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Platelet activation by *Histophilus somni* and its LOS induces endothelial cell pro-inflammatory responses and platelet internalization

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Abstract

Histophilus somni is a gram negative coccobacillus that causes respiratory and reproductive disease in cattle. The hallmark of systemic *H. somni* infection is diffuse vascular inflammation that can lead to an acute central nervous system disease known as thrombotic meningoencephalitis (TME).

Previously we demonstrated that *H. somni* and its LOS activate bovine platelets leading to expression of P-selectin, CD40L and FasL. Since activated platelets have been reported to induce endothelial cell cytokine production and adhesion molecule expression we sought to determine if bovine platelets induce pro-inflammatory and pro-coagulative changes in bovine pulmonary artery endothelial cells. Endothelial cells were incubated with platelets activated with ADP, *H. somni* or *H. somni* LOS. Incubation with activated bovine platelets significantly increased expression of adhesion molecules (ICAM-1, E-selectin) and tissue factor, as measured by flow cytometry, real-time PCR, and western blot analysis. Activated platelets also up-regulated expression of endothelial cell IL-1 β , MCP-1, and MIP-1 α as determined by real-time PCR and an IL-1 β ELISA. An interesting and surprising finding was that bovine platelets activated by *H. somni* or its LOS were internalized by bovine endothelial cells as visualized by transmission electron microscopy. This internalization appeared to correlate with endothelial cell activation and morphological changes indicative of cell stress. These findings suggest that activated platelets might play a role in promoting vascular inflammation during *H. somni* infection.

Keywords

Haemophilus; cytokine; E-selectin; tissue factor; ICAM-1; bovine

Introduction

Histophilus somni is a gram negative bacterial pathogen of cattle that causes respiratory and reproductive disease. A member of the *Pastuerallaceae* family, *H. somni* is one of the bacterial agents responsible for the bovine respiratory disease (BRD) complex, commonly known as “shipping fever”^{1, 2}. Infection with *H. somni* can lead to pneumonia, abortion, vascular inflammation, myocarditis, arthritis, multiple thrombotic lesions, and an acute form of vasculitis known as thrombotic meningoencephalitis (TME)^{3–7}. The molecular pathogenesis of *H. somni* is poorly understood. Known virulence factors of *H. somni* include resistance to serum-mediated killing, immunoglobulin binding proteins, and resistance to killing by phagocytes^{4, 6, 8–11}. *H. somni* produces a truncated form of lipopolysaccharide known as

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lipooligosaccharide (LOS), which is capable of undergoing phase variation¹². *H. somni* has also been reported to produce histamine and incorporate phosphorylcholine into its LOS, which may also influence its pathogenesis^{13, 14}.

How *H. somni* promotes vascular damage is poorly understood. Antigen-antibody complexes are not likely responsible, as colostrum-deprived calves without anti-*H. somni* antibodies can develop vasculitis⁵. Another possibility is direct interactions between *H. somni* and the endothelium¹⁵. Previously, our lab has reported that *H. somni* and its LOS can induce caspase activation and apoptosis in bovine endothelial cells^{16, 17}. Although *H. somni* is commonly found associated with lesions in the lung and sometimes in thrombi, *H. somni* is not found in many vascular lesions⁵. Because of this, we have investigated the participation of platelets in *H. somni* induced vasculitis. Besides their primary role in maintaining haemostasis, platelets also contribute to vascular inflammation and injury. It has been previously reported that activated human platelets can induce endothelial cell up-regulation of adhesion molecules (P-selectin, E-selectin, ICAM-1), chemokine release (MCP-1, MIP-1 α , IL-8), IL-1 β and tissue factor expression in co-culture systems^{18–20}.

We have reported previously that bovine platelets activated by *H. somni* or its LOS express pro-inflammatory molecules such as CD40L, P-selectin and FasL²¹. In addition, activated bovine platelets could also induce endothelial cell apoptosis in a co-culture model. In this study, we demonstrate that bovine platelets activated by *H. somni* and its LOS, can promote endothelial cell pro-inflammatory responses. Following incubation with activated platelets, bovine endothelial cells expressed increased levels of adhesion molecules (ICAM-1, E-selectin), chemokines and IL-1 β . Incubation with activated platelets also increased endothelial cell expression of tissue factor, which may promote coagulation and thrombus formation in vivo. We also observed that platelets activated by *H. somni* or its LOS demonstrated increased adherence to endothelial cells and significant numbers of platelets were also internalized. These data suggest that bovine platelets might play a role in promoting vascular inflammation and coagulation following activation by *H. somni* and its LOS.

Materials and Methods

Chemicals and Media

Adenosine diphosphate (ADP), Paraformaldehyde, thiamine monophosphate, penicillin, streptomycin, and Dulbecco's modified Eagle's medium (DMEM, containing phenol red, 25 mM HEPES, 4.5 g/L dextrose and 2 mM L-glutamine) were obtained from Sigma Chemical Co., (St. Louis, MO). Brain heart infusion broth (BHI) and yeast extract were obtained from Difco (Detroit, MI). Mouse monoclonal antibodies against ICAM-1 were purchased from Zymed (Carlsbad, California). FITC-conjugated anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Antibodies against von Willibrand Factor were purchased from Chemicon (Temecula, CA). Antibodies against CD146 were purchased from Serotec (Raleigh, NC). Antibodies against tissue factor were purchased from Enzyme Research (South Bend, IN). Karnovsky's fixative and other electron microscopy supplies were purchased from Electron Microscopy Sciences (Hatfield, PA).

Platelet preparation

Healthy donor cattle were housed and bled following an approved animal care committee protocol. Cattle were bled by coccygeal venipuncture using an 18 G needle and vacutainer tubes containing sodium citrate (0.38% v/v) as anticoagulant. To minimize platelet activation during blood collection, care was taken to obtain venipuncture at the first needle stick. Blood from the first collection tube was discarded because this tube was more likely to contain platelets activated by the process of venipuncture. The collected blood was transferred to 50

ml polypropylene conical tubes and centrifuged at $500 \times g$ for 10 minutes. The platelet rich plasma (PRP) was collected and the platelets enumerated with a hemocytometer after a 1:100 dilution in Tyrodes/HEPES buffer (138 mM NaCl, 2.9 mM KCl, 1 mM MgCl₂, 1 mM glucose, 0.5 mM NaH₂PO₄, 20 mM HEPES, 0.3% BSA, pH 7.4). For purposes of these experiments, PRP was diluted in Tyrodes/HEPES buffer to a concentration of 1×10^7 – 10^8 platelets/ml. The platelets did not appear to be activated by the process of blood collection or processing as determined by flow cytometry evaluation of surface markers. Leukocyte contamination of the platelet preparations, as determined by Diff-Quik staining (VWR; West Chester, PA) was less than 10^4 leukocytes per ml.

Cultivation of bacterial strains

The pathogenic *H. somni* strain 649 was originally obtained from an aborted fetus and has been shown to cause reproductive failure when administered intrabronchially or intravenously. *H. somni* was incubated for 16 hours at 37°C, without shaking, in 25 cm² tissue culture flasks containing 10 ml of BHI broth supplemented with 0.5% yeast extract and 0.05% thiamine monophosphate (BHI-YT). The number of colony forming units (CFU) per ml was determined by measuring absorbance (OD₆₀₀), and extrapolating from a standard curve of *H. somni* grown under the same conditions. Frozen stock cultures of each *H. somni* isolate were stored at –70°C in BHI-YT and 10% glycerol. A fresh aliquot was thawed for use in each experiment. Lipooligosaccharide (LOS) was isolated by enzymatic digestion and hot phenol extraction from pathogenic strain 649 of *H. somni* as described previously²².

Treatment of platelets

Approximately 1×10^7 – 10^8 platelets were added to 1.5 ml microcentrifuge tubes with Tyrodes/HEPES buffer (final volume 1 ml). Platelets were incubated with either *H. somni* (MOI 10:1) or *H. somni* LOS (500 ng/ml). Controls included untreated platelets and platelets stimulated with ADP (10 μM), as a positive control. Microcentrifuge tubes containing the platelet reactions were placed on a Labquake rotator (Barnstead International Dubuque, IA) and incubated at 37°C for 10 minutes. After incubation, 200 μl of 4% paraformaldehyde in PBS was added to each tube (final concentration 0.66% v/v) and the platelets were fixed for 30 minutes at 4°C. Following fixation, platelets were washed 3× in phosphate buffered saline. These conditions did not activate platelets as evaluated by expression of surface markers by flow cytometry.

Bovine pulmonary endothelial cells

The primary cultures of bovine endothelial cells used in this study were obtained by gentle scraping of the pulmonary artery collected from healthy steers at the time of slaughter, as described previously¹⁶. These endothelial cells were cultured in DMEM (Cellgro, Herndon, VA) containing penicillin and streptomycin (500 IU/ml, Cellgro) and 20% fetal bovine serum (Atlanta Biologicals, Norcross, GA). The endothelial cells were seeded at a density of 1×10^5 cells/ml in 6 well tissue culture plates, and allowed to adhere overnight.

Treatment of endothelial cells

Washed platelets were incubated with endothelial cells at a density of approximately 10:1. To encourage a close association between platelets and endothelial cells the tissue culture plates were centrifuged at $500 \times g$ for 10 minutes. Endothelial cells were then incubated at 37°C for 1–6 hours.

Real-time RT-PCR

Quantitative real time PCR was used to measure the changes in cytokine mRNA levels in endothelial cells following incubation with bovine platelets. The Qiagen RNeasy minikit system (Qiagen, Valencia, CA) was used to collect mRNA from endothelial cells, following

the manufacturers recommended protocol. The mRNA was converted to cDNA using the AMV Reverse Transcription System (Promega, Madison, WI) and cDNA samples were kept at -20°C until needed. Primers used to amplify IL-1 β , IL-8, MCP-1, MIP-1 α , E-selectin, ICAM-1, tissue factor and the housekeeping gene β -actin were designed using the Primer Express software program (Primer Express 2.0; Applied Biosystems, Foster City, CA) using bovine sequences for these genes (IL-1 β , GenBank accession M35589; MCP-1, GenBank accession M84602; MIP-1 α , GenBank accession AY077840; E-selectin, GenBank accession L12039; tissue factor, GenBank accession NM_173878 and β -actin, GenBank accession AY141970). Primers used in this study are listed below:

- 5' 3'
- IL-1 β forward: ATTGCCCAGGTTTCTGAAACA
- IL-1 β reverse: CTCGTCACTGTAGTAAGCCATCATT
- MCP-1 α forward: TAATCCTCTCGCTGCAACA
- MCP-1 α reverse: TGCTGAAGGCAGCTACTGTGA
- MIP-1 forward: GGTGTCATCTTCCAGACCAAAAA
- MIP-1 reverse: TCC TGG ACC CAG TCC TCA GT
- E-selectin forward: ACAAGCAGCCAACATGTAAAGCT
- E-selectin reverse: AACCACGGAGTGGCTACAGTTC
- Tissue factor forward: CGG TAC AAG ATG CAC GTA CGT
- Tissue factor reverse: AAT TCA AGT CCT TGC CAA AAA CA
- β -actin forward: AACCGTGAGAAGATGACCCAGAT
- β -actin reverse: GGGACAGCACAGCCTGGAT

All primers were designed to amplify approximately 100 bp of the desired gene, have a melting point of 60°C , and to minimize formation of primer-dimers. A nucleotide–nucleotide basic local alignment search tool (US National Library of Medicine, Bethesda, MD) was performed on the amplified regions to confirm the uniqueness of the PCR product. The forward and reverse primers (100 nM each) were combined with SYBR Green PCR Master Mix (Applied Biosystems), and cDNA template in a 96 well optical reaction plate (Applied Biosystems). The samples were cycled on a GeneAmp 7300 Real Time PCR System (Applied Biosystems). The cycle parameters were as follows: 50°C for 2 min, 95°C for 15 minutes, and for 40 cycles, 95°C for 15 seconds and 60°C for 1 minute. A cut-off of 1 was chosen for the threshold value for the amplicon levels. Fold increase in mRNA was calculated by the comparative threshold cycle (C_T) method using the formula $2^{\Delta\Delta C_T}$. Data were normalized for gene expression based on β -actin mRNA expression.

Western blot analysis

Endothelial cells ($1 \times 10^5/\text{ml}$) in 6 well plates were washed in PBS, then lysed with 100 μl of M-PER lysis buffer with HALT™ protease inhibitors (Pierce Biotechnology, Rockford, IL). The lysates were freeze-thawed 3 \times at -70°C for 10 minutes, centrifuged at $14,000 \times g$ for 15 minutes to pellet cellular debris, and the supernatants collected. A small sample was set aside for protein quantification. To the remaining sample, 50 μl of 4 \times loading buffer was added, and the lysates were boiled for 5 minutes. Lysates (50 μg) were resolved on 4–20% sodium dodecyl sulfate (SDS) polyacrylamide gels by electrophoresis (PAGE), under reducing conditions. Resolved proteins were electroblotted onto nitrocellulose membranes and then incubated with blocking buffer (5% skim milk powder and 0.05% Tween 20 in PBS (TBST) at room

temperature with agitation for 30 minutes. The membranes were then washed in TBST and incubated with agitation overnight at 4°C with antibodies for tissue factor or ICAM-1 in TBST with 1% skim milk powder. The membranes were washed again in TBST and incubated for 30 minutes with agitation at room temperature with the appropriate horseradish peroxidase conjugated antibodies (Jackson ImmunoResearch; West Grove, PA) in TBST with 1% skim milk. The membranes were washed once more in TBST and then incubated for 2 minutes in Super Signal Chemiluminescent substrate (Pierce). Protein bands were visualized using Kodak Biomax light film (Kodak, New Haven, CT). Afterwards, membranes were stripped using a commercial reagent (Restore™ western blot stripping reagent; Pierce), reblocked 1 hour and probed for β-actin expression (diluted 1:5000).

Flow cytometry

Endothelial cells were washed with PBS, and then detached by a brief incubation with Hanks-EDTA (calcium and magnesium-free Hanks, Sigma, St. Louis, MO; EDTA 2.5 mM, pH 7.4). The cells were incubated in 4% paraformaldehyde and fixed at 4°C for 30 minutes. Endothelial cells were then washed in 500 μl of PBS before being stained with antibodies for tissue factor or ICAM-1. After a 1 hour incubation, the cells were washed and incubated with FITC-conjugated secondary antibodies for 1 hour. Cells were then washed in PBS and analyzed by flow cytometry (FACScan, Becton-Dickinson) using WinMDI software (Scripps Research Institute, La Jolla, CA) and collecting 10,000 events for each sample.

Transmission Electron Microscopy

Endothelial cells were seeded at a density of 1×10^5 cells/ml on glass coverslips in 24 well tissue culture plates, and allowed to adhere overnight. Bovine platelets, prepared and treated as described above, were incubated with the endothelial cells for 12 hours. At that point, cell culture media was removed and the endothelial cells were fixed with a 25% v/v of Karnovsky's solution (2.5% glutaraldehyde, 4% paraformaldehyde) for 10 minutes at room temperature. The coverslips were washed with PBS, stained with 1% osmium tetroxide for 30 minutes, and then dehydrated through a graded series of ethanol washes. The endothelial cells were then embedded in 100% epoxy resin for 5 hours. Following embedding, the glass coverslip was dissolved by hydrofluoric acid, and samples were thin sectioned, stained with uranyl acetate and lead citrate, and examined with a Philips CM120 STEM transmission electron microscope (Phillips Electron Optics, Eindhoven, The Netherlands). Internalized and adherent platelets were quantified by examining endothelial cells at $\times 2650$ magnification. Platelets in direct contact with endothelial cells were recorded as adherent, while platelets that had been fully endocytosed were recorded as internalized. Approximately 50 endothelial cells from at least 5 separate fields were scored for each treatment.

Statistical analysis

An unbalanced one-way analysis of variance (ANOVA) was used to determine if significant variation existed between group means. To perform pair wise comparisons of all the means, we used Tukey's multiple comparison tests ($p < 0.05$) in the Prism 4 statistical package (GraphPad, San Diego, CA). The Tukey test is a conservative test because the critical value is an approximation to the exact value.

Results

Characterization of bovine pulmonary artery endothelial cells

Bovine pulmonary artery endothelial cells were isolated as described previously¹⁶. Endothelial cells were characterized by their "cobblestone" morphology, and their expression of CD146 and von Willebrand factor (vWF) as determined by flow cytometry (Data not shown).

Activated platelets induce endothelial cell surface molecule expression

To determine if activated platelets up-regulate endothelial cell adhesion molecules (E-selectin, ICAM-1) and tissue factor, bovine platelets (1×10^8) were incubated with either ADP (10 μ M), *H. somni* (1×10^8) or *H. somni* LOS (500 ng/ml) for 10 minutes. Previously, we reported that these conditions significantly increased bovine platelet expression of P-selectin, CD40L and FasL. Activated platelets were then fixed in 4% paraformaldehyde (1/10 vol/vol) for 30 minutes, then washed in PBS and incubated with bovine endothelial cells (MOI 10:1). Endothelial cell expression of E-selectin, ICAM-1 and tissue factor was determined by flow cytometry, real-time PCR and western blot analysis.

After a four hour incubation with activated platelets, we observed an increase in endothelial cell expression of ICAM-1 and tissue factor as determined by flow cytometry (Figure 1). ICAM-1 and tissue factor expression were significantly increased in endothelial cells incubated with platelets activated by ADP, *H. somni* LOS or *H. somni*, compared to control endothelial cells incubated with unactivated platelets ($p < 0.05$).

Using reverse-transcriptase real-time PCR, expression of mRNA transcripts for tissue factor and E-selectin were also examined. Endothelial cells incubated with platelets activated by *H. somni* or *H. somni* LOS had significantly increased E-selectin mRNA levels at two to four hours of incubation (Figure 2A). *H. somni* activated platelets also induced significant levels of tissue factor mRNA within 2 hours (Figure 2B).

We next measured endothelial cell E-selectin and ICAM-1 protein expression. Following a four hour incubation with activated bovine platelets, endothelial cell lysates were collected and analyzed by PAGE and western blot. Incubation with platelets activated by ADP, *H. somni* or *H. somni* LOS increased endothelial cell E-selectin (Figure 3A) and ICAM-1 (Figure 3B) as compared with endothelial cells incubated with unactivated platelets.

Endothelial cell expression of IL-1 β , MCP-1 and MIP-1 α following incubation with activated platelets

Endothelial cell expression of IL-1 β is reported to correlate with the severity of vascular damage during sepsis²³. IL-1 β also plays an important role as a pro-inflammatory molecule and promoter of coagulation. We found that endothelial cells incubated for four hours with platelets activated by *H. somni* or its LOS expressed increased levels of IL-1 β mRNA, as compared with untreated cells (Figure 4A). Secretion of IL-1 β from endothelial cells was also determined by ELISA. Significant increases in IL-1 β protein were detected in conditioned media from endothelial cells incubated for 6 hours with platelets activated by ADP, *H. somni*, and *H. somni* LOS, as compared with untreated endothelial cells or cells incubated with unactivated platelets (Figure 4). Bovine endothelial cells incubated for one hour with *H. somni* activated platelets also exhibited increased MIP-1 α (Figure 5A) and MCP-1 (Figure 5), which remained significantly elevated through three hours of incubation. In contrast, *H. somni* LOS-activated platelets induced a significant increase in endothelial cell MCP-1 mRNA levels only at two hours of incubation (Figure 5).

Transmission electron microscopy of endothelial cells interactions with activated platelets

We next used transmission electron microscopy (TEM) to visualize interactions between endothelial cells and platelets. Untreated bovine endothelial cells exhibited the typical polygonal morphology, as did endothelial cells incubated with unactivated platelets (Figure 6A, 6B). We rarely observed direct contact of unactivated platelets with endothelial cells (Figure 6C, 6D). An interesting but infrequent finding were endothelial cells with phagocytized platelets (Figure 6D).

Prior activation of platelets by *H. somni* LOS resulted in a substantial increase in platelet-endothelial cell adherence (Figure 7A, 7B). We also observed an increase in endothelial cells with internalized platelets (Figure 7A, 7C). Likewise, platelets incubated with ADP formed aggregates, some of which could be found internalized within endothelial cells (Figure 7D). In addition, we found that endothelial cells that had internalized and degraded platelets exhibited ruffled outer membranes (Figure 7D, 7E), suggestive of cell stress or damage.

When we incubated bovine endothelial cells with *H. somni* activated platelets, we observed *H. somni* in close proximity to both endothelial cells and platelets. Endothelial cells internalized significant numbers of *H. somni* activated platelets, but not *H. somni* cells (Figure 8A, 8B). This latter finding confirmed earlier reports, in which *H. somni* was rarely found internalized by endothelial cells as assessed by TEM^{16, 24}. One mechanism for the internalization of *H. somni* activated platelets may be through selective receptor-mediated interactions with endothelial cells leading to phagocytosis (Figure 8C).

Using transmission electron microscopy the number of *H. somni* activated platelets adherent to and internalized by bovine endothelial cells was quantified. A small number of endothelial cells endocytosed untreated platelets, and this number was significantly increased in endothelial cells incubated with platelets activated by *H. somni* or its LOS (Figure 9).

Discussion

Platelets are important for maintaining haemostasis and promoting inflammation. Activated platelets have been demonstrated previously to up-regulate endothelial cell adhesion molecules and inflammatory cytokine secretion and therefore may play an important role in vasculitis^{18–20}. It has been demonstrated that preventing platelet adherence to the endothelium reduces the severity of endothelial cell damage and vascular inflammation during sepsis^{25, 26}. In contrast, inhibiting leukocyte adherence had less of an effect. Whether the mechanisms that trigger these events are mediated principally by activated platelets or endothelial cells is not yet known. In this study, we demonstrated that activated bovine platelets induce endothelial cell expression of adhesion molecules (ICAM-1, E-selectin), tissue factor, IL-1 β , and chemokines (MCP-1, MIP-1 α). A surprising and exciting finding was that bovine pulmonary artery endothelial cells appeared to endocytose activated platelets. This is a novel finding that likely represents endothelial cell recognition of a surface molecule on activated platelets, because nearby bacteria were not phagocytized.

Platelets can be activated directly by bacteria, including several bacterial species linked to sepsis in humans, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Escherichia coli*^{27–30}. Endotoxin is also known to be a potent activator of platelets^{31–34}. For example, the activation of human platelets by the LOS of *Neisseria meningitidis* is thought to be important in microthrombosis and organ dysfunction observed in meningococcal septicemia³⁵.

We have demonstrated that *H. somni* activated bovine platelets induced endothelial cell expression of adhesion molecules and cytokines. Some endothelial cell factors are stored as pre-formed proteins in intracellular Weibel-Pallade bodies that are spontaneously released following endothelial cell activation, whereas others factors require active synthesis³⁶. As a result, we used a variety of methods to detect both surface expression and de novo synthesis. We observed that endothelial cell expression of ICAM-1, E-selectin and tissue factor were significantly up-regulated by *H. somni* activated platelets. The expression of these molecules in vivo could lead to enhanced leukocyte adherence to the endothelium and promote thrombus formation.

We also observed the production of the chemokines MCP-1 and MIP-1 α by endothelial cells incubated with activated platelets. These chemotactic proteins have been reported to be expressed by endothelial cells incubated with activated human platelets, and can promote leukocyte recruitment to areas of inflammation^{20, 37}. Using real-time PCR, we found both MCP-1 and MIP-1 α were significantly up-regulated following endothelial cell incubation with activated platelets. We also examined endothelial cell expression of IL-1 β , which can activate pro-inflammatory responses in leukocytes and endothelial cells. We found that platelets activated by *H. somni*, or its LOS, induced endothelial cell expression of IL-1 β , as measured by RT-PCR and ELISA.

The most novel and unexpected finding in this study was the engulfment of activated platelets by bovine endothelial cells. Unactivated bovine platelets were rarely internalized by endothelial cells. However, platelets activated by ADP, *H. somni* or its LOS were internalized at much higher numbers by a greater number of endothelial cells. We found that activated platelets were physically associated with endothelial cells which likely contributes to platelet endocytosis. In addition, internalized platelets exhibited signs of degradation, a process that also appeared to cause morphological changes indicative of cell stress in the endothelial cells. These events may perhaps contribute to the bovine endothelial cell apoptosis that has been previously reported²¹. Phagocytosis of platelets by monocytes has been shown to inhibit apoptosis of the latter, however, similar findings have not been reported for endothelial cells³⁸. In the present study, platelets appeared to be selectively phagocytized, because nearby *H. somni* cells were not internalized by endothelial cells. This observation confirms earlier reports that bovine endothelial cells rarely phagocytize *H. somni*^{16, 24}.

The mechanisms by which bovine endothelial cells phagocytize platelets are unclear at this time. It has been reported that human platelets can fuse their cytoplasmic membranes with those of human endothelial cells³⁹. In that study, platelet granule contents were released as the platelet and endothelial cell membranes merged together. Although a similar mechanism might have occurred in our study, because we used fixed platelets, simple fusion of the platelet cell membrane with that of the endothelial cell seems unlikely due to cross-linking of membrane components. However, human endothelial cells have been reported to phagocytize formaldehyde-fixed human platelets, an event that was associated with an increase in production of reactive oxygen species⁴⁰. The mechanism responsible could be mediated by CD40L, P-selectin or other proteins on the platelet surface binding to their cognate receptors on endothelial cells.

The hallmark of systemic *H. somni* infection in cattle is diffuse vascular inflammation, which on occasion leads to an acute and rapidly fatal cerebral vasculitis known as TME. Activated platelets are known to play critical roles in the pathogenesis of sepsis, by promoting vascular inflammation and coagulation. Previously, we have demonstrated that *H. somni* and its LOS can activate bovine platelets, and that activated bovine platelets also induce bovine endothelial cell apoptosis. In this study, we have shown that *H. somni* activated platelets can induce endothelial cell expression of surface molecules (E-selectin, ICAM-1 and tissue factor) IL-1 β and chemokines (MCP-1, MIP-1 α). We believe that these pro-inflammatory surface molecules and inflammatory mediators may contribute to the pathogenesis of vasculitis that occur during *H. somni* infection.

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References

1. Ellis JA. The immunology of the bovine respiratory disease complex. *Vet Clin North Am Food Anim Pract* Nov;2001 17(3):535–550. vi–vii. [PubMed: 11692507]
2. Loneragan GH, Dargatz DA, Morley PS, Smith MA. Trends in mortality ratios among cattle in US feedlots. *J Am Vet Med Assoc* Oct 15;2001 219(8):1122–1127. [PubMed: 11700712]
3. Andrews JJ, Anderson TD, Slife LN, Stevenson GW. Microscopic lesions associated with the isolation of *Haemophilus somnus* from pneumonic bovine lungs. *Vet Pathol* Mar;1985 22(2):131–136. [PubMed: 3984157]
4. Corbeil LB, Arthur JE, Widders PR, Smith JW, Barbet AF. Antigenic specificity of convalescent serum from cattle with *Haemophilus somnus*-induced experimental abortion. *Infect Immun* Jun;1987 55(6):1381–1386. [PubMed: 3570470]
5. Gogolewski RP, Leathers CW, Liggitt HD, Corbeil LB. Experimental *Haemophilus somnus* pneumonia in calves and immunoperoxidase localization of bacteria. *Vet Pathol* May;1987 24(3):250–256. [PubMed: 3300006]
6. Humphrey JD, Little PB, Barnum DA, Doig PA, Stephens LR, Thorsen J. Occurrence of “*Haemophilus somnus*” in bovine semen and in the prepuce of bulls and steers. *Can J Comp Med* Apr;1982 46(2):215–217. [PubMed: 7093816]
7. Harris FW, Janzen ED. The *Haemophilus somnus* disease complex (*Haemophilosis*): A review. *Can Vet J* Oct 1989;1984 30:816–822. [PubMed: 17423440]
8. Corbeil LB, Blau K, Prieur DJ, Ward AC. Serum susceptibility of *Haemophilus somnus* from bovine clinical cases and carriers. *J Clin Microbiol* Aug;1985 22(2):192–198. [PubMed: 4031034]
9. Gomis SM, Godson DL, Beskorwayne T, Wobeser GA, Potter AA. Modulation of phagocytic function of bovine mononuclear phagocytes by *Haemophilus somnus*. *Microb Pathog* Jan;1997 22(1):13–21. [PubMed: 9032758]
10. Czuprynski CJ, Hamilton HL. Bovine neutrophils ingest but do not kill *Haemophilus somnus* in vitro. *Infect Immun* Nov;1985 50(2):431–436. [PubMed: 4055025]
11. Corbeil LB, Bastida-Corcuera FD, Beveridge TJ. *Haemophilus somnus* immunoglobulin binding proteins and surface fibrils. *Infect Immun* Oct;1997 65(10):4250–4257. [PubMed: 9317034]
12. Inzana TJ, Hensley J, McQuiston J, et al. Phase variation and conservation of lipooligosaccharide epitopes in *Haemophilus somnus*. *Infect Immun* Nov;1997 65(11):4675–4681. [PubMed: 9353049]
13. Ruby KW, Griffith RW, Kaeberle ML. Histamine production by *Haemophilus somnus*. *Comp Immunol Microbiol Infect Dis* Jan;2002 25(1):13–20. [PubMed: 11831743]
14. Howard MD, Cox AD, Weiser JN, Schurig GG, Inzana TJ. Antigenic diversity of *Haemophilus somnus* lipooligosaccharide: phase-variable accessibility of the phosphorylcholine epitope. *J Clin Microbiol* Dec;2000 38(12):4412–4419. [PubMed: 11101573]
15. Behling-Kelly E, Vonderheid H, Kim KS, Corbeil LB, Czuprynski CJ. Roles of cellular activation and sulfated glycans in *Haemophilus somnus* adherence to bovine brain microvascular endothelial cells. *Infect Immun* Sep;2006 74(9):5311–5318. [PubMed: 16926425]
16. Sylte MJ, Corbeil LB, Inzana TJ, Czuprynski CJ. *Haemophilus somnus* induces apoptosis in bovine endothelial cells in vitro. *Infect Immun* Mar;2001 69(3):1650–1660. [PubMed: 11179340]
17. Sylte MJ, Kuckleburg CJ, Inzana TJ, Bertics PJ, Czuprynski CJ. Stimulation of P2X receptors enhances lipooligosaccharide-mediated apoptosis of endothelial cells. *J Leukoc Biol* Jun;2005 77(6):958–965. [PubMed: 15728716]
18. Henn V, Slupsky JR, Grafe M, et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* Feb 5;1998 391(6667):591–594. [PubMed: 9468137]
19. Danese S, de la Motte C, Sturm A, et al. Platelets trigger a CD40-dependent inflammatory response in the microvasculature of inflammatory bowel disease patients. *Gastroenterology* May;2003 124(5):1249–1264. [PubMed: 12730866]
20. Cha JK, Jeong MH, Bae HR, et al. Activated platelets induce secretion of interleukin-1beta, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1alpha and surface expression of intercellular adhesion molecule-1 on cultured endothelial cells. *J Korean Med Sci* Jun;2000 15(3):273–278. [PubMed: 10895967]

21. Kuckleburg CJ, Sylte MJ, Inzana TJ, Corbeil LB, Darien BJ, Czuprynski CJ. Bovine platelets activated by *Haemophilus somnus* and its LOS induce apoptosis in bovine endothelial cells. *Microb Pathog Jan*;2005 38(1):23–32. [PubMed: 15652292]
22. Inzana TJ, Iritani B, Gogolewski RP, Kania SA, Corbeil LB. Purification and characterization of lipooligosaccharides from four strains of “*Haemophilus somnus*”. *Infect Immun Nov*;1988 56(11):2830–2837. [PubMed: 3169988]
23. Peters K, Unger RE, Brunner J, Kirkpatrick CJ. Molecular basis of endothelial dysfunction in sepsis. *Cardiovasc Res Oct 15*;2003 60(1):49–57. [PubMed: 14522406]
24. Thompson KG, Little PB. Effect of *Haemophilus somnus* on bovine endothelial cell in organ culture. *Am J Vet Res May*;1981 42(5):748–754. [PubMed: 7020497]
25. Eipel C, Bordel R, Nickels RM, Menger MD, Vollmar B. Impact of leukocytes and platelets in mediating hepatocyte apoptosis in a rat model of systemic endotoxemia. *Am J Physiol Gastrointest Liver Physiol. Jan 8*;2004
26. Sun G, Chang WL, Li J, Berney SM, Kimpel D, van der Heyde HC. Inhibition of platelet adherence to brain microvasculature protects against severe *Plasmodium berghei* malaria. *Infect Immun Nov*;2003 71(11):6553–6561. [PubMed: 14573677]
27. Clawson CC, White JG. Platelet interaction with bacteria. I. Reaction phases and effects of inhibitors. *Am J Pathol Nov*;1971 65(2):367–380. [PubMed: 4400052]
28. Clawson CC, White JG. Platelet interaction with bacteria. II. Fate of the bacteria. *Am J Pathol Nov*;1971 65(2):381–397. [PubMed: 5002629]
29. Clawson CC. Platelet interaction with bacteria. 3. Ultrastructure. *Am J Pathol Mar*;1973 70(3):449–471. [PubMed: 4632008]
30. Clawson CC, Rao GH, White JG. Platelet interaction with bacteria. IV. Stimulation of the release reaction. *Am J Pathol Nov*;1975 81(2):411–420. [PubMed: 811123]
31. MacIntyre DE, Allen AP, Thorne KJ, Glauert AM, Gordon JL. Endotoxin-induced platelet aggregation and secretion. I. Morphological changes and pharmacological effects. *J Cell Sci Dec*;1977 28:211–223. [PubMed: 202608]
32. Nystrom ML, Barradas MA, Jeremy JY, Mikhailidis DP. Platelet shape change in whole blood: differential effects of endotoxin. *Thromb Haemost May*;1994 71(5):646–650. [PubMed: 8091394]
33. Saluk-Juszczak J, Wachowicz B, Kaca W. Stimulatory effects of endotoxin on the platelet secretory process. *Microbios 1999*;99(392):45–53. [PubMed: 10624010]
34. Saluk-Juszczak J, Wachowicz B, Kaca W. Endotoxins stimulate generation of superoxide radicals and lipid peroxidation in blood platelets. *Microbios 2000*;103(404):17–25. [PubMed: 11034442]
35. Mirlashari MR, Hagberg IA, Lyberg T. Platelet-platelet and platelet-leukocyte interactions induced by outer membrane vesicles from *N. meningitidis*. *Platelets Mar*;2002 13(2):91–99. [PubMed: 11897045]
36. Rondaij MG, Bierings R, Kragt A, van Mourik JA, Voorberg J. Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. *Arterioscler Thromb Vasc Biol May*;2006 26(5):1002–1007. [PubMed: 16469951]
37. Gerszten RE, Garcia-Zepeda EA, Lim YC, et al. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature Apr 22*;1999 398(6729):718–723. [PubMed: 10227295]
38. Lang D, Dohle F, Terstesse M, et al. Down-regulation of monocyte apoptosis by phagocytosis of platelets: involvement of a caspase-9, caspase-3, and heat shock protein 70-dependent pathway. *J Immunol Jun 15*;2002 168(12):6152–6158. [PubMed: 12055227]
39. Lou J, Donati YR, Juillard P, et al. Platelets play an important role in TNF-induced microvascular endothelial cell pathology. *Am J Pathol Nov*;1997 151(5):1397–1405. [PubMed: 9358766]
40. Gorog P, Pearson JD, Kakkar VV. Generation of reactive oxygen metabolites by phagocytosing endothelial cells. *Atherosclerosis Jul*;1988 72(1):19–27. [PubMed: 2850806]

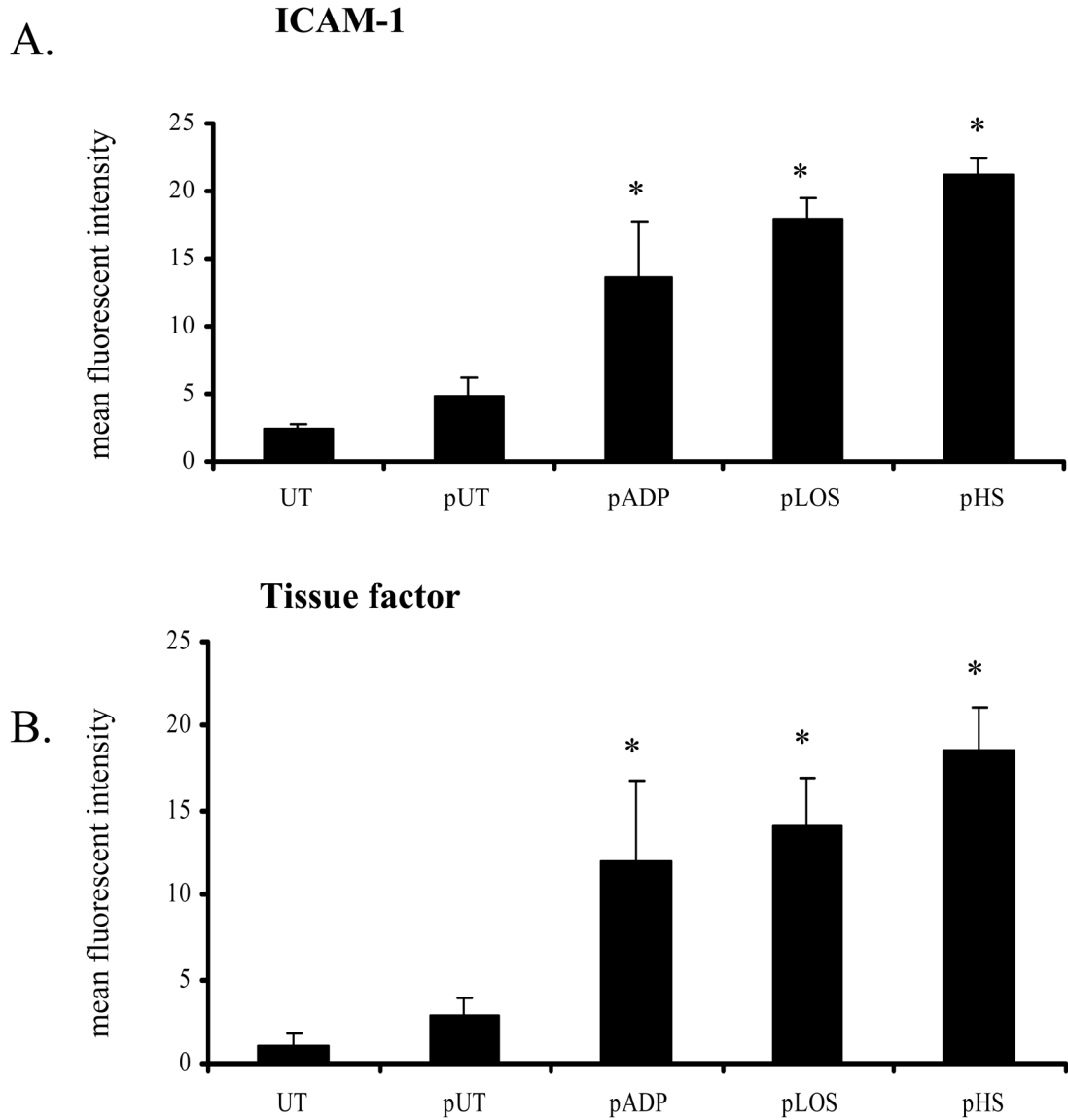


Figure 1. Activated platelets induce endothelial cell ICAM-1 and tissue factor expression

Bovine platelets (1×10^8 cells) were incubated with *H. somni* (pHS; MOI 1:1) or *H. somni* LOS (pLOS; 500 ng/ml) for 10 minutes before being briefly fixed in paraformaldehyde. Untreated platelets (pUT) and ADP activated platelets (pADP; 10 μ M) were used as negative and positive controls, respectively. Fixed platelets were then washed and incubated with bovine endothelial cells at an MOI of 10 to 1. After four hours, endothelial cells were collected and analyzed by flow cytometry for expression of ICAM-1 (A) or tissue factor (B). The data (A, B) illustrate the mean \pm SEM of 3 separate experiments (Tukey's Test * = $p < 0.05$ as compared to cells incubated with untreated platelets).

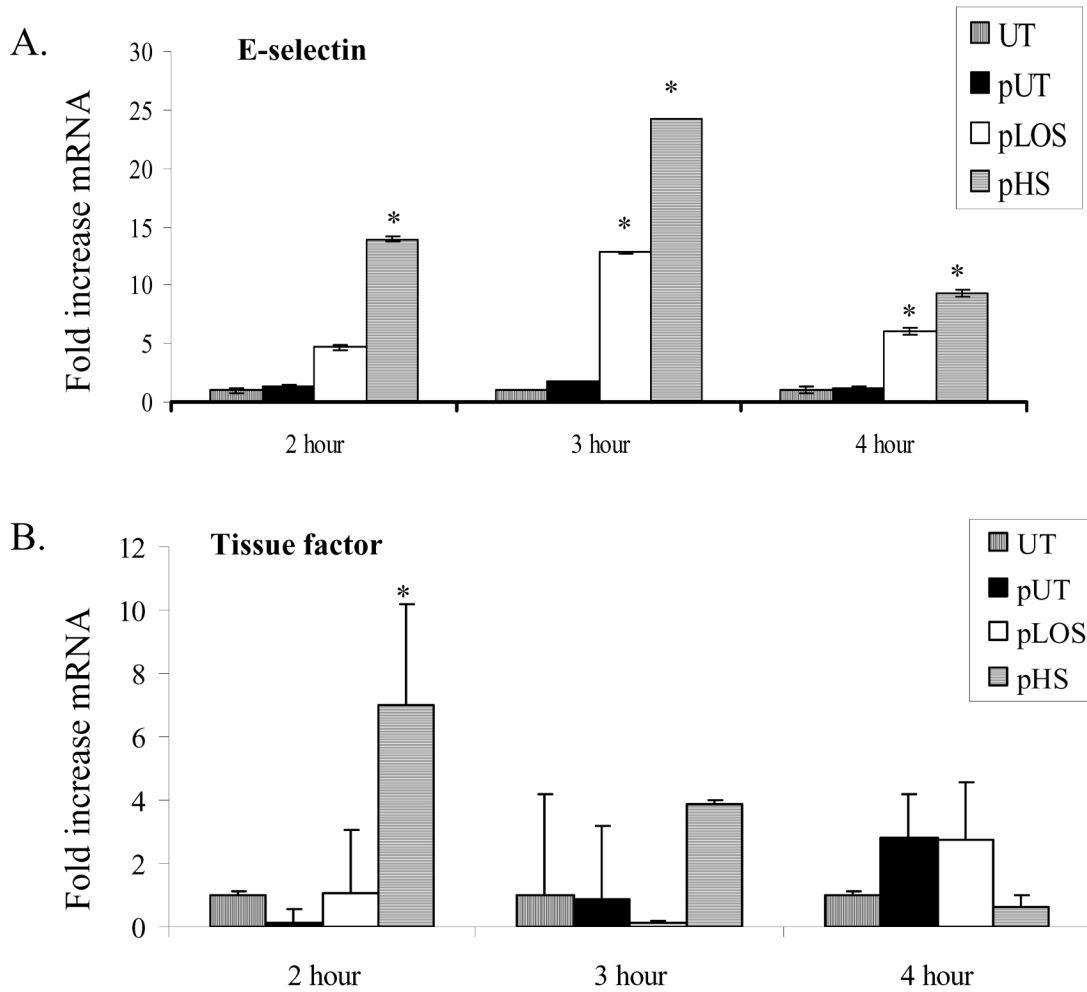


Figure 2. Activated platelets induce endothelial cell E-selectin and tissue factor mRNA production
 Bovine platelets (1×10^8 cells) were incubated with *H. somni* (pHS; MOI 1:1) or *H. somni* LOS (pLOS; 500 ng/ml) for 10 minutes before being fixed in paraformaldehyde. Untreated platelets (pUT) were used as negative a control. Fixed platelets were washed then incubated with bovine endothelial cells at an MOI of 10 to 1. At the indicated time points, endothelial cells were collected and mRNA isolated. Using real-time PCR, transcript levels of mRNA were standardized with our endogenous control (β -actin) and expressed as a fold increase in (A) E-selectin and (B) tissue factor as compared to untreated endothelial cells. These data are the mean \pm SEM from one representative experiment of three separate experiments performed (Tukey's test * = $p < 0.05$ as compared to cells incubated with untreated platelets).

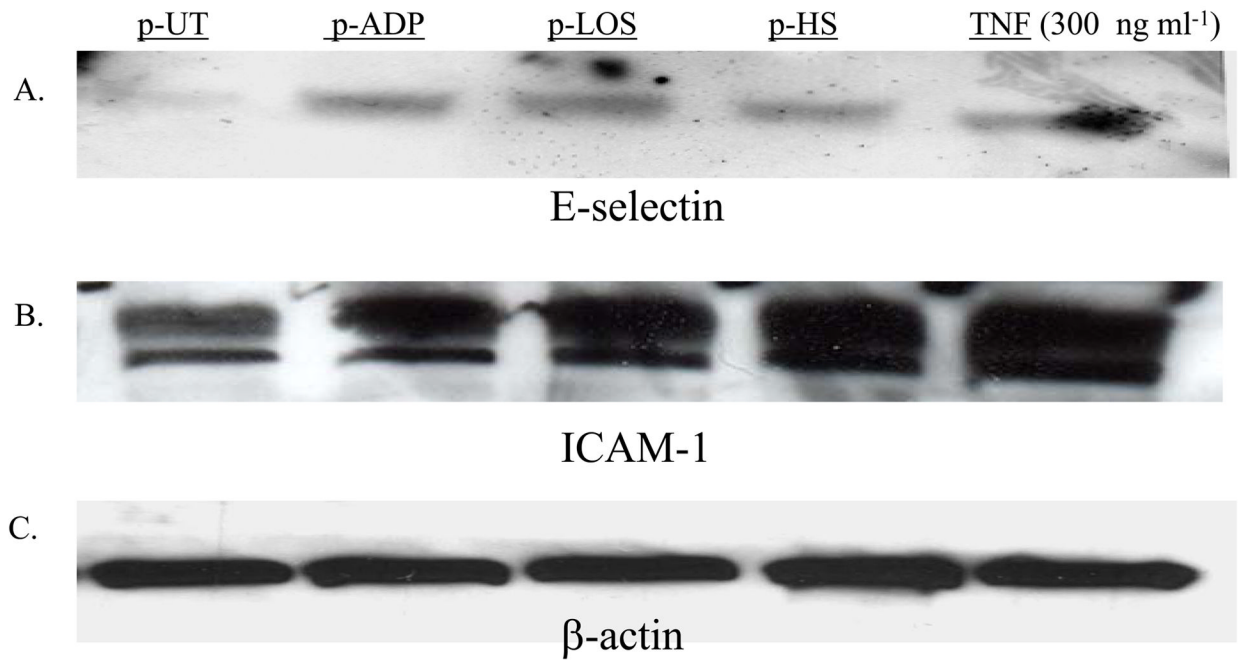


Figure 3. Western blot analysis of endothelial cell protein expression of E-selectin and ICAM-1
Bovine platelets (1×10^8 cells) were incubated with *H. somni* (pHS; MOI 1:1) or *H. somni* LOS (pLOS; 500 ng/ml) for 10 minutes before being fixed in paraformaldehyde. Untreated platelets (pUT), and platelets incubated with ADP (pADP; 10 μ M), were used as negative and positive controls, respectively. Endothelial cells were also incubated with TNF α (300 ng/ml) in the absence of platelets as an additional positive control. Fixed washed platelets were incubated with endothelial cells at an MOI of 10 to one for four hours at 37°C. The endothelial cells were collected and protein lysates prepared. Approximately 50 μ g of endothelial cell lysate were loaded into each lane of a polyacrylamide gel for electrophoresis and analysis by western blotting using antibodies for E-selectin (A), ICAM-1 (B) or β -actin (C). These data are representative of two separate experiments that were performed.

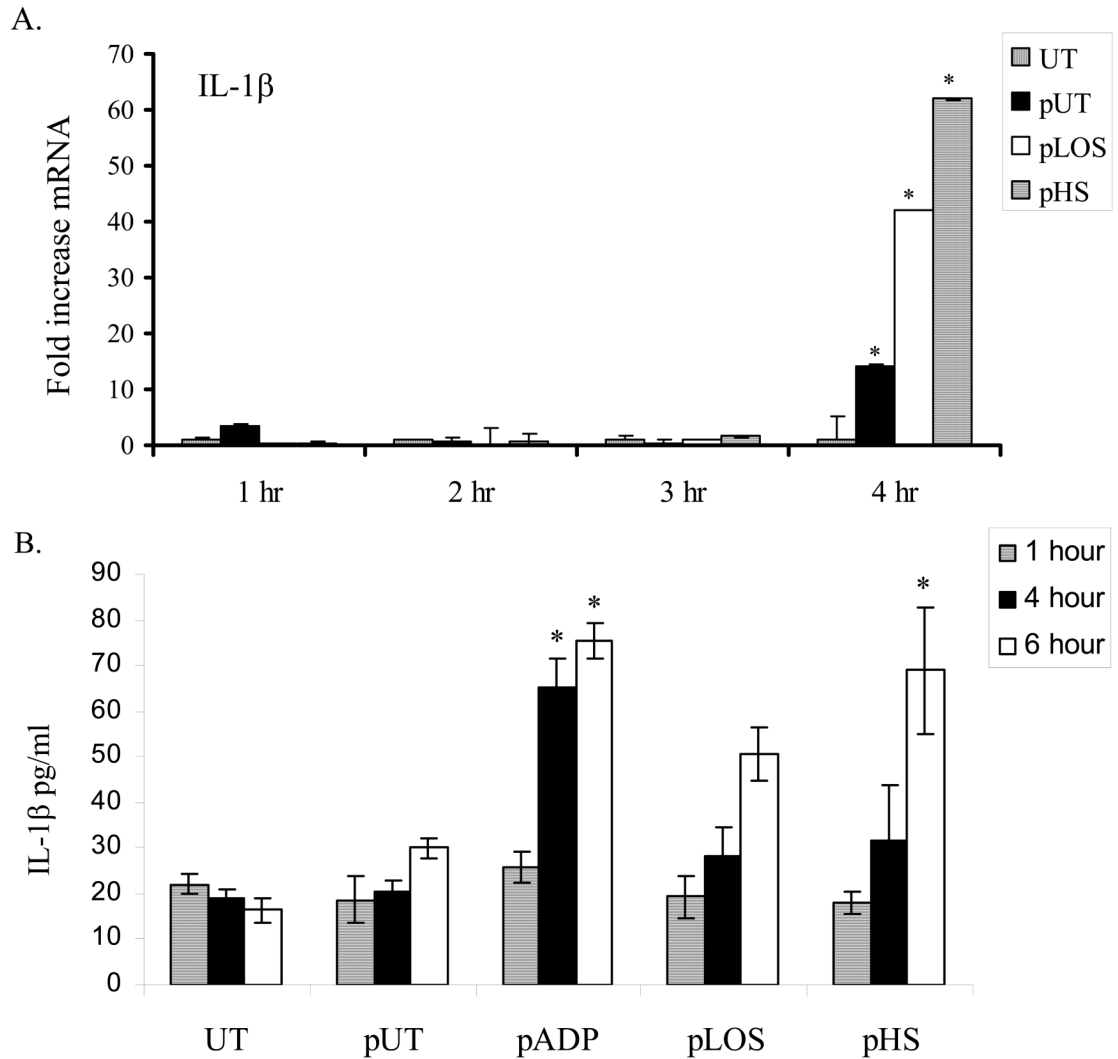


Figure 4. Incubation with activated bovine platelets increases bovine endothelial cell expression of IL-1 β

Bovine platelets (1×10^8 cells) were incubated with *H. somni* (pHS; MOI 1:1) or *H. somni* LOS (pLOS; 500 ng/ml) for 10 minutes before being fixed in paraformaldehyde. Untreated platelets (pUT) and ADP treated platelets (pADP; 10 μ M) were used as negative and positive controls, respectively. Incubation of endothelial cells with *H. somni* or LOS activated platelets resulted in increased levels of IL-1 β mRNA (A) as assessed by RT-PCR and protein (B) as quantified by ELISA. The data (A) are the mean \pm SEM from one representative experiment of three separate experiments performed, while the data (B) illustrate the mean \pm SEM of three separate experiments (Tukey's Test * = $p < 0.05$ as compared to untreated endothelial cells).

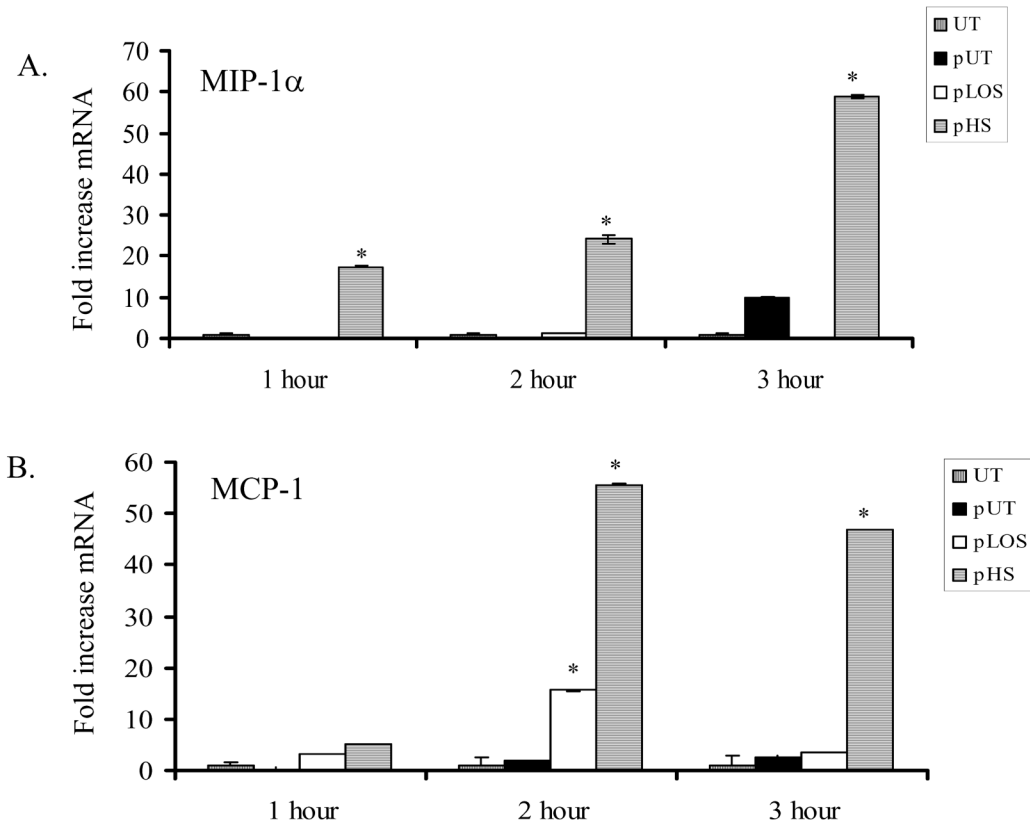


Figure 5. Endothelial cell MIP-1 α and MCP-1 mRNA production is induced by activated bovine platelets

Bovine platelets (1×10^8 cells) were incubated with *H. somni* (pHS; MOI 1:1) or *H. somni* LOS (pLOS; 500 ng/ml) for 10 minutes before being fixed in paraformaldehyde. Untreated platelets (pUT) were used as a negative control. The platelets were added to endothelial cells and incubated for 1 to 3 hours at 37°C. Activated platelets induced significant increases in MIP-1 α (A) and MCP-1 (B) mRNA expression by one and two hours, respectively. These data are the mean \pm SEM from one representative experiment of three separate experiments performed (Tukey's Test * = $p < 0.05$ as compared to cells incubated with untreated platelets).

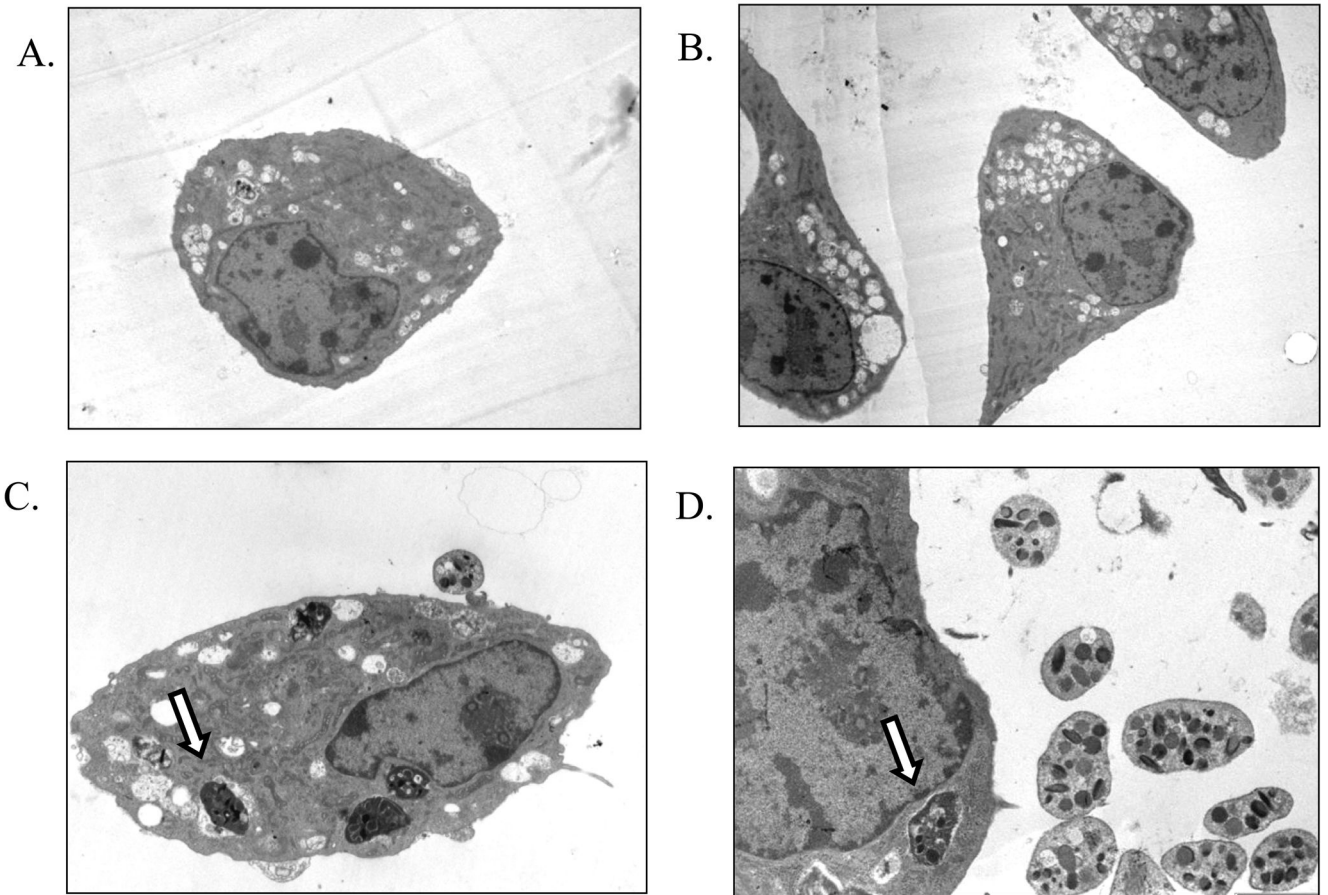


Figure 6. Transmission electron microscopy of endothelial cells incubated with unactivated platelets

Bovine pulmonary artery endothelial cells were examined by transmission electron microscopy following incubation with unactivated bovine platelets (10 platelets per endothelial cell). Untreated cells demonstrated typical endothelial cell morphology (A, B; $\times 2650$ magnification). Following incubation with unactivated platelets, we observed no significant alterations in endothelial cell morphology (C; $\times 5560$ magnification) and little platelet adherence. We were surprised to observe what appeared to be bovine platelets inside of endothelial cells (C, D; $\times 7100$ magnification) as indicated by the arrow. These photomicrographs are representative of three separate experiments.

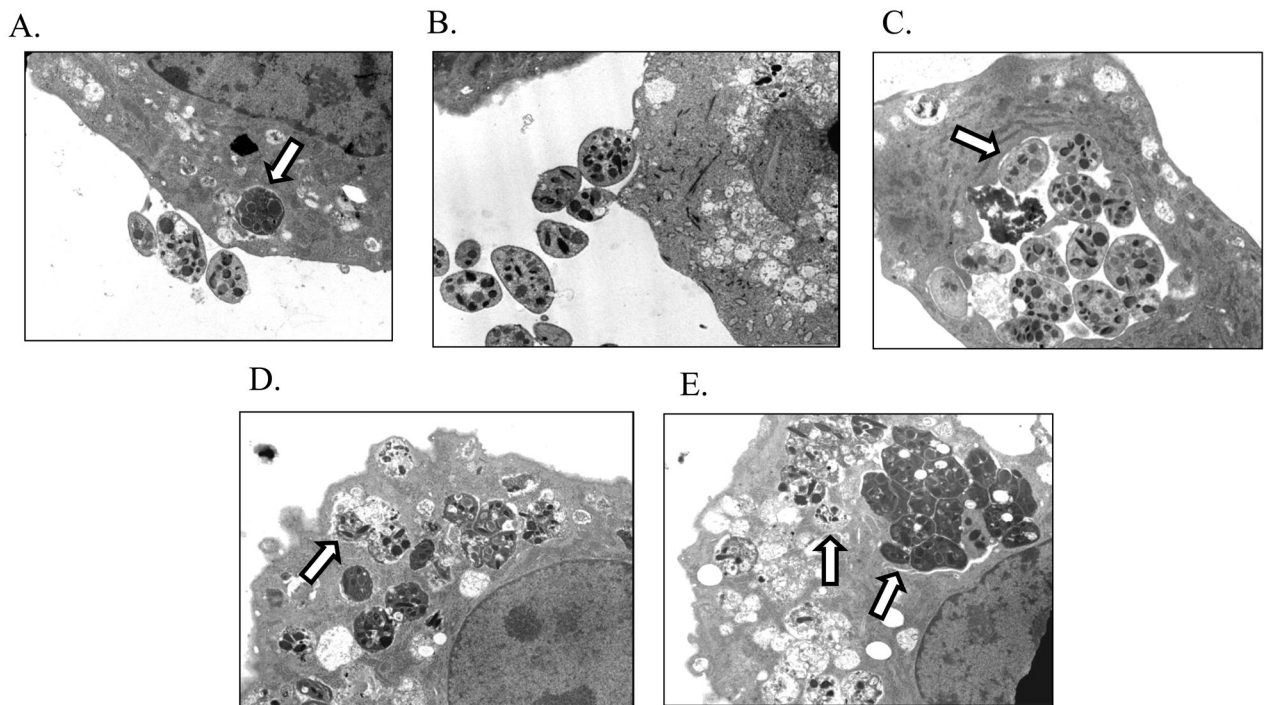


Figure 7. Transmission electron microscopy of endothelial cell interactions with ADP or LOS activated platelets

Bovine pulmonary artery endothelial cells were incubated for 12 hours with bovine platelets that had been activated by *H. somni* LOS (pLOS; 500 ng/ml) or ADP (10 μ M). Platelets incubated with *H. somni* LOS were in close association with endothelial cells (A, B; $\times 5560$ magnification). As indicated by the arrows (B, C) platelets were also found inside of endothelial cells, as were platelets activated with ADP (D, E; $\times 5650$ magnification). In addition, the decomposition of platelets inside of endothelial cells appears to be associated with membrane ruffling suggestive of endothelial cell injury (D, E). These photomicrographs are representative of three separate experiments.

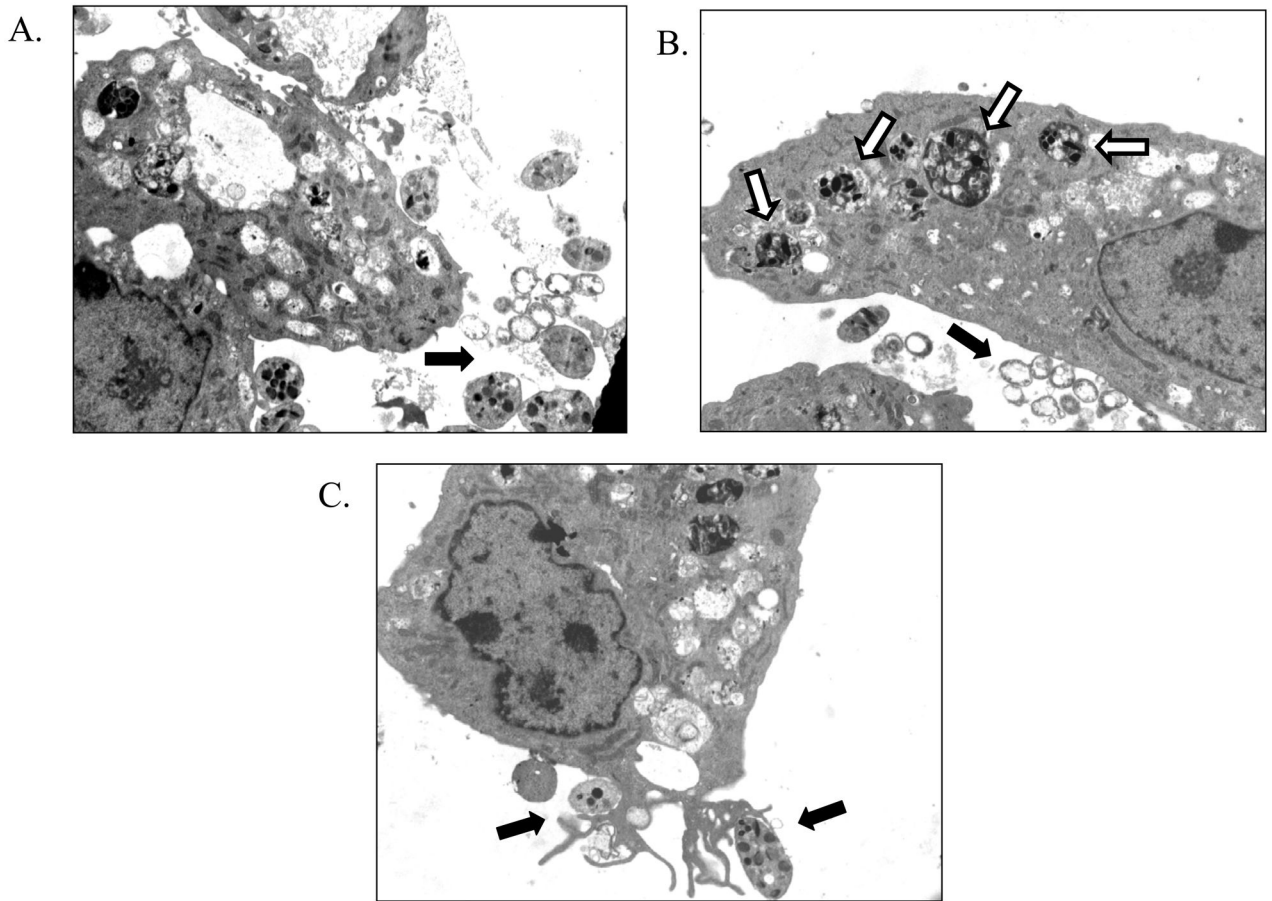


Figure 8. Transmission electron microscopy of endothelial cell interactions with *H. somni* activated platelets

Platelets activated by prior incubation with *H. somni* (A–C; $\times 2650$ magnification) were found to be in close association with the endothelial cells. Although *H. somni* cells could be found in close contact with both platelets and endothelial cells (black arrows), they were rarely found inside of endothelial cells. In contrast, endothelial cells appeared to readily ingest activated platelets (A, B; white arrows). An endothelial cell with pseudopods interacting with *H. somni*-activated platelets is illustrated in (C, black arrows). These photomicrographs are representative of three separate experiments.

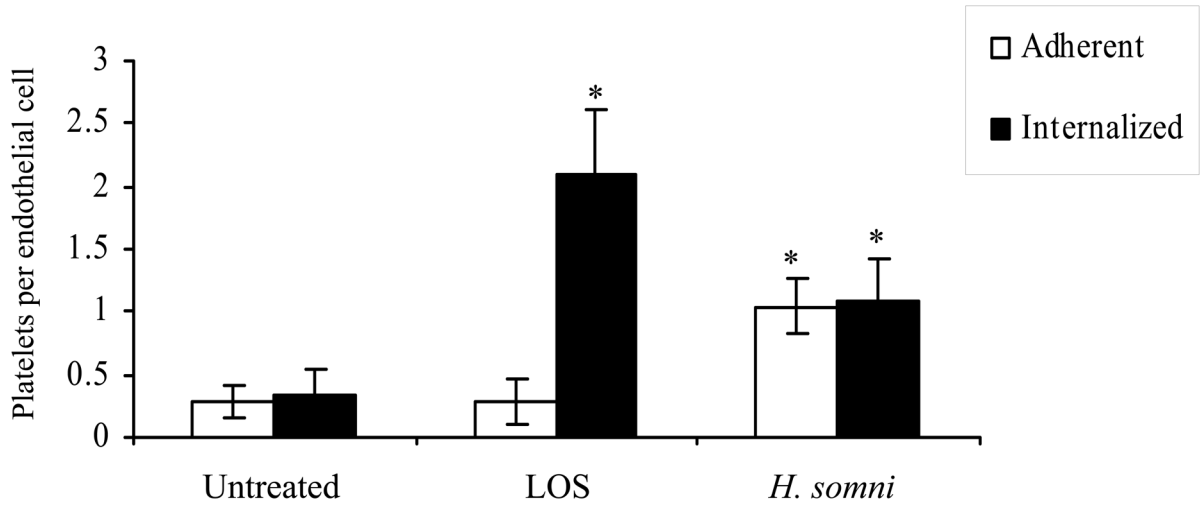


Figure 9. *H. somni* activated bovine platelets adhere to and are internalized by bovine pulmonary artery endothelial cells

Using transmission electron microscopy the number of platelets adherent to and internalized by bovine endothelial cells was quantified. Approximately 50 endothelial cells from 5 separate fields were examined. Platelets in direct contact with endothelial cells, but not internalized, were recorded as adherent. Platelets found inside endothelial cells were recorded as internalized. These data are the mean \pm SEM of two separate experiments (Tukey's Test * = $p < 0.05$ as compared to endothelial cells incubated with untreated platelets).