Acquisition of plasmin activity and induction of arachidonic acid release by *Streptococcus suis* in contact with human brain microvascular endothelial cells

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**Abstract**

Infections caused by *Streptococcus suis*, a major swine pathogen, include meningitis, arthritis, pneumonia and septicemia. In this study, we investigated interactions that may occur between human brain microvascular endothelial cells (HBMEC), the main constituent of the blood–brain barrier, and *S. suis*. We show that *S. suis* acquires plasmin activity in a time-dependent manner when in contact with cultured HBMEC. Cell-associated plasmin activity reached a plateau following a 48 h co-incubation period. Zymography analysis revealed that HBMEC produce urokinase, which is probably involved in activation of plasminogen bound to *S. suis*.

We also show that a *S. suis* culture supernatant which possesses both phospholipase C and haemolysin (suilysin) activities was able to induce the release of arachidonic acid from the membrane of HBMEC. Evidence suggests that the action of suilysin on HBMEC may be a prerequisite for the action of additional molecules such as phospholipase C. These new biological effects associated with *S. suis* may play an important role in the migration of *S. suis* through the blood–brain barrier and in the modulation of local inflammation.

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**Keywords:** *Streptococcus suis*; Human brain microvascular endothelial cells; Plasmin; Phospholipase; Suilysin; Arachidonic acid

**1. Introduction**

Several bacterial species in the upper respiratory tract of pigs can initiate severe infections leading to the death of the animal. One early colonizer, *Streptococcus suis*, is a major swine pathogen world-wide [1] that causes meningitis, arthritis, pneumonia, and septicemia [2]. *S. suis* is also an important zoonotic agent that can cause infections in workers in the pig industry [3,4]. Thirty-five serotypes (1–34 and 1/2) have been identified so far and serotype 2 is the one most commonly isolated from diseased pigs [5]. While several virulence factors (capsule, haemolysin, adhesin) of *S. suis* have been identified and characterized [5–7], the exact mechanisms by which...
this bacterium invades the host and causes infections are still unclear.

Brain microvascular endothelial cells are the main constituent of the blood–brain barrier (BBB). This barrier plays an important role in the regulation of macromolecule and cell trafficking through the tight junctions between cells and contributes to the maintenance of the biochemical homeostasis of the central nervous system (CNS) [8]. Pathogens use several pathways to invade the BBB, including transcellular penetration, paracellular entry, and transmigration with infected leucocytes [9,10]. Paracellular entry of S. suis may rely on the secretion of suilysin, a potent haemolysin produced by virulent strains [11]. Damage to infected brain microvascular endothelial cells has been associated with the presence of suilysin which, combined with the ability of S. suis to attach to these cells, can alter the permeability of the BBB [12]. S. suis can also stimulate the production of interleukin-6, interleukin-8, and monocyte chemotactic protein-1 by human endothelial cells, which may modulate the integrity of the BBB [13].

The acquisition of plasmin activity is a mechanism by which invasive bacteria can enhance their capabilities to destroy cell integrity and penetrate more deeply into tissues [14]. We recently showed that S. suis can bind porcine and human plasminogens on its surface, which can then be activated into plasmin by an endogenous plasminogen activator [15]. This capacity may affect BBB permeability and contribute to the invasive potential of S. suis, as has been shown for Candida albicans [16]. Host-derived lipids and prostaglandins are important inflammatory mediators that can also modulate BBB permeability [17]. Arachidonic acid found in the eukaryotic cell membranes is a prostaglandin precursor [17]. The release of arachidonic acid from the cell surface can be mediated by either bacterial or mammalian phospholipases [18,19]. In this study, we investigated interactions that may occur between human brain microvascular endothelial cells (HBMEC) and S. suis. More specifically, we demonstrated the ability of S. suis to acquire plasmin activity and to induce arachidonic acid release following contact with HBMEC.

2. Materials and methods

2.1. Bacteria and growth conditions

S. suis S735 (reference strain; ATCC 43765) and 31533 (virulent European strain), which belong both to serotype 2, were routinely grown in Todd–Hewitt broth (THB; BBL Microbiology Systems, Cockeysville, MA, USA) or on Todd–Hewitt agar (THA) at 37 °C under aerobiosis. A suilysin-negative mutant (SX911) derived from strain 31533 and generated by allelic replacement [11] was grown in presence of 200 µg/ml of spectinomycin. The bacteria used to stimulate HBMEC were cultivated in THB until they reached the mid-log growth phase (optical density at 660 nm of 0.5–0.6). The bacteria were washed once in phosphate-buffered saline (PBS 50 mM, pH 7.4) and suspended in half the initial volume. The bacterial concentration (cfu/ml) of the suspension was determined by plating on THA. Heat-treated bacteria were prepared by incubating a bacterial suspension at 60 °C for 45 min, which has been reported to kill S. suis [20]. Heat-treated bacterial suspensions were stored at 4 °C until used. A culture supernatant of S. suis grown for 18 h in THB was collected following centrifugation, and filtered through a 0.22 µm pore filter to remove any remaining bacteria. The supernatant was concentrated 5-fold with an Amicon Ultratube (Millipore, Bedford, MA) with a 10 kDa molecular weight cutoff membrane. The supernatant was stored at 4 °C until used.

2.2. Suilysin preparation

Recombinant S. suis 31533 suilysin was produced in Escherichia coli and purified as described previously [11]. The purified protein was kept at −20 °C at a concentration of 800 haemolytic units/µg as determined using equine blood cells (HU/µg).

2.3. Cultivation of HBMEC

HBMEC line produced from a brain biopsy of an adult female with epilepsy was kindly provided by Dr. K. Kim (Johns Hopkins University School of Medicine, Baltimore, MD, USA). The cells, which had been immortalized by transfection with simian virus 40 large T antigen [21], maintained their morphological and functional characteristics. HBMEC were grown in RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% heat inactivated foetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO, USA), 10% Nu-serum IV supplement (BD Biosciences, Bedford, MA, USA), penicillin (50 µg/ml), and streptomycin (50 µg/ml). Cultures were incubated at 37 °C in a 5% CO₂ humid atmosphere. The cells were used before passage 35 for all experiments.

2.4. Stimulation of HBMEC

For stimulation assays, 48-h HBMEC cultures in flasks were trypsinized (0.5% trypsin [w/v]) for 3 min at 37 °C, diluted in culture medium to a concentration of 1.5 × 10⁵ cells/ml, and 1 ml of the suspension was placed in each well of 24-well culture plates. The plates were incubated for 24 h to allow the cells to reach confluence. The medium was removed and 1 ml of heat-killed S. suis suspended in supplemented RPMI 1640 medium was added to give a ratio of 1000 bacteria per cell. The plates were incubated at 37 °C in a 5% CO₂-humid atmosphere for up to 60 h.
2.5. Determination of plasmin activity

At different time intervals (24, 36, 48, and 60 h), the wells containing HBMEC and S. suis were scraped and the suspension was centrifuged for 5 min at 200g to remove the HBMEC. The supernatant was then centrifuged for a further 20 min at 12,000g, to recover S. suis and determine surface-associated plasmin activity. The bacterial pellet was resuspended in 150μl of PBS, and 50μl of this suspension was added to 50μl of PBS. After 15 min preincubation at 37°C, 20μl of the plasmin chromogenic substrate Val-Leu-Lys-nitroanilide (2 mg/ml; Bachem, Torrance, CA, USA) was added. The assay mixtures were incubated for 24 h at 37°C and then centrifuged to remove bacteria. The absorbance of the supernatant at 415 nm (A415) was recorded using a microplate reader. A standard curve was constructed by incubating known concentrations of commercial plasmin with its specific substrate using the same assay conditions as for S. suis. The A415 values obtained with S. suis were compared to the standard curve to determine the number of equivalent plasmin units (EU) of plasmin activity. The plasmin activity acquired by S. suis was confirmed by a zymography analysis. Gelatin (final concentration 0.03%) was incorporated into a 10% polyacrylamide gel containing sodium dodecyl sulphate (0.1%). Samples of bacteria incubated with HBMEC were mixed with electrophoresis sample buffer and the electrophoresis was carried out on ice at a constant voltage of 100 V using the buffer system of Laemmli [22]. The gels were washed in 0.05 M Tris–HCl (pH 7.5) and then incubated (2 × 15 min) at 37°C in equilibration buffer (0.05 M Tris–HCl, 0.1 M NaCl, 2.5% Triton X-100, pH 7.5). The gels were rinsed in 0.05 M Tris–HCl (pH 7.5) for 15 min and placed in reaction buffer (0.05 M Tris, 0.1 M NaCl, 0.01 M CaCl2, pH 7.5) overnight at 37°C with gentle shaking. Proteolysis zones were detected following staining with Coomassie blue and destaining (2:3:15/methanol:acetic acid:water).

2.6. Determination of plasminogen activator activity

The production of plasminogen activators by HBMEC was investigated by zymography as described previously [23]. Briefly, supernatants from HBMEC cultures that had been stimulated or not with S. suis cells were subjected to electrophoresis on a 11% polyacrylamide gel containing 0.05% gelatin or 0.05% gelatin and 0.007 mg/ml plasminogen (human, Sigma). The gels were then washed with 2.5% Triton X-100 and incubated for 5 h at 37°C in a 0.1 M glycine–NaOH buffer (pH 8.3). The gels were stained with Coomassie blue, destained, and compared. Activation of the plasminogen incorporated into the gel was revealed by the production of a clear band that was not present in the gel containing only gelatin.

2.7. Determination of arachidonic acid release

A HBMEC suspension (100 μl, 1.5 × 10^5 cell/ml) prepared as described above was distributed into the wells of 96-well plates and incubated for 24 h in the presence of 1 μCi/ml of ^3H-arachidonic acid (204 Ci/mmol; MP Biochemicals, Irvine, CA). The cells were washed twice with fresh culture media and then stimulated for 24 h with heat-treated S. suis (1000 bacteria per HBMEC), S. suis culture supernatant, or recombinant suilysin (4000 HU/ml). Detached HBMEC were removed from the cell culture media by centrifugation (5 min, 200g). The quantity of ^3H-arachidonic acid released into the supernatants was determined using a scintillation counter. Unstimulated HBMEC were used as a negative control. All assays were performed in triplicate.

2.8. Determination of phospholipase activity

Phospholipase activity was determined using ^14C-labelled phosphatidylcholine (0.025 μCi/ml; Amersham, Baie d’Urfé, QC) as previously described [24]. S. suis (S735, 31533 SX911) was grown overnight in THB at 37°C. The cells were harvested by centrifugation and suspended at an OD600 of 1.8 in PBS. Culture supernatants concentrated 8-fold by ultrafiltration through an Amicon Ultratube with a 10 kDa molecular weight cutoff membrane were also tested. The phospholipase activity of the recombinant suilysin (17.6 and 440 HU) was also tested. Twenty-five μl of each sample was added to 30 μl of PBS containing ^14C-labelled phosphatidylcholine (2 μl) and 6.5 ng of non-radioactive phosphatidylcholine. The mixtures were vortexed for one minute and incubated for 48 h at 37°C with gentle agitation. Twenty μl of each assay mixture was applied to a silica gel (TLC) plate (Fisher, Nepean, ON, Canada) and the migration was carried out at room temperature in a solvent system composed of petroleum–ethanol–acetic acid (50:50:1; v/v/v) until the solvent front was within 0.5 cm of the top of the plate. The plate was air-dried and exposed to a Kodak X-Omat S film (Kodak, Rochester, NY) for six days at −80°C in presence of a transcreen (BioMAX, Kodak). Commercial phospholipase C and D (23.2 μg; Sigma) were used as controls.

2.9. Statistical analysis

The data was analysed using the Statistix 7 Analytical Software.

3. Results

As shown in Fig. 1A, S. suis in contact with HBMEC in culture acquired cell surface plasmin activity in a time-dependent manner, as determined with the specific
chromogenic substrate. Cell-associated plasmin activity reached a plateau following a 48 h co-incubation of S. suis with HBMEC. Based on the standard curve (slope = 0.4 EU per A_{415} unit; \( r^2 = 0.98 \)), the plasmin activity associated with \( 10^9 \) S. suis cells was estimated at 8.4 ± 0.3 EU after a 48 h contact period. The time of incubation was correlated with EU of plasmin activity associated to S. suis (Pearson \( r \) of 0.93). The acquisition of plasmin activity by S. suis was confirmed by zymography on a gelatin-containing polyacrylamide gel. As shown in Fig. 1B, lysates of S. suis cells co-incubated with HBMEC for 24 and 48 h produced a gelatinase band that migrated at a molecular mass of 78 kDa, which is the same as the commercial plasmin used as a control.

Since we previously showed that S. suis cannot activate plasminogen by itself [15], the production of plasminogen activators by HBMEC was investigated by zymography on a polyacrylamide gel containing both plasminogen and gelatin. The culture media of stimulated and unstimulated HBMEC were analysed at time 0 and 48 h. None of the samples tested produced a clear zone in the control zymograms containing only gelatin (data not shown). However, the zymograms containing both gelatin and plasminogen showed two major gelatinase bands corresponding to plasminogen activators (Fig. 2). The most intense band had a molecular mass estimated at 52 kDa whereas the weak band had a molecular mass of approximately 91 kDa. Stimulation of HBMEC with S. suis did not increase the intensity of the bands.

In the second part of the study, the capacity of S. suis to induce arachidonic acid release from the surface of HBMEC was tested. Bacterial cells showed no capacity to mediate the release of \( ^3 \)H-arachidonic acid from HBMEC (data not shown). However, treating the HBMEC with the culture supernatants of S. suis S735 and S. suis 31533 resulted in a twofold increase in the release of \( ^3 \)H-arachidonic acid (Fig. 3). No significant induction of release of \( ^3 \)H-arachidonic acid was observed following treatment of HBMEC with a purified preparation of recombinant suilysin or with a supernatant of the suilysin-deficient mutant of S. suis 31533 (SX911). Furthermore, the presence of dithiothreitol, a known...
activator of suilysin activity, had no inducing effect in the assay using the recombinant suilysin (data not shown).

In order to identify potential factors involved in arachidonic acid release, the supernatant of \( S. suis \) was analysed for phospholipase activity. The degradation profile of \(^{14}\text{C}-\text{phosphatidylcholine} \) by the culture supernatants of \( S. suis \) 31533 and S735 suggested that a phospholipase C was present (Fig. 4). Indeed, diacylglycerol (1,3-diacylglycerol and 1,2-diacylglycerol) and choline phosphate, which are major cleavage products of phospholipase C, were detected, whereas no phosphatidic acid, a characteristic degradation product of phospholipase D, was detected. The suilysin-deficient mutant SX911 appeared to have weaker phospholipase activity than the wild-type strain. Weak phospholipase C activity was observed with the recombinant suilysin.

Fig. 4. Detection of phospholipase activity by TLC and autoradiography in \( S. suis \) culture supernatants and a preparation of recombinant suilysin using \(^{14}\text{C}-\text{phosphatidylcholine} \) as the substrate. Lane 1: commercial phospholipase D; lane 2: commercial phospholipase C; lane 3: \( S. suis \) 31533 culture supernatant; lane 4: \( S. suis \) SX911 culture supernatant; lane 5: \( S. suis \) culture S735 culture supernatant; lane 6: 10 \( \mu \text{g/ml} \) suilysin (440 HU\(_E\)); lane 7: 0.4 \( \mu \text{g/ml} \) suilysin (17.6 HU\(_E\)).

4. Discussion

Little is known about the mechanisms by which \( S. suis \) invades tissues and modulates inflammation to induce meningitis. In this study, we show that \( S. suis \) acquired plasmin activity when co-incubated with HBMEC and that it induced the release of arachidonic acid from the cell surface of HBMEC. These biological effects may play an important role in the migration of \( S. suis \) through the BBB and in the modulation of the local inflammatory response.

Plasminogen is an important component of the fibrinolytic system. Its activation into plasmin, a broad spectrum serine protease, is tightly regulated by the equilibrium between urokinase (uPA), tissue plasminogen activator (tPA) and inhibitors (\( \alpha\)-antiplasmin, \( \alpha\)-2-macroglobulin) [14]. We recently reported that \( S. suis \) was able to bind plasminogen on its cell surface, although it cannot produce a plasminogen activator [15]. Results from the present study indicated that \( S. suis \), when co-incubated with HBMEC, could acquire plasmin activity. In our experimental procedure, the plasminogen came from the FBS added to the HBMEC culture medium and was activated into plasmin by plasmin activators constitutively secreted by HBMEC. A zymography analysis revealed that the major plasminogen activator produced by the HBMEC had a molecular mass of approximately 52 kDa, which is consistent with that reported for uPA [14]. The second plasminogen activator detected had a molecular mass of 91 kDa and was produced in lower amounts. This high molecular mass form may represent uPA complexed with plasminogen activator inhibitors, as previously reported [25]. The production of plasminogen activators by HBMEC was not upregulated by the presence of \( S. suis \). This observation differs from the results obtained with \( Staphylococcus aureus \) [26] and \( Borrelia burgdorferi \) [27], which stimulate the production of plasminogen activators by epithelial cells and monocytes, respectively. The acquisition of plasmin activity on the bacterial cell surface is a major advantage for invasive bacteria since it may contribute to the destruction of the extracellular matrix and promote the bacterial tissue penetration [28]. Interestingly, the penetration of endothelial cell monolayers by \( B. burgdorferi \) is mediated by surface-associated plasmin activity [29]. Future studies will look at whether the acquisition of cell surface plasmin activity by \( S. suis \) contributes to its capacity to invade human and porcine brain microvascular endothelial cells.

Arachidonic acid is the main precursor of prostaglandins, which are small lipids that enhance BBB vascular permeability and nitric oxide production by HBMEC, thus promoting tissue destruction and cell migration [17]. The release of membrane-associated arachidonic acid by HBMEC stimulated with \( S. suis \) culture supernatants appears to involve, at least in part, suilysin. Strain SX911, which has a single insertion of a Kan\(^\text{r}\) cassette, rendering it deficient in suilysin production, failed to induce the release of arachidonic acid. However, no release of arachidonic acid was observed when HBMEC were stimulated with purified recombinant suilysin. On the one hand, while the recombinant suilysin produced in \( E. coli \) retained its haemolytic property, it may not have all properties of native suilysin. On the other hand,
the action of suilysin on HBMEC may be a prerequisite for the action of other molecules such as phospholipase, which may disrupt the membrane and induce the release of arachidonic acid. The haemolysis induced by pathogenic bacteria is frequently associated with phospholipases [18]. In this study, we showed that S. suis produces a phospholipase C. Phospholipase C hydrolyses phosphatidylcholine into choline phosphate and diacylglycerol, a messenger that activates protein kinase C, an important metabolite of arachidonic acid production [30]. An haemolysin (pneumolysin) produced by Streptococcus pneumoniae was found to induce the production of phospholipase A in lung artery endothelial cells and the release of arachidonic acid by stimulated lung artery endothelial cells [31]. Such a phenomenon may also occur for the S. suis-mediated release of arachidonic acid.

The phospholipase C activity of S. suis identified in this study appears to involve at least two distinct molecules, one of which is suilysin. This is supported by the observation that the mutant lacking suilysin activity still possesses phospholipase C activity. In addition, the phospholipase activity is found both on the surface of S. suis and in the culture supernatant, which is not the case for suilysin, being only found in culture supernatant [27]. The non-haemolytic mutant produced a truncated form of suilysin lacking haemolytic activity, which means that the phospholipase C might not have folded properly. This would result in weaker phospholipase C activity than the wild strain, as indicated by the TLC results. Additional studies are required to better characterize the phospholipase C produced by S. suis.

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References


