Endothelial cell apoptosis induced by bacteria-activated platelets requires caspase-8 and -9 and generation of reactive oxygen species

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Summary
A common feature of severe sepsis is vascular inflammation and damage to the endothelium. Because platelets can be directly activated by bacteria and endotoxin, these cells may play an important role in determining the outcome of sepsis. For example, inhibiting platelet interactions with the endothelium has been shown to attenuate endothelial cell damage and improve survival during sepsis. Although not entirely understood, the interactions between bacteria-activated platelets and the endothelium may play a key role in the vascular pathology of bacterial sepsis. *Haemophilus somnus* is a bacterial pathogen that causes diffuse vascular inflammation and endothelial damage. In some cases *H. somnus* infection results in an acute and fatal form of vasculitis in the cerebral microvasculature known as thrombotic meningoencephalitis (TME). In this study, we have characterized the mechanisms involved in endothelial cell apoptosis induced by activated platelets. We observed that direct contact between *H. somnus*-activated platelets and endothelial cells induced significant levels of apoptosis; however, Fas receptor activation on bovine endothelial cells was not able to induce apoptosis unless protein synthesis was disrupted. Endothelial cell apoptosis by *H. somnus*-activated platelets required activation of both caspase-8 and caspase-9, as inhibitors of either caspase inhibited apoptosis. Furthermore, activated platelets induced endothelial cell production of reactive oxygen species (ROS) and disrupting ROS activity in endothelial cells significantly inhibited apoptosis. These findings suggest that bacterial activation of platelets may contribute to endothelial cell dysfunction observed during sepsis, specifically by inducing endothelial cell apoptosis.

Keywords
Histophilus, FasL, ROS, TNFα, *Haemophilus somnus*

Introduction
Platelets have well-defined roles in blood clotting and maintenance of haemostasis, and are also important in promoting inflammation and regulating immune responses. Activated platelets express surface markers and secrete molecules that contribute to leukocyte activation and vascular inflammation. Platelet expression of chemotactic factors (platelet factor 4, lipoxygenase products, RANTES), cytokines (IL-1β), and platelet activating factor (PAF) can contribute to inflammation. In addition, activated platelets express surface molecules such as CD40L (CD154), FasL (CD95) and P-selectin (CD62P) that can interact with leukocytes and endothelial cells (1–4).

During sepsis, platelets are thought to play a major role in the immune response to bacteria and bacterial products such as lipopolysaccharide (LPS). Platelet activation to bacteria is mediated through the expression of toll-like receptors (TLRs) (5, 6). In addition, platelets may also capture and present molecules like LPS to cells such as neutrophils which can lead to TNFα production, inflammation and tissue injury (7, 8). Therefore, platelet responses to bacteria can be important in regulating the immune response in the host and in contributing to tissue injury.

*Haemophilus somnus* is a gram negative coccobacillus that causes respiratory and reproductive disease in cattle. During septicemic *H. somnus* infections the organism can cause diffuse vascular inflammation, arthritis, myocarditis, and an acute and fatal form of vasculitis known as thrombotic meningoencephalitis (TME) (9–12). It is speculated that *H. somnus* and its lipooligosaccharide (LOS) interact directly with the endothelium in vivo to promote pro-inflammatory changes and vascular damage (i.e. endothelial cell apoptosis). *H. somnus* is commonly observed within lesions in the respiratory tract, and within thrombi. However, in
some areas of vascular inflammation, there is no evidence of bacterial antigen (10). Therefore, other mechanisms may also contribute to endothelium injury during H. somnus infection.

Because H. somnus activates bovine platelets, and activated platelets play important roles in mediating vascular inflammation and endothelial cell damage, we decided to investigate the contribution of activated bovine platelets in the pathogenesis of H. somnus infection. In the present study, we demonstrate that H. somnus activated platelets induced endothelial cell apoptosis which was dependent on the production of reactive oxygen species (ROS), and caspase-8 and caspase-9 activation. We also found that, although bovine platelets express FasL, signaling through the Fas receptor on bovine endothelial cells does not induce apoptosis unless protein synthesis was interrupted. Our findings demonstrate that bacteria-activated platelets may induce endothelial cell apoptosis through a contact-dependant process requiring ROS production.

### Materials and methods

#### Chemicals and media

Acetovanillone, adenosine diphosphate (ADP), allopurinol, ascorbic acid, catalase, paraformaldehyde, thiamine monophosphate, penicillin, sepharose 4B, streptomycin, superoxide dismutase, Escherichia coli lipopolysaccharide (LPS) 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, USA). Brain heart infusion broth (BHI) and yeast extract were obtained from Difco (Detroit, MI, USA). Anti-Fas activating IgM was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY, USA). Stauosporin was purchased from ICN Biomedicals (Costa Mesa, CA, USA). Hoechst 33342 and BCECF were purchased from Molecular Probes (Eugene, OR, USA). Bovine TNFα was purchased from Endogen (Rockford, IL, USA). Soluble TNFα receptor antagonist was a gift from Amgen (Thousand Oaks, CA, USA). Antibodies for the detection of TNFα by ELISA and Western blotting were purchased from Endogen and AbD Serotec (Raleigh, NC, USA), respectively.

#### Platelet preparation

Bovine platelets were collected in vacutainer tubes containing sodium citrate (0.38% v/v) as anticoagulant as described previously (13). For purposes of these experiments, platelet-rich plasma (PRP) was diluted in Tyrodes/HEPES buffer to a concentration of 1x10^8 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-Quick staining, was less than 10^4 leukocytes per ml.

#### Cultivation of bacterial strains

H. somnus was cultured as described previously (14). For this study, the pathogenic H. somnus strain 649 was used, which was originally obtained from an aborted fetus and has been shown to cause reproductive failure when administered intrabrachially or intravenously. H. somnus LOS was isolated by enzymatic digestion and hot phenol extraction as described previously (15, 16).

### Cultivation of bovine pulmonary artery endothelial cells (BCECF)

The primary cultures of bovine endothelial cells used in this study were obtained by gentle scraping of the pulmonary artery from healthy steers euthanized at approximately 12–18 months of age. These endothelial cells were cultured in DMEM (Cellgro, Herndon, VA, USA) containing penicillin and streptomycin (500 IU/ml, Cellgro) and 20% fetal bovine serum (Atlanta Biologics, Norcross, GA, USA). Endothelial cells were characterized by their “cobblestone” morphology and by flow cytometry using antibodies for CD146 and vWF.

#### Human platelet isolation and endothelial cell cultivation

Human platelets were collected using similar methods as those used for bovine platelets. Primary isolated human umbilical vein endothelial cells (HUVECs) were used between passages 4–6 for our experiments and were cultured in DMEM containing penicillin and streptomycin (500 IU/ml) with 20% fetal bovine serum.

#### BCECF platelet labeling

To observe platelet adherence to endothelial cells, in some experiments platelets were labeled with BCECF. Bovine platelets in PRP were passed through a Sepharose 4B column in Tyrodes-HEPES buffer, with a 4.5 micron filter to minimize contaminating leukocytes. Purified platelets were incubated with BCECF (1.5 mM) at 37°C for 30 minutes (min). Platelets were then passed again through the sepharose 4B column to eliminate extracellular dye.

#### Platelet treatments

Bovine platelets (1x10^8 platelets/ml) were incubated with H. somnus (5x10^8) or H. somnus LOS (500 ng/ml) for 10 min at 37°C. Untreated platelet and platelets incubated with ADP (10 µM) were used as negative and positive controls, respectively. Platelets were fixed in 4% paraformaldehyde (1/10 v/v) for 30 min at 4°C, then washed 3x in phosphate-buffered saline (PBS) before being incubated with endothelial cells. For some experiments, platelet supernatants were collected before fixation. Supematants were centrifuged for 10 min at 1,500 g to pellet the cells. The supernatants were then carefully removed and passed through a 0.22 micron filter, before being incubated in polymyxin B (10 µg/ml) to marginalize the contribution of LOS carryover.

#### Platelet incubation with endothelial cells

Bovine endothelial cells were untreated, incubated with unactivated or activated platelets (MOI 10:1) or staurosporine (200 nM) as a positive control. In circulation platelets are normally in close association with the endothelium. To replicate this close contact, platelet-endothelial cell co-cultures were briefly centrifuged at 500 x g for 10 min before being incubated at 37°C for 12 hours (h). In some experiments, endothelial cells were pre-incubated 30 min with TNFα (100 ng/ml) or cycloheximide (CHX, 10 µg/ml).

#### Caspase activity assays

Caspase-3 activity was detected in endothelial cells using the Apo-ONE™ detection assay (Promega) as described previously.
(13). Caspase-8 and caspase-9 activity were detected using caspase specific Caspase-Glo™ luminescent assays (Promega). For these experiments, endothelial cells were grown overnight on white LuminiNunc 96 well plates (Nalge Nunc International, Rochester, NY, USA). To each well of a 96-well plate, 100 µl of Caspase-Glo™ reagent, containing a pro-luminojenic substrate, was added and the plates incubated for 60 min at room temperature. Caspase-8 and Caspase-9 activity results in cleavage at specific peptide sequences (LETD and LEHD, respectively) releasing the substrate for luciferase (aminoluciferin) resulting in the production of light which was detected using a luminometer. For some experiments bovine endothelial cells were incubated with a pan-caspase inhibitor (Z-VAD-FMK; 10 µM) or selective inhibitors of caspase-8 (Z-LETD-FMK; 10 µM) or caspase-9 (Z-LEHD-FMK; 10 µM) (R&D systems, Minneapolis, MN, USA).

Hoechst 33342 staining

Endothelial cells were seeded at a density of 1x10^5 cells/ml on glass cover slips in 24-well tissue culture plates and allowed to adhere overnight. Endothelial cells were incubated with bovine platelets that had been labeled with BCECF and treated with H. somnus or other activation agents. After a 12-h incubation at 37°C, the cell culture medium was removed and the endothelial cells fixed with 4% paraformaldehyde for 30 min at 4°C. Cells were then washed in PBS, and stained for 10 min at 4°C with Hoechst 33342 (10 µg/ml in PBS Molecular Probes, Eugene, OR, USA). The coverslips were again washed in distilled H₂O, mounted onto glass slides, and viewed by UV fluorescent microscopy with a DAPI (4',6'-diamidino-2-phenylidole) filter block set at 355 nm excitation and 465 nm emission wavelengths.

Detection of TNFα

Following incubation with activated platelets, endothelial cells were analyzed for the production of TNFα mRNA by real-time PCR. Bovine endothelial cells were washed once in Hanks-EDTA (Hanks without calcium or magnesium, Sigma, St. Louis, MO, USA; 2.5 mM EDTA, pH 7.4), then incubated with trypsin for 5 min to remove adherent cells. Endothelial cells were collected and washed 2x in PBS. The Qiagen RNeasy minikit system (Qiagen, Valencia, CA, USA) was used to collect mRNA from endothelial cells, following the manufacturers recommended protocol. The mRNA was converted to cDNA using the Reverse Transcription System (Promega, Madison, WI, USA) and cDNA samples were kept at -20°C until needed. Primers used to amplify TNFα and the housekeeping gene β-actin were designed using the Primer Express software program (Primer Express 2.0; Applied Biosystems, Foster City, CA, USA) using bovine sequences for these genes (TNFα, GenBank accession Z48808; β-actin, GenBank accession AY141970). Primers used in this study are listed below:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>5’ TNFα forward</td>
<td>CCGTGTGGGACTGCTAT</td>
</tr>
<tr>
<td>3’ TNFα reverse</td>
<td>AACTCTGACACTCTGGTGTGC</td>
</tr>
<tr>
<td>5’ β-actin forward</td>
<td>AACTCTGACACTCTGGTGTGC</td>
</tr>
<tr>
<td>3’ β-actin reverse</td>
<td>GGGCAGGACAGCAGCTGGAT</td>
</tr>
</tbody>
</table>

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 (U.S. National Library of Medicine, Bethesda, MD, USA) 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 min, and for 40 cycles, 95°C for 15 seconds and 60°C for 1 min. After completion of the PCR cycles, a melting/disassociation curve was generated to verify the absence of primer dimer formation and other extraneous double stranded DNA products. A cut-off of 1 was chosen for the threshold value for the amplicon levels. Fold increase in mRNA was calculated using the comparative threshold cycle (Ct) method using the formula 2^ΔΔT. Data were normalized for gene expression based on β-actin mRNA expression. To detect TNFα production by ELISA, endothelial cell conditioned media was collected and stored at −20°C until analyzed. Cytokine production was quantified using a commercially available bovine TNFα ELISA, following the instructions of the manufacturer (Pierce Biotechnology, Inc., Rockford, IL, USA). To detect TNFα, protein lysates were prepared from cell culture supernatants and whole cells. Supernatants were concentrated using Centricron Spin columns (YM-10; Millipore, Billerica, MA, USA) following the instructions of the manufacturer. Lysates were prepared for Western blotting as described previously (17). Recombinant bovine TNFα was used as positive control.

Detection of ROS

Bovine endothelial cells were washed 2x in pre-warmed PBS and then incubated with serum-free DMEM containing 100 nM di-fluorodihydrofluorescein (DFF). DFF passively diffuses across the cell membrane, where non-specific esterases cleave lipophilic blocking groups, producing a charged form of the dye that does not readily diffuse out of the cell. After a 1 h incubation at 37°C, endothelial cells were washed 2x in pre-warmed PBS, before growth media was added. For some treatments, endothelial cells were pre-incubated 1 h with ROS inhibitors before inoculation. The inhibitors used were allopurinol (500 µM) a xanthine oxidase inhibitor, apocynin (100 µM) an inhibitor of NAD(P)H, catalase (1,000 U/ml), superoxide dismutase (1,000 U/ml), and the free radical scavengers DMSO (2%) and ascorbic acid (1 mM). Cells were then incubated with the platelet treatment groups, or with PMA (100 nM) as a positive control. ROS production was quantified using a fluorescent plate reader (485 emission, 525 absorption).

Statistical analysis

An unbalanced one-way analysis of variance (ANOVA) was used to determine if significant variation existed between group means. Pairwise comparisons of all the means were done with the Tukey test (significance set at p<0.05) using the Prism 4 statistical package (GraphPad, San Diego, CA, USA).
Figure 1: Activated platelets induce endothelial cell caspase-3 activity. Bovine pulmonary artery endothelial cells (2 x 10^4) were incubated in a 96-well plate with various numbers of H. somnus activated bovine platelets (◆), untreated platelets (▲), or equivalent amounts of conditioned media from platelets stimulated with H. somnus (■). After a 12-h incubation, caspase-3 activity was detected using the Apo-ONE™ detection assay, as described previously in the Methods. These data represent the ± SEM of three separate experiments. * p < 0.05 compared to untreated endothelial cells.

Figure 2: Platelet association with apoptotic endothelial cells. Bovine platelets were pre-loaded with the green fluorescent dye BCECF, before being activated with H. somnus or H. somnus LOS. Platelets (5 x 10^6/ml) were then fixed in paraformaldehyde, washed in PBS, and incubated with bovine endothelial cells (1 x 10^5/ml) on glass coverslips. After a 12-h incubation, the endothelial cells were fixed in paraformaldehyde as described in the Materials and methods, and stained with Hoechst-33342. Cells were then examined for condensed chromatin (blue) by fluorescent microscopy to identify apoptotic endothelial cells with attached platelets (green). The circles indicate areas of condensed chromatin. A) Untreated cells exhibited almost no chromatin condensation; however, we found endothelial cells with condensed chromatin in close apposition to platelets activated by prior incubation with H. somnus (B) or H. somnus (C) LOS.

Figure 3: Activated platelets induce endothelial cell caspase-8 and caspase-9 activity. Bovine platelets previously activated with ADP (pADP; 10 µM), H. somnus (pHS; MOI 1:1) or H. somnus LOS (pLOS; 500 ng/ml) were added to bovine endothelial cells and incubated for 12 h at 37°C. Unactivated platelets (pUT) were used as a negative control. Endothelial cell caspase-8 and caspase-9 activity were quantified using a luminescent caspase detection assay. Activated bovine platelets induced a significant increase in bovine endothelial cell caspase-8 and caspase-9 activity, while unactivated platelet did not. These data represent the ± SEM of three separate experiments. * p < 0.05 compared with untreated endothelial cells.
Results

Endothelial cell apoptosis induced by H. somnus-activated platelets

Significant levels of caspase-3 activity were found when platelets were incubated with endothelial cells at a ratio of 10:1 (Fig. 1). To induce a similar level of caspase-3 activity using conditioned media required the equivalency of 100 platelets per endothelial cell. H. somnus culture filtrates did not induce endothelial cell apoptosis, suggesting that products produced by platelets, and not bacteria, were responsible for endothelial cell apoptosis.

Activated platelets are associated with apoptotic endothelial cells

We next determined if activated platelets were attached to apoptotic endothelial cells. Bovine platelets were loaded with the green fluorescent dye, BCECF, before being incubated with H. somnus or H. somnus LOS. Activated platelets were then fixed, washed and incubated with endothelial cells for 12 h. The endothelial cells were fixed in paraformaldehyde and counter-stained with Hoechst 33342. Unactivated platelets could be seen attached to endothelial cells; however, chromatin condensation was not observed in these cells (Fig. 2A). In contrast, endothelial cells incubated with platelets activated by H. somnus (Fig. 2B) or H. somnus LOS (Fig. 2C) exhibited both attached platelets and chromatin condensation.

Platelet induced endothelial cell apoptosis is dependent on caspase-8 and caspase-9 activity

Because apoptosis was greater when activated platelets were in close association with endothelial cells, we speculated that platelets induced apoptosis through the receptor-mediated caspase-8 pathway. To test this possibility, bovine platelets were incubated for 10 min with ADP (10 µM), H. somnus (MOI 10:1) or H. somnus LOS (500 ng/ml). After incubation, platelets were fixed, washed, and then incubated for 12 h with bovine endothelial cells. Using a luminescent detection assay, significant levels of caspase-8 and caspase-9 activity were observed in endothelial cells incubated with activated platelets, as compared to untreated endothelial cells or cells incubated with unactivated platelets (Fig. 3).

We next investigated whether caspase-8 or caspase-9 were required for endothelial cell apoptosis induced by activated platelets. Endothelial cell apoptosis can be induced by CD40L or FasL, both of which we detected on the surface of H. somnus activated platelets (13). Therefore, we expected that caspase-8, rather than caspase-9, would be the dominant caspase responsible for endothelial cell apoptosis induced by adherent platelets. Because caspase-3 is the central executioner caspase, activated by either caspase-8 or caspase-9, we quantified the activity of this enzyme as a measure of apoptosis. Bovine endothelial cells were pre-incubated for 1 h with a pan-caspase inhibitor (Z-VAD), or with inhibitors of caspase-8 (Z-IEDT-FMK) and caspase-9 (Z-LEHD-FMK) before being incubated with activated platelets for 12 h. Caspase-3 activity was significantly increased in endothelial cells incubated with activated platelets, and was reduced to baseline levels in endothelial cells pre-incubated with the pan-caspase inhibitor, Z-VAD (Fig. 4). The addition of either a caspase-8 or caspase-9 inhibitor also significantly decreased endothelial cell caspase-3 activity in cells incubated with activated platelets. These results suggest that endothelial cell apoptosis induced by activated platelets requires initiation of both caspase-8 and caspase-9 apoptotic pathways.

Fas induced endothelial cell apoptosis

Because activated platelets express FasL, we investigated the sensitivity of endothelial cells to Fas-mediated apoptosis. Endothelial cells were incubated with an anti-Fas antibody that has been reported previously to promote apoptosis through the Fas receptor (18, 19). After a 12-h incubation, endothelial cells incubated with the anti-Fas activating antibody alone did not exhibit a significant increase in caspase-3 activity as compared to untreated endothelial cells (Fig. 5). However, if endothelial cells were first pre-incubated with the protein synthesis inhibitor cycloheximide, before addition of the anti-Fas antibody, caspase-3 activity was significantly increased. This finding suggests that bovine pulmonary artery endothelial cells are resistant to Fas-mediated apoptosis.

Role of TNFα in endothelial cell apoptosis

We reasoned that activated platelets might stimulate endothelial cells to release factors which could promote endothelial cell death in an autocrine or paracrine manner. Two factors that are known to sensitize endothelial cells to undergo apoptosis are TNFα and ROS. To test the role of TNFα, endothelial cells were incubated with activated bovine platelets and TNF production was determined by real-time PCR and ELISA. We observed a significant increase in TNFα mRNA in endothelial cells incubated with platelets activated by H. somnus or its LOS (Fig. 6). However, using a TNFα ELISA we did not detect a significant increase in TNFα release through 6 h of incubation (data not shown). Because we considered that TNFα may be cell associated, we prepared protein lysates from whole cells or cell culture supernatants 12 h after incubation with activated platelets. Using Western blot analysis, we were not able to detect any TNFα production from our endothelial cells, despite the increase in TNFα mRNA that we observed.

To determine if TNFα could promote Fas-mediated apoptosis, endothelial cells were pre-incubated with 100 ng/ml of TNFα for 30 min before addition of an activating anti-Fas antibody. TNFα alone induced a significant level of caspase-3 activity, which was increased further in the presence of cycloheximide (data not shown). Endothelial cells co-incubated with anti-Fas antibody and TNFα did not exhibit greater caspase-3 activity than endothelial cells incubated with TNFα alone. Although addition of cycloheximide significantly increased endothelial cell apoptosis in response to Fas (Fig. 5) or TNFα, it did not enhance caspase-3 activity in endothelial cells incubated with TNFα and Fas in combination (data not shown). To determine if endothelial cell TNFα production was required for apoptosis in response to activated platelets, we used a recombinant human soluble TNFα receptor antagonist. Pre-incubation of endothelial cells with the soluble TNFα receptor prior to incubation with H. somnus activated platelets did not diminish apoptosis induced by the platelets (data not shown).
Role of ROS in endothelial cell apoptosis

Endothelial cells have been reported to produce ROS when incubated with platelets or CD40L (20, 21). Because apoptosis can be induced by the accumulation of ROS, we investigated the ability of *H. somnus*-activated platelets to cause endothelial cell apoptosis by this pathway. We observed a significant increase in endothelial cell ROS production within 20 min of the addition of activated platelets (Fig. 7A). To determine if ROS contributed to platelet-induced apoptosis, we pre-incubated endothelial cells with inhibitors of ROS production or free-radical scavengers. Pretreatment with inhibitors of xanthine oxidase (i.e. allopurinol) or NAD(P)H (i.e. apocynin) resulted in a modest decrease in
endothelial cell caspase-3 production (Fig. 7B). Addition of catalase or superoxide dismutase did not inhibit apoptosis (data not shown). However, the free radical scavengers, dimethyl sulfoxide (DMSO) and ascorbic acid, both significantly inhibited endothelial cell caspase-3 activation. These results demonstrate that inhibiting ROS generation, or scavenging ROS, may protect endothelial cells from platelet-induced apoptosis.

**Human platelets incubated with E. coli LPS induce endothelial cell apoptosis**

We next considered the possibility that human platelets stimulated with LPS may also be able to induce endothelial cell apoptosis. Human platelets were incubated with ADP (10 µM) or LPS from the human pathogen *E. coli* 0111:B4, which has previously been demonstrated to activate platelets (22). Following a 10-min incubation, platelets were then incubated with HUVECs at a ratio of 10:1 for 12 h. Caspase-3 activity was detected using the Apo-ONE™ detection assay, as described previously. We observed that, similar to bovine platelets, ADP- or LPS-activated human platelets could also induce a significant increase in endothelial cell caspase-3 activity compared to cells incubated with untreated platelets (Fig. 8).

**Discussion**

The ability of platelets to regulate endothelial cell function is well-known. Activated platelets can promote endothelial cell proliferation, pro-coagulative changes and regulate pro-inflammatory responses (1, 23, 24). Platelets can also contribute to vascular damage and endothelial cell apoptosis (25–28). For example, platelets have been shown to induce endothelial cell apoptosis by direct cell contact during liver reperfusion (25, 26). Likewise, in models of sepsis, platelet interactions with the endothelium can lead to blood vessel damage (29–31). The removal of circulating platelets, but not leukocytes, has been shown to prevent endothelial cell damage during experimental endotoxemia (29). In *Plasmodium berghei* infection, inhibition platelet adherence to the endothelium protects against damage to the cerebral microvasculature (32). These studies and others suggest that platelets can play a significant role in promoting damage to the vasculature.

In this study we have demonstrated that *H. somnus* activation of bovine platelets leads to bovine endothelial cell apoptosis through a process requiring both caspase-8 and caspase-9 activity. Although we have previously reported that bovine platelets induce endothelial cell apoptosis in response to *H. somnus*, the mechanisms by which this occurred are still under investigation.
Apoptosis was greater when the endothelial cells were in close proximity with fixed activated platelets; however, conditioned media from activated platelets also induced some endothelial cell caspase-3 activity. Therefore, soluble factors released from bovine platelets may also contribute to apoptosis. Previously it has been reported that secreted factors from platelets, such as TGFβ, can promote apoptosis in brain endothelial cells (27). Platelet-derived microparticles from septic patients, which possess NAD(P)H oxidase activity, can also induce apoptosis in both endothelial and vascular smooth muscle cells (28). Similarly, platelet surface-expressed molecules, such as FasL, have been reported to induce apoptosis in human Fas-expressing cells (2). CD40L has also been shown to induce endothelial cell apoptosis, and can sensitize cells to FasL-mediated apoptosis by up-regulating Fas (33–36). Therefore, platelets may be able to induce endothelial cell apoptosis through a variety of mechanisms.

The expression of FasL on activated platelets is of particular interest. Signaling through Fas-FasL is cited as a major contributor to vascular damage during sepsis and hemorrhagic shock (37–39). In addition, vascular endothelial cell expression of Fas and caspase-8 are significantly elevated during sepsis in response to IFNγ, leading to increased sensitivity to FasL-mediated apoptosis (35). Despite these findings, the contribution of platelet FasL toward endothelial cell apoptosis has not been thoroughly investigated, which is surprising given the documented importance of Fas-FasL signaling in sepsis. Our laboratory has previously demonstrated that bovine platelets activated by ADP, or the bacterial pathogen H. somnus, also express FasL (13). We have therefore suspected that this molecule might contribute to endothelial cell apoptosis as well.

Due to a lack of bovine-specific reagents, we could not directly address the question of whether platelet FasL directly activates Fas signaling in endothelial cells. However, we did find that bovine endothelial cells appear to be resistant to Fas-mediated apoptosis, which is similar to reports for human endothelial cells (40). Although bovine endothelial cells appeared to be resistant to apoptosis induced by Fas receptor signaling, we found that ROS production was necessary for apoptosis induced by activated platelets. These findings highlight a novel pathway for endothelial cell apoptosis induced by activated platelets.

We therefore decided to investigate if other molecules (TNFα and ROS) might promote endothelial cell apoptosis. Because platelets have been reported to sensitize brain microvascular cells to cell injury induced by TNFα (41), we sought to determine if platelets could induce endothelial cell TNFα production. We found that activated platelets stimulated endothelial cell TNFα mRNA expression; however, soluble TNFα was not detected by ELISA. We speculated that TNFα might remain cell surface-associated, where it could still contribute to endothelial cell apoptosis, or that concomitant release of soluble TNF receptors from endothelial cells may have confounded our ability to detect TNFα. However, we did not detect TNFα in whole cell lysates or concentrated cell culture supernatants by Western blot analysis. Using a soluble TNFα receptor antagonist to investigate the contribution of TNFα not detected by our ELISA we also found no inhibition in endothelial cell apoptosis induced by activated platelets. From these results we infer that activated platelets do not cause bovine endothelial cell apoptosis through a TNFα-dependent pathway. We believe that TNFα mRNA produced by our endothelial cells may be post-transcriptionally regulated to prevent its translation to active protein. This type of regulation has been previously described and it is not surprising that expression of this inflammatory cytokine would be tightly regulated (42–44).

Signaling through endothelial cell CD40 has been shown to cause ROS generation, which in turn has been linked to mitochondrial damage and apoptosis (20, 45). Similar to human platelets, we previously reported that bovine platelets express CD40L (13). Therefore, we considered the possibility that activated platelets may be able to induce ROS synthesis in endothelial cells. We found that platelets significantly increased ROS production in bovine endothelial cells, and that inhibition of ROS greatly diminished endothelial cell apoptosis. It is tempting to speculate that CD40L–CD40 interactions may contribute to events downstream of the Fas receptor, perhaps due to ROS production.

There are two pathways of Fas-mediated apoptosis that are dependent on the availability of active caspase-8 at the death inducing signaling complex (DISC). In type I responses, active caspase-8 can directly process caspase-3, leading to activation and apoptosis. In type II responses, only a small quantity of activated caspase-8 is produced at the DISC. Apoptosis in these cells requires cleavage of Bid to tBid, which then promotes Bax accumulation on the mitochondrial membrane. This in turn leads to cytochrome C release, and activation of caspase-9, that subsequently activates caspase-3 to cause apoptosis (46). An important characteristic of type II Fas-mediated apoptosis is the regulation of anti-apoptotic proteins of the Bcl-2 family (Bcl-2 or Bcl-xL). Because Bcl-2 or Bcl-xL can render type II cells resistant to Fas-mediated apoptosis, down-regulation of these proteins may need to occur. Although we did not quantify the expression of anti-apoptotic proteins, the generation of ROS is reported to decrease expression of Bcl-2 and Bcl-xL (47). We found that disrupting caspase-8 activity inhibited endothelial cell apoptosis.
cell apoptosis, which is consistent with a surface receptor (e.g. Fas or CD40) triggering apoptosis. However, inhibition of caspase-9 also significantly decreased caspase-3 activity induced by activated platelets. These observations suggest that bovine endothelial cell apoptosis induced by platelets required both caspase-8 and caspase-9 activation, similar to a type II receptor-mediated caspase-9 pathway (48–51). This observation, coupled with the importance of ROS production in promoting apoptosis, implies a unique mechanism for endothelial cell apoptosis induced by activated platelets. It has been reported that human endothelial cells are similarly resistant to Fas-mediated apoptosis, while porcine endothelial cells are not (40). We found in our study that LPS-activated human platelets could also promote endothelial cell apoptosis. This is similar to a recently published study demonstrating the role of ROS in human platelet-induced endothelial cell apoptosis (52). Therefore, our findings support the concept that bovine endothelial cells may be a useful model for understanding apoptosis induced by platelets in human cells.

The ability of platelets activated by bacteria to induce endothelial cell apoptosis suggests a possible role for platelets in the pathogenesis of bacterial sepsis. Platelet activation could contribute to vasculitis and thrombus formation by inducing endothelial cell apoptosis and exposing the underlying extracellular matrix. Furthermore, apoptotic endothelial cells are reported to be pro-adhesive for unactivated platelets which could further amplify the local coagulation and inflammatory responses (53). We believe that by investigating the interactions between bacteria, platelets and endothelial cells in our model, it may be possible to better understand the complex associations and vascular complications that occur during sepsis in other organisms as well.

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References


