflammation. We applied the animal model of *Helicobacter pylori* LPS-induced gastritis in conjunction with prophylactic and therapeutic administration of a specific PAF antagonist, BN52020, to investigate the role of PAF in gastric mucosal responses to *H. pylori* infection. Prophylactic BN52020 administration produced up to 73.6% reduction in the severity of the LPS-induced inflammatory changes, whereas up to 38.4% increase in the severity of mucosal involvement occurred with BN52020 administered therapeutically. The prophylactic effects of BN52020 were accompanied by a drop in apoptosis and the expression of TNF-α and NOS-2, while BN52020 administered therapeutically caused a marked upregulation in apoptosis, TNF-α, and NOS-2. The untoward therapeutic effects of BN52020, moreover, were potentiated further in the presence of COX-2 inhibitor, whereas NOS-2 inhibitor caused a reduction in the extent of inflammatory changes. Our findings point to PAF as a key mediator of gastric mucosal inflammatory responses to *H. pylori* and suggest its modulatory role in the expression of COX-2 derived anti-inflammatory prostaglandins that are involved in controlling the extent of NOS-2 induction.

Keywords: *Helicobacter pylori* LPS; Acute gastritis; PAF; NOS-2; COX-2; TNF-α

Helicobacter pylori, a spiral Gram-negative bacterium colonizing the gastric mucosa, is now recognized as a primary cause of chronic active gastritis, duodenal ulcers, and over 80% of gastric ulcers in the world [1,2]. Among the factors implicated in the virulent action of *H. pylori* is its cell wall lipopolysaccharide (LPS) [3,4]. Indeed, gastric mucosal responses associated with *H. pylori* infection in humans as well as those characterizing mucosal inflammatory changes in the animal model of *H. pylori* LPS-induced gastritis are manifested by up-regulation in NO and prostaglandin production, massive enhancement in apoptosis, and the induction of TNF-α that triggers transcriptional factor NF-κB activation [5–7]. The activated NF-κB translocates to the nucleus where it activates genes mediating various aspects of inflammatory responses, including the induction of inducible COX-2 and NOS-2 enzymes [8,9].

Although the increase in gastric mucosal production of NO and prostaglandins is viewed by some as one of the beneficial effects of *H. pylori* infection contributing to mucosal resistance to injury, a mounting evidence suggests that overexpression of COX-2 and NOS-2 is intimately implicated as the promoting event in *H. pylori*-associated atrophic gastritis, intestinal metaplasia, and Barrett's esophagus [5,10,11]. Moreover, studies indicate that the induction in NOS-2 expression leads to the excessive formation of NO-related species that evoke transcriptional disturbances, cause alteration in the extent of apoptosis and prostaglandin production, and lead to up-regulation in TNF-α [12,13].

While the involvement of TNF-α in NF-κB activation is well documented, more recent data suggest that the most proximal mediator released in response to bacterial LPS is a phospholipid-derived messenger, platelet-activating factor (PAF) [14,15]. There are also indications that the initial inflammatory responses to LPS resulting in the release of PAF lead to the early phase of NF-κB

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activation that induces TNF-α expression, the effects of which are magnified and perpetuated further by the secondary and prolonged activation of NF-κB [15,16]. Interestingly, the late phase of NF-κB activation is associated with the expression of COX-2-derived anti-inflammatory prostaglandin, 15d-PGJ2, and the inhibition of COX-2 during this phase of NF-κB activation protracts the resolution of inflammation [16,17]. Moreover, it has been shown recently that inhibitors of NF-κB when given therapeutically during the resolution of inflammation cause prolongation of the inflammatory responses [17].

In this study, using the animal model of *H. pylori* LPS-induced acute gastritis [3], in conjunction with prophylactic and therapeutic administration of a specific PAF antagonist, BN52020, we investigated the role of PAF in gastric mucosal inflammatory responses to *H. pylori* infection.

Materials and methods

Animals. The study was conducted with Sprague–Dawley rats in compliance with the Institutional Animal Care and Use Committee. The animals were divided into groups and subjected to two different types of treatment regimen. In one, prophylactic, the animals at 16 and 4 h, before intragastric surface epithelial application of *H. pylori* LPS at 50 μg/animal [3,7], were administered at 0–30 mg/kg with BN52020 (Calbiochem) or vehicle consisting of 5% gum arabic in saline, whereas in the second type of treatment, therapeutic, the intragastric administration of BN52020 or the vehicle was performed 16 and 24 h after the LPS application. The rats in each group were then maintained for two days on the twice daily doses of COX-2 inhibitor, NS-398 (Sigma) at 0–30 mg/kg or NOS-2 inhibitor, 1400W (Calbiochem) at 0–15 mg/kg, and killed 16 h after the last treatment on the fourth day following the LPS application. The stomachs were dissected and the gastric mucosal tissue was used for histologic and biochemical measurements.

Helicobacter pylori LPS. H. pylori ATCC No. 4350 clinical isolate was used for LPS preparation [3]. The bacterium was homogenized with liquid phenol–chloroform–petroleum ether and centrifuged, and the LPS contained in the supernatant was precipitated with water, washed with 80% phenol solution, and dried with ether. The dry residue was dissolved in a small volume of water at 45 °C and centrifuged at 100,000*g* for 4 h, and the resulting LPS sediment was subjected to lyophilization.

Mucosal histology. The sections of gastric mucosa were cut into 4 μm strips, fixed in 10% buffered formalin, and stained with hematoxylin and eosin [3]. The morphological pattern of gastritis was graded in accordance with the Sydney system and the changes in mucosal histology were quantified, as described earlier [3].

Apoptosis and NOS-2 activity assays. Measurements of apoptosis were conducted in accordance with the manufacturer's (Boehringer Mannheim) instruction, using epithelial cells prepared from gastric mucosal scrapings [7]. The cytoplasmic histone-associated DNA fragments, reacted with immobilized anti-histone antibody, were incubated with anti-DNA peroxidase and probed with ABTS reagent for spectrophotometric quantification. The NOS-2 activity in gastric mucosal homogenates was measured by monitoring the conversion of L-[2,3,4,5-^{3}H]arginine to L-[^{3}H]citrulline using NOS-Detect Assay Kit (Stratagene). The formed L-[^{3}H]citrulline was quantified by scintillation counting [6].

TNF-α expression assay. TNF-α was quantified with an enzyme-linked immunosorbent assay system (Genzyme), using gastric mucosal homogenates and horseradish peroxidase-conjugated anti-TNF-α. The complex was then probed with TMB reagent for spectrophotometric TNF-α quantification [6].

Data analysis. All experiments were carried out in duplicate and the results are expressed as means ± SD. Analysis of variance (ANOVA) was used to determine significance and the significance level was set at $p < 0.05$. The protein content of samples was measured with BCA protein assay kit (Pierce).

Results

The role of PAF as a mediator of gastric mucosal inflammatory reaction to *H. pylori* infection and the underlying mechanism of its action were assessed in the animal model of *H. pylori* LPS-induced gastritis using rats subjected to intragastric administration of a specific PAF antagonist, BN52020, either before (prophylactic) or after (therapeutic) the LPS application. The results of histologic examination of the mucosa 4 days after exposure of the animals to the LPS in the absence of BN52020 administration revealed a pattern of inflammatory changes resembling that of acute gastritis with the mean grade of mucosal pathologic condition of 5.3 (Fig. 1), and characterized by the infiltration of lamina propria with lymphocytes and plasma cells, edema, hyperemia, and epithelial hemorrhage extending from the lamina propria to the surface of the mucosa. Prophylactic administration of BN52020 at 16 and 4 h before the lipopolysaccharide led to dose-dependent reduction in the severity of mucosal inflammatory involvement, which with BN52020 at 20 mg/kg decreased by 73.6% (Fig. 1). A marked increase in the severity of mucosal changes, however, occurred with BN52020 administered therapeutically at 16 and 24 h following the LPS. The effect was dose-dependent and manifested by an increase

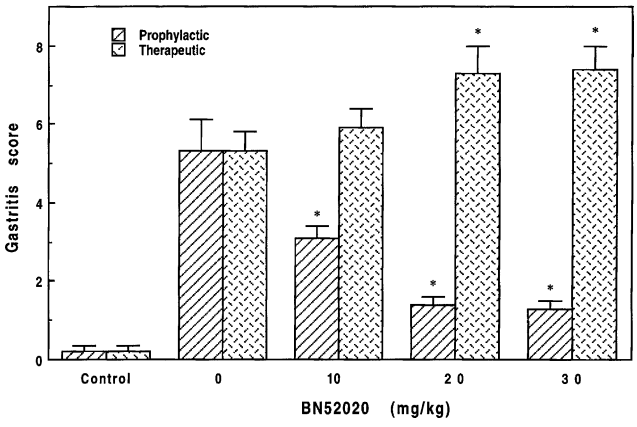

Fig. 1. Prophylactic and therapeutic effects of PAF antagonist, BN52020, on the scores of acute gastritis elicited in rats by *H. pylori* LPS. Prophylactic administration of BN52020 was performed at 16 and 4 h before the LPS application, while therapeutic administration of BN52020 was carried out at 16 and 24 h after the LPS application. Values represent means ± SD obtained with six animals in each group. *$P < 0.05$ compared with that of LPS alone.

in the density of the inflammatory infiltrate, neutrophils, and granulocytes, and superficial erosions, which with BN52020 at 20 mg/kg produced a 38.4% increase in the severity of inflammatory changes (Fig. 1).

The data on the effect of COX-2 inhibitor, NS-398, and NOS-2 inhibitor, 1400W, on the LPS-induced pattern of mucosal inflammatory changes in the presence of prophylactic and therapeutic administration of BN52020 are summarized in Fig. 2. Inhibition of COX-2 or NOS-2 activity in the presence of prophylactic administration of PAF antagonist, BN52020, elicited only 4–6% additional reduction in the extent of the LPS-induced inflammatory involvement. By contrast, the inhibition of COX-2 activity in the presence of therapeutic administration of BN52020 led up to 22.1% enhancement of the PAF antagonist effect in the inflammatory involvement elicited by *H. pylori* LPS (Fig. 2A). The untoward effects of therapeutic BN52020 administration on the extent of mucosal inflammatory responses to *H. pylori* LPS, however, were countered by NOS-2 inhibitor, 1400W, which at 10 mg/kg dose elicited a 53.4% reduction in the severity of mucosal inflammatory involvement (Fig. 2B).

The analysis of gastric mucosal NOS-2 activity revealed that *H. pylori* LPS-induced inflammatory changes were accompanied by a massive induction in the expression of NOS-2 activity (Fig. 3). The prophylactic administration of BN52020 was associated with a 38.1% reduction in the mucosal NOS-2 activity, while a 53.9% increase in NOS-2 expression was attained with the therapeutic administration of BN52020. Moreover, the inhibitory effect of prophylactic BN52020 administration on NOS-2 activity was potentiated further not only by 1400W, an inhibitor of NOS-2, but also by NS-398, an inhibitor of COX-2. On the other hand, the enhancement of the LPS-induced expression of NOS-2 activity associated with the therapeutic effects of BN52020 was further amplified by a 22.1% in the presence of NS-398, while 1400W exerted the inhibitory (70.5%) effect.

The influence of prophylactic and therapeutic administration of PAF antagonist, BN52020, on the extent of *H. pylori* LPS-induced epithelial cell apoptosis is presented in Fig. 4. A decrease in gastric mucosal inflammatory involvement in the presence of prophylactic BN52020 administration was reflected in a 41.5% decline in the rate of apoptosis, whereas a 67.1% higher rate of apoptosis accompanied the increase in the severity of mucosal changes in the presence of therapeutic BN52020 administration. The antiapoptotic effects of prophylactic BN52020 administration showed further increase in the presence of the inhibitors of COX-2 and NOS-2 activity. The proapoptotic effects of therapeutic PAF antagonist

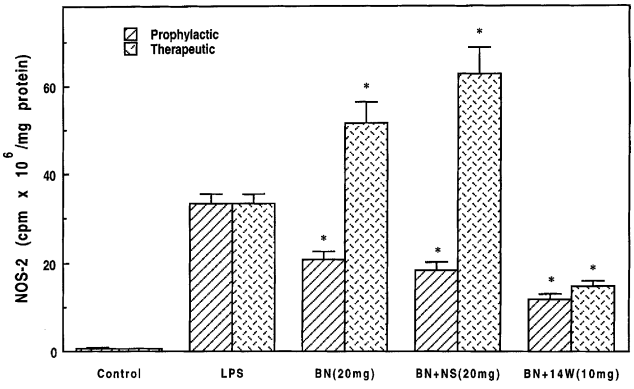

Fig. 3. Effect of NS-398 and 1400W on the LPS-induced changes in gastric mucosal expression of NOS-2 activity in the presence of prophylactic and therapeutic administration of BN52020. $^*P < 0.05$ compared with that of LPS alone.

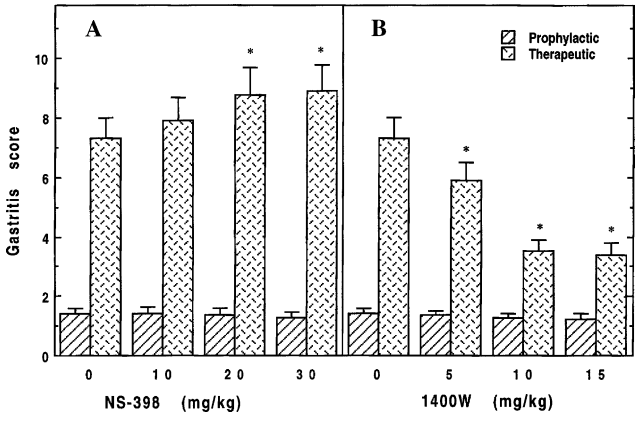

Fig. 2. Effect of COX-2 inhibitor, NS-398 (A), and NOS-2 inhibitor, 1400W (B) on the LPS-induced acute gastritis in the presence of prophylactic and therapeutic administration of PAF antagonist, BN52020. Following BN52020 (at 20 mg/kg) administration, the animals were maintained for 2 days on the twice daily doses of NS-398 or 1400W. $^*P < 0.05$ compared with that of LPS + BN52020.

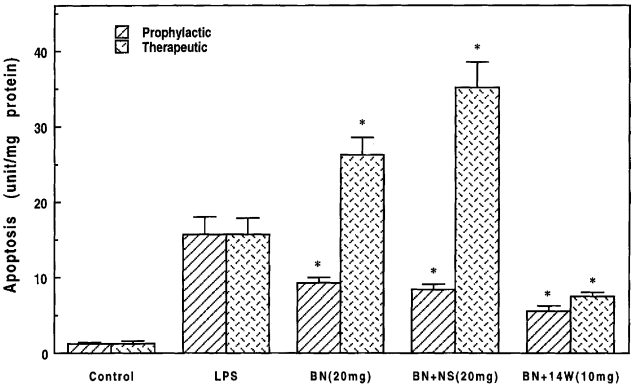

Fig. 4. Effect of NS-398 and 1400W on the LPS-induced changes in the rate of gastric epithelial cell apoptosis in the presence of prophylactic and therapeutic administration of BN52020. $^*P < 0.05$ compared with that of LPS alone.

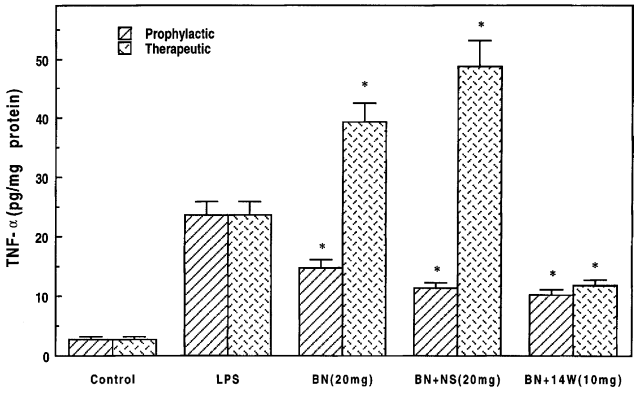

Fig. 5. Effect of NS-398 and 1400W on the LPS-induced changes in gastric mucosal expression TNF-α in the presence of prophylactic and therapeutic administration of BN52020. *$P < 0.05$ compared with that of LPS alone.

administration were potentiated by a 34.1% in the presence of COX-2 inhibitor, NS-398, while the NOS-2 inhibitor, 1400W, exerted a 72.4% inhibitory effect.

The pattern of changes in gastric mucosal expression of TNF-α is summarized in Fig. 5. Compared to the LPS alone, a 37.2% reduction in the LPS-induced mucosal level of TNF-α was attained in the presence of prophylactic BN52020 administration, and the inhibitory effect of BN52020 was potentiated (25–30%) further by NS-398 and 1400W. A 65.9% increase in gastric mucosal expression of TNF-α occurred in the presence of therapeutic BN52020 administration and the inclusion of NS-398 increased the expression of TNF-α by an additional 23.6%, whereas the NOS-2 inhibitor, 1400W, caused a significant (69.1%) decline in TNF-α.

Discussion

Enhancement in gastric mucosal TNF-α production, excessive NO and prostaglandin generation, and alteration in the extent of epithelial cell apoptosis are well-recognized features of gastritis associated with *H. pylori* infection in humans as well as characterize mucosal inflammatory responses in the animal model of *H. pylori* LPS-induced gastritis [3,4,9]. Studies indicate that upregulation in NO and prostaglandins with *H. pylori* infection is the result of TNF-α-mediated activation of the transcriptional factor NF-κB, recognized for its ability to rapidly elicit transcriptional responses to a variety of pro-inflammatory stimuli that lead to the induction of COX-2 and NOS-2 genes [8,9]. The emerging new insight into the pathways for NF-κB activation, moreover, indicates that the most proximal mediator released in response to bacterial infection is PAF [14,15].

Although normally not present in the cells, the PAF is rapidly generated from membrane alkylacyl-glycerophosphorylcholine through the concerted action of phospholipase A2 and acetyl-transferase enzymes in response to inflammatory stimulus [14,15]. Once released, PAF elicits a rapid induction of TNF-α expression through the activation of NF-κB target genes encoding proinflammatory cytokines, chemokines, growth factors as well as inducible enzymes such as COX-2 and NOS-2 [16,17]. Therefore, the use of PAF antagonists to abrogate the consequences of NF-κB activation in response to microbial infection offers tempting target for accelerated resolution of inflammation. However, the results remain at variance, with an apparent PAF antagonist efficacy in resolution of inflammation in animal model of colitis [18], and the clinical data showing no beneficial effect of PAF antagonist over placebo in patients with sepsis [19]. Yet another study with mice revealed that LPS-induced anaphylactic shock-like symptoms can be prevented by prophylactic pretreatment with PAF antagonist, due to abrogation of the LPS-induced early phase of NF-κB activation [20]. However, in the same animal, therapeutic administration of NF-κB inhibitors during the resolution of inflammation protracted the inflammatory responses [17].

In the study presented herein we assessed the effect of a specific PAF antagonist, BN52020, on the course of mucosal inflammatory responses to *H. pylori* LPS by analyzing the interplay between the extent of mucosal pathology, apoptotic activity, and the expression of TNF-α and NOS-2 enzyme activity. The results revealed that prophylactic administration of BN52020 elicited a marked reduction in the severity of the LPS-induced mucosal inflammatory involvement, whereas exacerbation in the severity of mucosal inflammatory changes occurred with therapeutic 52020 administration. Moreover, the prophylactic effects of BN52020 were associated with a drop in the rate of apoptosis and the mucosal expression of TNF-α and NOS-2. On the other hand, an increase in the severity of mucosal changes attained with BN52020 administered therapeutically, and manifested by a marked upregulation in apoptosis, TNF-α and NOS-2, were potentiated further in the presence of COX-2 inhibitor NS-398, while NOS-2 inhibitor 1400W elicited a profound reduction in the severity of BN52020 proinflammatory effect.

The findings thus point to PAF as a key mediator of the early phase of inflammatory events elicited in gastric mucosa by *H. pylori* LPS, and manifested by the increased expression of TNF-α, enhanced apoptotic activity, and the induction of NOS-2. Our results are supported by the data with the in vitro and in vivo systems demonstrating that PAF is a critical mediator in the early activation of NF-κB activities that trigger early response genes encoding for TNF-α and NOS-2 that initiate inflammatory reaction [15]. The fact that proinflammatory effects of therapeutic PAF antagonist administration were potentiated further in the presence

of COX-2 inhibitor, moreover, implies that the interference with PAF actions during the late phase of gastric mucosal responses to *H. pylori* LPS affects the formation of prostaglandins associated with the resolution of inflammation. Indeed, recent studies revealed that the resolution of inflammation is accompanied by the production of anti-inflammatory 15d-PGJ2 and that selective COX-2 inhibitors when administered therapeu tically protract the inflammatory reaction [17,21,22].

Equally pertinent to the interpretation of the results of our study are the data indicating that 15d-PGJ2 and related anti-inflammatory prostaglandins, produced during the resolution of inflammation, exert inhibitory effect on the NOS-2-dependent NO generation [22,23]. Indeed, we observed that increase in the severity of mucosal involvement with the use of COX-2 inhibitor in conjunction with therapeutic administration of BN52020 was associated with the induction in mucosal NOS-2 activity, and that reduction in the inflammatory effect of BN52020 attained in the presence of NOS-2 inhibitor was also reflected in down-regulation in the expression of TNF-α and apoptosis. As the increase in apoptosis and up-regulation in NOS-2 expression also accompanies *H. pylori*-induced gastritis, [5,10], our results infer that PAF may play a pivotal role in affecting the nature of pathologic consequences of *H. pylori* infection. This contention is further supported by the data indicating that induction of NOS-2 leads to proapoptotic caspase-3 activation and the excessive formation of NO-related species that evoke transcriptional disturbances, cause alterations in prostaglandin formation, and lead to up-regulation of proinflammatory cytokine production [12,13,24].

In summary, our findings point to PAF as a key mediator of gastric mucosal inflammatory responses to *H. pylori* LPS and demonstrate that the interference with its actions following the initiation of inflammatory reaction protracts the resolution of inflammation. The results imply the role of PAF in controlling the expression of COX-2-derived anti-inflammatory prostaglandins, including 15d-PGJ2, that play a crucial role in modulation of the extent of NOS-2 induction and hence the mucosal NO production.

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