Expression of Phosphorylcholine by *Histophilus somni* Induces Bovine Platelet Aggregation[▽]

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***Histophilus somni*-induced platelet aggregation was inhibited by antagonists of the platelet-activating factor (PAF) receptor but not inhibitors of PAF synthesis. In addition, *H. somni* cells expressing phosphorylcholine (ChoP) induced aggregation, while ChoP[−] *H. somni* cells did not. This suggests that *H. somni* ChoP may induce platelet aggregation via interactions with the PAF receptor.**

Histophilus somni is a gram-negative pleomorphic bacillus that causes respiratory disease primarily in cattle. Clinical signs of *H. somni* infection can include pneumonia, abortion, arthritis, septicemia, myocarditis, vasculitis, and an acute condition known as thrombotic meningoencephalitis (1, 3, 8, 10, 13).

The ability of *H. somni* to modify its lipooligosaccharide (LOS) composition and structure is thought to play an important role in its virulence. *H. somni* can incorporate sialic acid into the outer core of the LOS molecule, and this event has been shown to be critical for resistance to serum-mediated killing (14). It has been reported that some strains of *H. somni* that do not incorporate sialic acid are unable to produce disease (5). Another modification is the addition of phosphorylcholine (ChoP) to the outer core of the LOS (12).

H. somni exhibits extensive intrastrain variability in ChoP incorporation into the LOS molecule (12). The possible contribution of ChoP to *H. somni* pathogenesis is unknown. For other species of bacteria, ChoP can be incorporated into bacterial structures such as fibrillar proteins and cell wall components that are important for bacterial adherence to host cells. For example, the expression of ChoP by *Streptococcus pneumoniae* has been reported to contribute to pneumococcal adherence and invasion in the lung (6, 30) and the brain (20). Similarly, the expression of ChoP on the LOS of *Haemophilus influenzae* contributes to its binding and internalization by human epithelial cells (27, 28). This adherence was demonstrated to be due to an interaction between ChoP expressed on LOS and the platelet-activating factor (PAF) receptor on epithelial cells. ChoP has also been found in *Actinobacillus actinomycesetemcomitans*, which facilitates binding to the PAF receptor on human vascular endothelial cells, followed by internalization of the bacteria (7, 23, 24). ChoP has also been detected in *Bacillus* spp. and *Clostridium* spp., and the lipopolysaccharide of *Escherichia coli* O26:B6 was found to activate human platelets through a PAF receptor-dependent pathway (9, 19).

Our laboratory has previously reported that *H. somni* and its

LOS can activate bovine platelets (16). We also found that *H. somni*, but not its LOS, could induce platelet aggregation. The mechanism by which *H. somni* induces platelet aggregation is unknown. It has previously been demonstrated that endotoxin and bacteria can adhere to and activate platelets from several different mammalian species (2, 11, 18, 21, 22, 31–33). For this study, we sought to investigate the interaction between *H. somni* and bovine platelets and determine if bacterial expression of ChoP affects platelet activation.

We first wanted to ascertain whether platelet aggregation was induced by ChoP-expressing *H. somni* cells. Using colony immunoblotting with an anti-phosphorylcholine antibody (15), two *H. somni* variants of strain 7735 were selected for either high or low expression of ChoP. These populations were enriched through selective passage in culture. Bovine platelets (2.5×10^8 platelets) (isolation procedures were described previously [16]) were incubated with one of the two *H. somni* variants (multiplicity of infection [MOI] of 5:1) for 10 min in a Chronolog aggregometer. As a positive control, platelets were treated with PAF (10^{-6} M; Calbiochem) to induce irreversible aggregation within 5 min. It was found that ChoP⁺ *H. somni* induced platelet aggregation, while ChoP[−] *H. somni* did not (Fig. 1A and B). ChoP⁺ *H. somni* consistently induced approximately 15% aggregation, which was not reversible within a 30-min incubation period (data not shown). Upon microscopic examination, platelet aggregates could be observed following incubation with ChoP⁺ but not with ChoP[−] *H. somni* (Fig. 1C and D). In addition, we observed ChoP⁺ *H. somni* within bovine platelet aggregates. Pretreatment of ChoP⁺ *H. somni* with polymyxin B (10 µg/ml; Sigma) for 10 min resulted in a modest decrease in the ability of *H. somni* to induce aggregation (Fig. 1E).

We next considered the possibility that *H. somni* may interact with the PAF receptor on platelets. To exclude the contribution of platelet-derived PAF, we incubated platelets with previously described selective inhibitors of phospholipase A₂, AACOCF₃ (10 µM; Calbiochem) or cPLA (10 µM; Calbiochem), for 10 min prior to the addition of ChoP⁺ *H. somni* (17, 26). Some platelets were preincubated with the PAF receptor antagonist WEB 2170 (10 µg/ml) at a concentration that was previously demonstrated to inhibit platelet activation by PAF (29). Platelets preincubated with WEB 2170 but not inhibitors

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FIG. 1. ChoP⁺ but not ChoP⁻ *H. somni* induces bovine platelet aggregation. Platelets (500 μ l, 2.5×10^8 platelets/ml) were placed in siliconized glass cuvettes and incubated with ChoP⁺ or ChoP⁻ *H. somni* (MOI of 5:1) or with PAF (10^{-6} M) as a positive control. Platelet aggregation was measured for 10 min using the turbidimetric method in a Chrono-Log Model 560-Ca Dual Sample Lumi-Ionized Calcium aggregometer, and the percent aggregation was calculated using AGGRO/LINK software. The aggregation plot in A is a representative experiment of four separate experiments that were performed. The data in B illustrate the means $\pm$ standard errors of the means (SEM) of four separate experiments showing the percent aggregation induced at 10 min (*, $P < 0.05$ compared to ChoP⁺ *H. somni*-treated platelets). For some aggregation experiments, platelets were removed from the cuvette and transferred onto glass coverslips using a Cytospin centrifuge before being fixed and stained with Diff-Quick. These platelets were then observed by light microscopy for the presence platelet-bacterium aggregates (C and D). We observed such aggregates in the presence of ChoP⁺ but not ChoP⁻ *H. somni*. Preincubation of ChoP⁺ *H. somni* for 10 min with polymyxin B (PB) (10 μ g/ml) inhibited platelet aggregation (E). These data represent the means $\pm$ SEM of four separate experiments (*, $P < 0.05$ compared to ChoP⁺-treated platelets). PBS, phosphate-buffered saline.

FIG. 2. Platelet aggregation is inhibited by the PAF receptor antagonist WEB 2170. Platelets ($500 \mu\text{l}$, 2.5×10^8 platelets/ml) were pretreated for 10 min with the PAF receptor antagonist WEB 2170 ($10 \mu\text{g/ml}$) or the PAF synthesis inhibitor AACOCF ($10 \mu\text{M}$) or cPLA ($10 \mu\text{M}$) before incubation with ChoP⁺ *H. somni* (MOI of 5:1). Platelet aggregation was then measured as described in the legend of Fig. 1. The data represent the means $\pm$ SEM of four separate experiments (*, $P < 0.05$ compared to platelets incubated with ChoP⁺ *H. somni* alone).

of PAF synthesis demonstrated a significant diminution in platelet aggregation following incubation with ChoP⁺ *H. somni* (Fig. 2).

To study PAF receptor signaling, we used an inhibitor of downstream signaling through phospholipase C β (D609, $200 \mu\text{M}$; Calbiochem) and the PAF receptor antagonist WEB 2170 ($10 \mu\text{g/ml}$). Using an antibody against P-selectin as a marker of platelet activation (BD Bioscience), we observed low baseline P-selectin levels in unstimulated platelets. Following incubation with PAF (10^{-7} M) or ChoP⁺ *H. somni* (MOI of 5:1), we observed a significant increase in platelet activation (Fig. 3A and B). However, neither PAF antagonist resulted in a significant decrease in platelet P-selectin expression induced by ChoP⁺ *H. somni*, suggesting that PAF receptor activation is not required for platelet activation by ChoP⁺ *H. somni*. In addition, D609 inhibited PAF-induced aggregation but not aggregation induced by ChoP⁺ *H. somni* (data not shown). We next compared platelet activation in response to ChoP⁺ or ChoP⁻ *H. somni* as determined by flow cytometry evaluation of P-selectin expression and fibrinogen binding. The latter event, which is required for platelet aggregation, was detected using an antibody against platelet-bound fibrinogen (American Diagnostica, Stamford, CT). We found that both ChoP⁺ and ChoP⁻ *H. somni* cells induced significant platelet P-selectin expression (Fig. 3C). Surprisingly, ChoP⁺ *H. somni* did not induce a significant level of platelet fibrinogen binding compared to platelets stimulated with PAF. The lack of fibrinogen binding suggests that *H. somni* may induce platelet aggregate formation using a mechanism distinct from activation induced by PAF.

Transmission electron microscopy (TEM) was used to visualize whether the expression of ChoP by *H. somni* affected the adherence of the bacteria to platelets or platelet morphology. Bovine platelets were incubated with ChoP⁺ or ChoP⁻ *H. somni* for 10 min before being fixed and embedded for TEM. Unactivated platelets demonstrated their characteristic discoid shape, with no significant changes in morphology (Fig. 4A). Following incubation with ChoP⁺ or ChoP⁻ *H. somni*, bovine platelets exhibited spreading, pseudopod formation, and changes in morphology indicative of platelet activation (Fig. 4B). Although platelet spreading and pseudopod formation were observed following exposure to either ChoP⁺ or ChoP⁻

FIG. 3. *H. somni*-induced platelet activation is independent of PAF receptor signaling. Bovine platelets (2.5×10^8) were incubated for 10 min at 37°C with ChoP⁺ or ChoP⁻ *H. somni* (MOI of 5:1) or PAF (10^{-7} M). The platelets were then fixed in paraformaldehyde (0.4% final volume) and stained with antibodies for P-selectin or fibrinogen before being analyzed by flow cytometry. The results reflect the means $\pm$ SEM of percent positive platelets from at least three separate experiments. (A) Platelets preincubated with the PAF receptor antagonist WEB 2170 or the phospholipase C β inhibitor D609 expressed significantly less P-selectin after PAF stimulation than platelets treated with PAF alone (*, $P < 0.05$ compared to untreated platelets). (B) Pretreatment of platelets with WEB 2170 or D609 resulted in a modest decrease in P-selectin expression in platelets challenged with *H. somni*; however, this decrease was not significant ($P > 0.05$ compared to platelets incubated with ChoP⁺ *H. somni* alone). Panel C illustrates that platelets incubated with either ChoP⁺ or ChoP⁻ *H. somni* significantly up-regulated platelet P-selectin expression. However, neither strain was able to induce significant levels of platelet fibrinogen binding compared to untreated platelets (*, $P < 0.05$ compared to untreated platelets).

H. somni, only ChoP⁺ *H. somni* cells were capable of inducing platelet aggregation (Fig. 4C and D). In addition, obvious binding of platelets to ChoP⁺ *H. somni* only was observed. Closer examination of this interaction revealed surface structures on ChoP⁺ *H. somni* that appeared to facilitate the adherence of the bacteria to the platelets (Fig. 4E and F).

In this study, we demonstrated that ChoP expression by *H. somni* plays a role in its interactions with bovine platelets. Preincubation of platelets with the PAF receptor antagonist

FIG. 4. Transmission electron microscopy of platelets activated by *H. somni*. Platelets ($500\ \mu\text{l}$, 2.5×10^8 platelets/ml) were placed in siliconized glass cuvettes and incubated with ChoP⁺ or ChoP⁻ *H. somni* (MOI of 5:1) in a Chrono-Log Model 560-Ca Dual Sample Lumi-Ionized Calcium aggregometer. After a 10-min incubation, the platelet-rich plasma was fixed with a 25% volume of Karnovsky's solution (2.5% glutaraldehyde, 4% paraformaldehyde) for 10 min at room temperature and then centrifuged onto glass coverslips at $1,500 \times g$ for 10 min to facilitate TEM processing. (A) Unactivated bovine platelets exhibit the typical discoid or "plate" morphology. (B) Shape change and pseudopod formation but no aggregate formation by bovine platelets incubated with ChoP⁻ *H. somni*. (C and D) Aggregates of bovine platelets incubated with ChoP⁺ *H. somni*. (E and F) Platelets incubated with ChoP⁺ *H. somni* at greater magnification, with arrows indicating areas of attachment between bovine platelets and *H. somni*. These results are representative of three separate experiments.

WEB 2170 prevented platelet aggregation. However, the ineffectiveness of downstream PAF receptor signaling inhibitors suggests that PAF receptor signaling was not required for platelet activation or aggregation. One possible explanation

may reflect the location of the ChoP molecule on *H. somni* LOS, where it is coupled to the primary glucose residue that is bound to heptose I (4). Steric interference from sugars in the outer core may make ChoP less accessible to interactions with

the extracellular environment. In contrast, the ChoP group on *H. influenza* LOS is located on a terminal glucose on either heptose I or heptose III, where it may be more accessible for PAF receptor binding (25). Our findings suggest that *H. somni* may interact with the PAF receptor, but the resulting events are dissimilar to those by which PAF activates platelets. Upon careful examination, we conclude that aggregation reflected cross-linking between platelets and bacteria, a response that was not observed in bacteria that lacked ChoP (Fig. 1C and D and 4B to D). Future work will be directed at determining if *H. somni* ChoP interacts directly with the PAF receptor or through another platelet receptor and how these interactions may contribute to platelet activation.

Statistical analysis. An unbalanced one-way analysis of variance was used to determine if significant variation existed between group means. Pairwise comparisons of group means were performed using Tukey's test ($P < 0.05$) with the Prism 4 statistical package (GraphPad, San Diego, CA).

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