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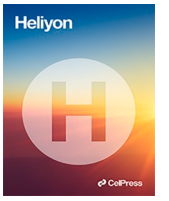

Baicalin inhibited PANX-1/P2Y6 signaling pathway activation in porcine aortic vascular endothelial cells infected by *Glaesserella parasuis*

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ABSTRACT

Glaesserella parasuis can induce endothelial barrier damage in piglets, although the mechanism by which this pathogen triggers inflammatory damage remains unclear. Baicalin possesses anti-inflammatory and anti-oxidant activities. However, whether baicalin can relieve endothelial barrier damage caused by *Glaesserella parasuis* infection has not yet been studied. Hence, we evaluated the ability of baicalin to counteract the changes induced by *Glaesserella parasuis* in porcine aortic vascular endothelial cells. The results showed that *Glaesserella parasuis* could upregulate the expression of pannexin 1 channel protein and promote the release of adenosine triphosphate, adenosine diphosphate, adenosine 3′-monophosphate, uridine triphosphate, uridine diphosphate, and uridine monophosphate in porcine aortic vascular endothelial cells. The expression level of purinergic receptor P2Y6 was upregulated in porcine aortic vascular endothelial cells triggered by *Glaesserella parasuis*. In addition, *Glaesserella parasuis* could activate phospholipase C-protein kinase C and myosin light chain kinase-myosin light chain signaling pathways in porcine aortic vascular endothelial cells. Baicalin could inhibit pannexin 1 channel protein expression, reduce adenosine triphosphate, adenosine diphosphate, adenosine 3′-monophosphate, uridine triphosphate, uridine diphosphate, and uridine monophosphate release, and attenuate the expression level of P2Y6 in porcine aortic vascular endothelial cells induced by *Glaesserella parasuis*. Baicalin could also reduce the activation of phospholipase C-protein kinase C and myosin light chain kinase-myosin light chain signaling pathways in porcine aortic vascular endothelial cells triggered by *Glaesserella parasuis*. Our study report that *Glaesserella parasuis* could promote pannexin 1 channel protein expression, induce nucleosides substance release, and P2Y6 expression in porcine aortic vascular endothelial cells and baicalin could inhibit the expression levels of pannexin 1, nucleosides substance, and P2Y6 in the porcine aortic vascular endothelial cells induced by *Glaesserella parasuis*, which might be served as some targets for treatment of inflammation disease caused by *Glaesserella parasuis*.

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1. Introduction

Glaesserella parasuis (*G. parasuis*) is a common bacterial that colonises the upper respiratory tract of pigs [1]. This pathogen causes Glässer's disease, the typical symptoms of which include fibrinous polyserositis, arthritis, and meningitis [2]. With the change in the swine breeding mode to all-in/all-out, the economic losses due to *G. parasuis* have increased. To date, 15 serotypes of *G. parasuis* have been identified by using the heat-stable antigen extract test [3]. However, up to 20 % isolates have not been identified by the Kielstein–Rap–Gabrielson (KRG) scheme [4]. *G. parasuis* serovar 5 is considered to be highly virulent [5]. Currently, *G. parasuis* is difficult to control on pig farms and causes massive disruptions.

G. parasuis infection induces a marked inflammatory response, although the exact mechanisms remain unclear. We have shown that *G. parasuis* induced vascular endothelial cells apoptosis by activating the receptor of advanced glycation endproducts (RAGE), mitogen-activated protein kinase (MAPK), and AP-1 signaling pathways [6]. Moreover, *G. parasuis* activates the nuclear factor kappa B (NF-κB) and NLRP3 inflammasome signaling pathways in porcine aortic vascular endothelial cells (PAVECs) [7] and induces the release of high mobility group protein B1 (HMGB1) in peripheral blood monocytes [8]. Although *G. parasuis* can activate several important inflammatory signaling pathways, little is known about the mechanism by which this bacterium induces vascular endothelial barrier injury. P2Y receptors are G protein–coupled receptors that can be activated by extracellular nucleotides [9]. Researchers have reported that the P2Y6 receptor can be specifically activated by uridine diphosphate (UDP), leading to protein kinase C (PKC) and phospholipase C (PLC) activation [10]. The P2Y6 receptor has an important role in mediating the inflammatory response [11]. However, whether it participates in *G. parasuis*–induced inflammation has not been studied.

Baicalin is extracted from the Chinese herb *Scutellaria baicalensis*; it has been shown to have several important biological functions [12]. Baicalin inhibits *Staphylococcus saprophyticus* formation by influencing its primary adhesion and aggregation phases [13]. Baicalin attenuates necroptosis in macrophages triggered by lipopolysaccharide (LPS) by inhibiting oligomerization of phosphorylated mixed lineage kinase domain like pseudokinase (MLKL) and reducing caerulein-induced acute pancreatitis in mice [14]. Baicalin alleviates endometrial inflammatory injury by regulating the expression of tight junction proteins [15]. In addition, baicalin reduces HMGB1 release, attenuates apoptosis, and inhibits MAPK signaling activation induced by *G. parasuis* [16]. However, whether baicalin affects activation of the P2Y6 signaling pathway triggered by *G. parasuis* has not been investigated.

We investigate the effect of baicalin on *G. parasuis*–induced P2Y6 signaling pathway activation in PAVECs. We found that *G. parasuis* induced pannexin 1 (PANX-1) expression; adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine 3′-monophosphate (AMP), uridine triphosphate (UTP), UDP, and uridine monophosphate (UMP) release; and P2Y6 and MLCK expression in PAVECs. Baicalin inhibited PANX-1 and P2Y6 expression; reduce ATP, ADP, AMP, UTP, UDP, and UMP release; and attenuated activation of PLC-PKC and myosin light chain kinase (MLCK)-myosin light chain (MLC) in PAVECs triggered by *G. parasuis*. Our results might provide some targets to control *G. parasuis* infection.

2. Materials and methods

2.1. Bacterial strain and growth conditions

G. parasuis strain SH0165 serovar 5 was isolated from the lung of a commercially produced pig with Glässer's disease; it showed the typical disease characteristics, namely arthritis, fibrinous polyserositis, haemorrhagic pneumonia, and meningitis [16]. This strain was cultured at 37 °C in tryptic soy broth (TSB) (Difco Laboratories, USA) or on tryptic soy agar (TSA) (Difco Laboratories, USA) supplemented with 10 μg/ml nicotinamide adenine dinucleotide (NAD) (Ameresco, USA) and 10 % foetal bovine serum (FBS; Sijiqing, China) [17].

2.2. Piglets and drugs

This study was conducted in strict accordance with the recommendations of the China Regulations for the Administration of Affairs Concerning Experimental Animals 1988 and the Hubei Regulations for the Administration of Affairs Concerning Experimental Animals 2005. The protocol was approved by Hubei Province Science and Technology Department of China (permit number SYXK[ER] 2010-0029). The experiments and euthanasia procedure were approved by the Animal Care and Use Committee of Wuhan Polytechnic University, Hubei Province, China (WPU202303001). All experimental animals were euthanized at the end of the experiment.

Five 30-day-old naturally farrowed early-weaned piglets (Duroc × Landrace × large white), weighing 9–11 kg, were purchased from Wuhan Wannianqing Animal Husbandry Co., Ltd (Wuhan, China) for PAVEC isolation.

Baicalin was obtained from Sichuan Taikang Pharmaceutical Co., Ltd (China; lot number: 50802007). Before use, baicalin was dissolved and diluted in RPMI-1640 medium (Gibco, USA). MRS2578 (a P2Y6 receptor inhibitor) and probenecid (a PANX-1 inhibitor) were purchased from Selleck Chemicals (China).

AMP, ADP, ATP, UMP, UDP, and UTP were purchased from Stanford Chemicals (Lake Forest, CA, USA). Stable isotope–labelled adenosine-$^{13}C_{10}$-triphosphate (ATP-$^{13}C_{10}$), adenosine-$^{15}N_5$-monophosphate (AMP-$^{15}N_5$), and *N*-(*tert*-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA, 95 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. PAVEC isolation and culture

PAVECs were isolated and characterized as described previously [18,19]. Briefly, after treatment of the aortic lumen with 0.1 %

type I collagenase (Sigma-Aldrich) in M − 199 medium (Gibco) and penicillin-streptomycin solution (HyClone, USA) for 30 min at 37 °C, PAVECs were collected in small sheets. The cell suspension was centrifuged at 100 *g* for 30 min and then was resuspended in 5 mL of M − 199 medium supplemented with 10 % FBS (Gibco). PAVECs were cultured in a T-25 tissue culture flask (Costar, USA) at 37 °C. Their viability was evaluated with Trypan blue exclusion.

2.4. Determining the effect of MRS2578 and probenecid on PANX-1 and P2Y6 expression in G. parasuis–infected PAVECs

To determine the appropriate MRS2578 and probenecid concentration, 5×10^5 cells were seeded on culture plates and pretreated with different concentrations of MRS2578 (1–20 μM) or probenecid (25–200 μM) for 1 h. *G. parasuis* (5×10^5 colony-forming units [CFU]/mL) was added to each well and co-cultured with PAVECs for 12 h at 37 °C with 5 % CO_2. PANX-1 and P2Y6 messenger RNA (mRNA) expression was determined with reverse transcription–quantitative polymerase chain reaction (RT-qPCR) [20]. Briefly, total RNA from PAVECs was isolated with the TRIzol Reagent (Invitrogen, USA). Then complementary DNA (cDNA) was synthesized with the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Beijing, China) according to the manufacturer's instructions. The primers utilized in this study are presented in Table 1. The thermal cycling conditions were denaturation for 95 °C at 30 s, annealing at the temperature indicating for 30 s, and extension at 72 °C for 30 s. The relative expression levels were determined based on the threshold cycle (CT) method. The $2^{-\Delta\Delta CT}$ method was used to determine the fold change.

2.5. The effect of baicalin on PANX-1, P2Y6, and MLCK expression in G. parasuis–infected PAVECs

To determine the optimal baicalin concentration that affects PANX-1, P2Y6, and MLCK expression, 5×10^5 cells were seeded on the culture plates and pretreated with baicalin (25–100 μM) for 1 h. Then 5×10^5 CFU/mL *G. parasuis* was added to each well and co-cultured with PAVECs for 12 h. RNA was isolated from PAVECs as described in section 2.5 to evaluate PANX-1, P2Y6, and MLCK mRNA expression.

2.6. Establishment of a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method to determine the effect of baicalin on the release of 'find me' signals from G. parasuis–infected PAVECs

To explore the effect of baicalin on the release of 'find me' signals, 5×10^5 PAVECs were pretreated with 100 μM probenecid or 50 μM baicalin for 1 h. Then 5×10^5 CFU/mL *G. parasuis* was added to each well and co-cultured with PAVECs for 12 h. The cell supernatant were collected and stored at −80 °C until use.

The supernatant was treated with 85 % cold methanol (9:1, v/v) containing 2 μM AMP-$^{15}N_5$ and 20 μM ATP-$^{13}C_{10}$, then vortexed for 1 min. After centrifugation (13,000 *g*) at 4 °C for 10 min, the supernatant was transferred to another tube. Derivatization was initiated by adding 75 μL of MTBSTFA to 200 μL of supernatant with 85 % methanol and completed over 5 min with constant vortexing. The derivatized samples were centrifuged at 13,000 *g* for 10 min at 4 °C, and the supernatant was injected into the LC-MS/MS system for analysis.

To explore the effect of baicalin on the release of 'find me' signals, a LC-MS/MS method was established based on a previous study [21]. Briefly, chromatography of nucleotides was conducted with an UltiMate 3000 UPLC system (Thermo Fisher, San Jose, CA, USA) equipped with an Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm; 1.7 μm particle size; Waters, Milford, MA, USA). The mobile phase included A, 5 mM ammonium acetate aqueous solution, and B, 100 % acetonitrile. The column was maintained at 35 °C, and the system was set up to operate at a flow rate of 0.2 mL/min at the following linear gradient conditions: 0–5 min, 5%–30 % B; 5–10 min, 30%–95 % B; 10–13 min, 95 % B; 13–14 min, 95%–5% B; 14–20 min, 5 % B. MS spectra were acquired in the negative ionization mode with a hybrid quadrupole orbitrap mass spectrometer (Q Exactive, Thermo Fisher). The sheath gas flow rate was 25 mL/min; the auxiliary gas flow rate was 15 mL/min; the spray voltage was 3.2 kV; and the temperature of the capillary tube was 320 °C. Full scan and product ion modes with the *m/z* range of 100–900 were performed, and the quantitative data were acquired in the multiple reaction monitoring (MRM) mode. The Xcalibur version 4.0 software was used for data collection.

Table 1
Primer sequences for reverse transcription–quantitative polymerase chain reaction.

Gene name	Nucleotide sequence (5'→3')		Melting temperature (°C)	Length (base pairs)
β-actin	Forward	TGCGGGACATCAAGGAGAAG	57.4	216
	Reverse	AGTTGAAGGTGGTCTCGTGG	57.4	
P2Y6	Forward	CACCAAGACAGCCTACCT	54.9	142
	Reverse	GGTGAAGTAGAAGAGGATGG	55.4	
PANX-1	Forward	GTATCGCAAGCCTCTGAA	52.6	189
	Reverse	TTACCAGCACTATTCCTCTC	53.4	
MLCK	Forward	CCTGCTTTCATTTTGCCC	52.6	118
	Reverse	GCTGTCCGTTTCTGTGCC	57.2	
PLCβ3	Forward	CCAGCCCTGCTCATCTAC	53	150
	Reverse	AGCCTCACTCTCCCCAAT	53.6	
PKCα	Forward	ACCACTGCGGGTCCTTGC	59.5	142
	Reverse	TAGATGCGCCCCCTCTTC	57.2	

PANX-1, pannexin 1; MLCK, myosin light chain kinase; PLCβ3, phospholipase C β3; PKCα, protein kinase Cα.

2.7. Determination of the effect of baicalin on PLC-PKC and MLCK-MLC signaling pathway activation in G. parasuis–infected PAVECs with RT-qPCR

To explore whether baicalin affects activation of the PLC-PKC and MLCK-MLC signaling pathways, 5×10^5 PAVECs were plated and pretreated with MRS2578 (10 μM), probenecid (100 μM), or baicalin (50 μM) for 1 h. Next, 5×10^5 CFU/mL *G. parasuis* was added to each well and co-cultured with PAVECs for 12 h. Meanwhile, an equal amount of medium or baicalin was added to isolated cells as the control groups. The mRNA expressions of relevant genes were determined by RT-qRCR [19]. The primers are present in Table 1.

2.8. Determination of the effect of baicalin on PLC-PKC and MLCK-MLC signaling pathway activation in G. parasuis–infected PAVECs by Western blot

After co-culturing PAVECs and *G. parasuis* for 12 h, the cells were collected for Western blot [7]. Briefly, a total protein extraction kit (Beyotime Biotechnology, Shanghai, China) was used to extract protein. The protein concentration was determined with a BCA protein assay kit (Beyotime Biotechnology). Then, proteins were separated with 12 % SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with 5 % non-fat milk for 1 h at 25 °C to block nonspecific protein binding. The membrane was washed three times with TBST and then incubated with the primary antibody (Cell Signaling Technology, USA) for 10 h at 4 °C. Following washing with TBST three times, the membranes were incubated with HRP-linked goat anti-rabbit antibody (Proteintech, USA) under 25 °C for 30 min and visualized by using the Immobilon kit (Millipore, USA). Protein expression was determined with the FluorChem FC2 AIC system (Alpha Innotech, USA).

2.9. Statistical analysis

The data are presented as the mean ± standard deviation. Differences between two groups were analyzed with ANOVA. A p-value <0.05 was considered significant.

3. Results

3.1. Determination of the appropriate probenecid and MRS2578 concentrations for G. parasuis–infected PAVECs

PANX-1 mRNA expression was increased in *G. parasuis*–infected PAVECs compared with the control group ($p < 0.01$) (Fig. 1A). Treatment with 100–200 μM probenecid downregulated PANX-1 expression in PAVECs induced by *G. parasuis* compared with the infection group ($p < 0.01$) (Fig. 1A). Therefore, we used a probenecid concentration of 100 μM for the subsequent experiments.

P2Y6 mRNA expression was significantly upregulated in *G. parasuis*–infected PAVECs compared with the control group ($p < 0.01$) (Fig. 1B). Treatment with 1–20 μM MRS2578 significantly inhibited P2Y6 mRNA expression compared with the infection group (1 μM, $p < 0.05$; 5–20 μM, $p < 0.01$) (Fig. 1B). Thus, we chose 10 μM as the MRS2578 concentration for the subsequent experiments.

3.2. Determination of the concentration of baicalin on PANX-1, P2Y6, and MLCK expression in PAVECs infected by G. parasuis

We next determined the optimum baicalin concentration based on PANX-1, P2Y6, and MLCK mRNA expression. We found that 25–100 μM baicalin significantly attenuated the expression of PANX-1, P2Y6, and MLCK compared with the infection group ($p < 0.05$)

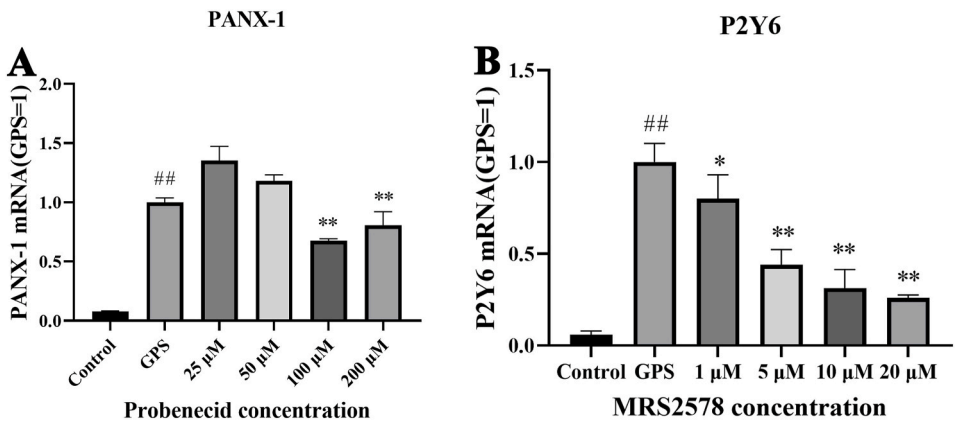

Fig. 1. The effects of probenecid on PANX-1 expression and MRS2578 on P2Y6 expression in *Glaesserella parasuis*–infected porcine aortic vascular endothelial cells (PAVECs). (A) PANX-1 messenger RNA (mRNA) expression determined with reverse transcription–quantitative polymerase chain (RT-qPCR). **(B)** P2Y6 mRNA expression determined with RT-qPCR. Each experiment was repeated at least three times. GPS, *G. parasuis*; PANX-1, pannexin 1. $^{\#\#}p < 0.01$ versus control; $^{*}p < 0.05$; $^{**}p < 0.01$.

(Fig. 2A–C). Based on these results, we chose 50 μM as the baicalin concentration for the subsequent experiments.

3.3. Baicalin inhibited G. parasuis–induced PANX-1 expression in PAVECs

PANX-1 mRNA expression was increased significantly *G. parasuis*–infected PAVECs compared with the control group ($p < 0.01$) (Fig. 3). Treatment of *G. parasuis*–infected PAVECs with MRS2578, probenecid, or MRS2578 plus probenecid significantly down-regulated PANX-1 expression compared with the infection group ($p < 0.01$) (Fig. 3). Baicalin could also inhibit PANX-1 mRNA expression in *G. parasuis*–infected PAVECs compared with the infection group ($p < 0.01$) (Fig. 3).

3.4. Baicalin reduced 'find me' signal substance release from PAVECs triggered by G. parasuis

We found that ATP, ADP, AMP, UTP, UDP, and UMP could be completely derivatized with MTBSTFA (Supplemental Fig. 1). In addition, these derivatives displayed some other characteristic fragments, including base ions, bases, and base and pentose binding ions (Supplemental Fig. 1). After stimulating PAVECs with *G. parasuis*, the concentration of ATP, ADP, AMP, UTP, UDP, and UMP was upregulated significantly compared with the control group (Table 2). Treatment of *G. parasuis*–infected PAVECs with probenecid or baicalin significantly inhibited the release of ATP, ADP, AMP, UTP, UDP, and UMP compared with the infection group (Table 2).

3.5. Baicalin inhibited P2Y6 expression in G. parasuis–infected PAVECs

P2Y6 mRNA expression increased significantly in *G. parasuis*–infected PAVECS compared with the control group ($p < 0.01$) (Fig. 4A). Treatment of *G. parasuis*–infected PAVECs with MRS2578, probenecid, MRS2578 plus probenecid, or baicalin reduced P2Y6 mRNA expression compared with the infection group ($p < 0.01$) (Fig. 4A). We also evaluated P2Y6 protein expression by Western blot. *G. parasuis* significantly increased P2Y6 expression compared with the control group ($p < 0.01$) (Fig. 4B). Treatment of *G. parasuis*–infected PAVECs with MRS2578, probenecid, MRS2578 plus probenecid, or baicalin reduced P2Y6 protein expression ($p < 0.01$) (Fig. 4B).

3.6. Baicalin inhibited G. parasuis–induced activation of the PLC-PKC signaling pathway in PAVECs

We found that co-culturing PAVECs with *G. parasuis* for 12 h could not induce PLCβ3 and PKCα protein and mRNA expression compared with the control group (Figs. 5A and 6A). Moreover, treatment with MRS2578, probenecid, MRS2578 plus probenecid, or baicalin did not significantly change PLCβ3 and PKCα protein expression compared with the infection group (Figs. 5B and 6B). However, compared with the control group, *G. parasuis* significantly upregulated phosphorylated PLCβ3 (*p*-PLCβ3) and phosphorylated PKCα (*p*-PKCα) expression in PAVECs ($p < 0.01$) (Fig. 5C, D, 6C, 6D). Treatment with MRS2578, probenecid, MRS2578 plus probenecid, or baicalin reduced *p*-PLCβ3 and *p*-PKCα expression compared with the infection group ($p < 0.05$) (Fig. 5C, D, 6C, 6D).

3.7. Baicalin inhibited G. parasuis–induced activation of the MLCK-MLC signaling pathway in PAVECs

We also evaluated whether *G. parasuis* could activate the MLCK-MLC signaling pathway in PAVECs. *G. parasuis* promoted MLCK mRNA and protein expression compared with the control group (Fig. 7A and B). Treatment of *G. parasuis*–infected PAVECs with MRS2578, probenecid, or MRS2578 plus probenecid significantly reduced MLCK mRNA and protein compared with the infection group ($p < 0.05$) (Fig. 7A and B). 50 μM baicalin could inhibit the MLCK expression compared the infection group ($p < 0.01$) (Fig. 7A and B). *G. parasuis* could not induce MLC expression in PAVECs compared with the control group (Fig. 7C). *G. parasuis* enhanced phosphorylated MLC (*p*-MLC) expression in PAVECs compared with the control group ($p < 0.01$) (Fig. 7D and E). Treatment of

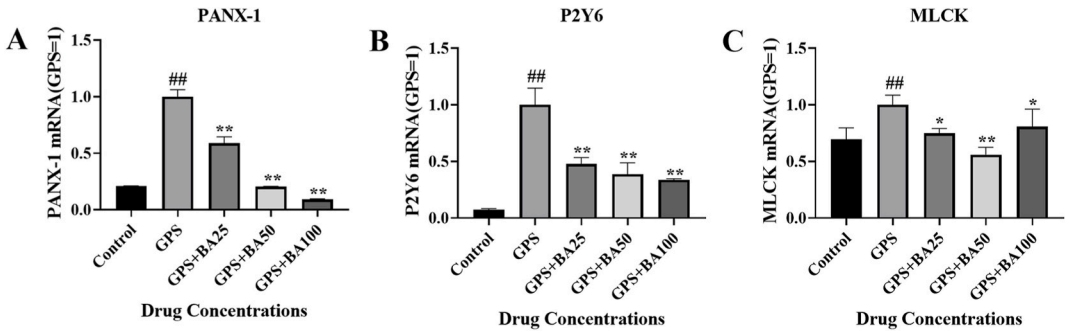

Fig. 2. The effects of baicalin on PANX-1, P2Y6, and MLCK expression in *Glaesserella parasuis*–infected porcine aortic vascular endothelial cells (PAVECs). (A) PANX-1, **(B)** P2Y6 and **(C)** MLCK messenger RNA (mRNA) expression determined with reverse transcription–quantitative polymerase chain. Each experiment was repeated at least three times. PANX-1, pannexin 1; BA, baicalin; GPS, *G. parasuis*; MLCK, myosin light chain kinase. $^{\#\#}p < 0.01$ versus control; $^{*}p < 0.05$; $^{**}p < 0.01$.

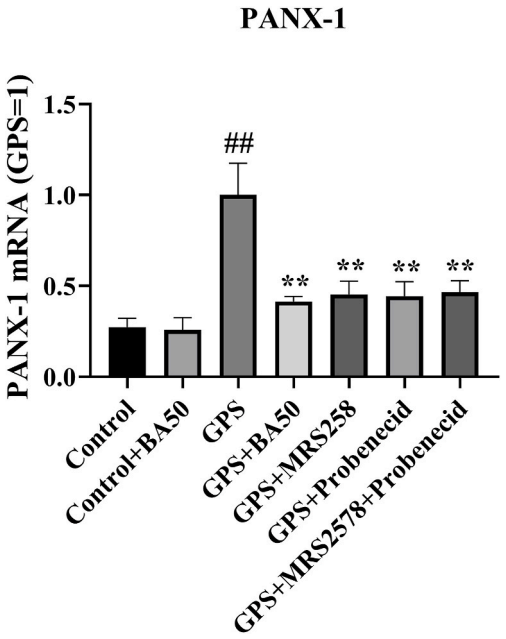

Fig. 3. The effects of baicalin on PANX-1 expression in *Glaesserella parasuis*–infected porcine aortic vascular endothelial cells (PAVECs). PAVECs were pretreated with MRS2578 (10 μM), probenecid (100 μM), MRS2578 plus probenecid, or baicalin (50 μM) for 1 h, and then co-cultured with *G. parasuis* for 12 h. Meanwhile, an equal amount of medium or baicalin was added to isolated cells for the control groups. PANX-1 messenger RNA (mRNA) expression was determined with reverse transcription–quantitative polymerase chain. Each experiment was repeated at least three times. PANX-1, pannexin 1; BA, baicalin; GPS, *G. parasuis*. $^{\#\#}p < 0.01$ versus control; $^{**}p < 0.01$.

Table 2
Nucleotide content of the analyzed samples from each group.

Analyte	Control (μM)	GPS (μM)	Baicalin (μM)	Probenecid (μM)
ATP	ND	5.09 ± 0.19	ND	ND
ADP	ND	1.70 ± 0.04	0.26 ± 0.06	ND
AMP	1.03 ± 0.09	1.31 ± 0.02	0.22 ± 0.04	0.24 ± 0.04
UTP	ND	8.59 ± 0.39	ND	ND
UDP	ND	3.83 ± 0.20	0.16 ± 0.03	0.20 ± 0.07
UMP	0.08 ± 0.03	0.38 ± 0.04	0.08 ± 0.03	0.09 ± 0.01

ADP, adenosine diphosphate; AMP, adenosine 3′-monophosphate; ATP, adenosine triphosphate; GPS, *Glaesserella parasuis*; ND, not detected; UDP, uridine diphosphate; UMP, uridine monophosphate UTP, uridine triphosphate.

G. parasuis–infected PAVECs with MRS2578, or MRS2578 plus probenecid significantly attenuated *p*-MLC expression in PAVECs compared with the infection group ($p < 0.05$) (Fig. 7D and E). 50 μM baicalin could significantly reduce the *p*-MLC expression in PAVECs ($p < 0.01$) (Fig. 7D and E).

4. Discussion

The mechanism by which *G. parasuis* induces an inflammatory response in piglets is not entirely clear and has caused great difficulties for the pig industry. Moreover, the mechanism of endothelial barrier damage in piglets remains unclear. It has been reported that bacteria infected–host cells involved in regulating innate immunity could release nucleotides as 'find-me' signals, and then display 'eat-me' signals such as phosphatidylserine and calreticulin on their surface, leading to downregulation of 'don't-eat-me' signals that protect them from phagocytosis [22]. The best-characterized 'find-me' signals are the nucleotides ATP and UTP [23]. In this study, we used a LC-MS/MS method to detect the release of ATP, ADP, AMP, UTP, UDP, and UMP from PAVECs. Our detection method has good precision and rate of recovery, and could detect lower levels of extracellular nucleotides, which provides a method for detecting the release of extracellular nucleosides.

PANX-1 is a membrane channel protein that is involved in the pathogenesis of many pathogenic bacteria and considered as a promising therapeutic target [24]. The PANX-1 channel is critical for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection [25]. Upregulation of PANX-1 enhances migration and invasion of testicular cancer cells [26]. PANX-1 downregulation reduces pro-inflammatory cytokine expression induced by LPS in reactive astrogliosis [27]. High levels of extracellular ATP released from PANX1 channels could contribute to the inflammatory response in mice [28]. In this study, we found that PAVECs could release

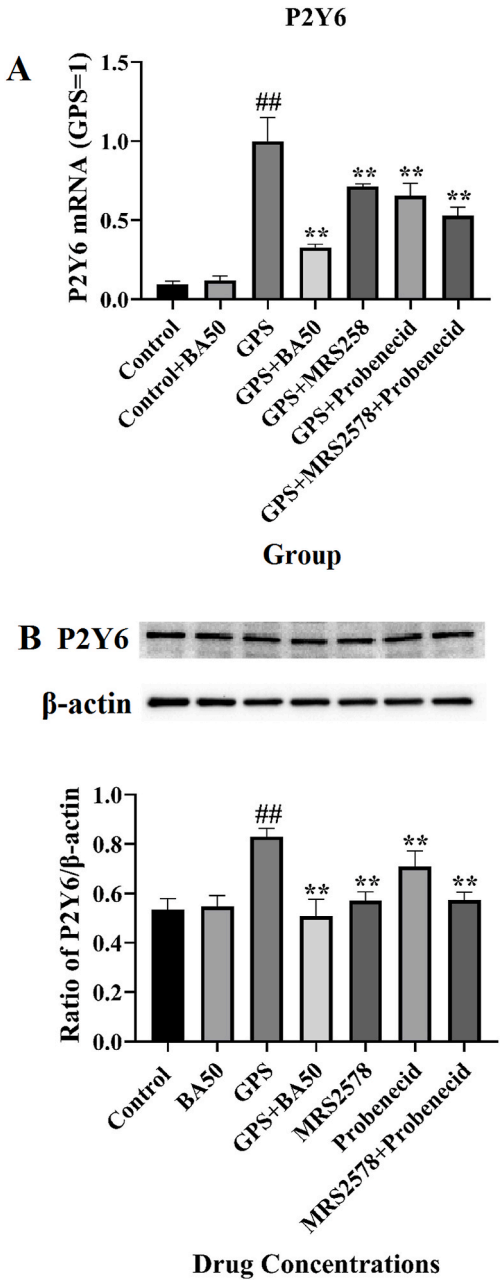

Fig. 4. The effects of baicalin on P2Y6 expression in *Glaesserella parasuis*–infected porcine aortic vascular endothelial cells (PAVECs). (A) P2Y6 messenger RNA (mRNA) expression determined with reverse transcription–quantitative polymerase chain. **(B)** P2Y6 protein expression determined with Western blot. Each experiment was repeated at least three times. BA, baicalin; GPS, *G. parasuis*. $^{\#\#}p < 0.01$ versus control; $^{**}p < 0.01$.

ATP to the extracellular medium via PANX-1 channels. Thus, we speculate that high extracellular ATP levels released by PANX-1 channels contribute to the inflammatory response in *G. parasuis*–infected PAVECs. However, the specific mechanism requires further investigation.

Metabotropic purinergic (P2Y) receptors can recognize extracellular ATP/ADP, which might propagate via chemotaxis and inflammation [29]. The P2Y receptors send 'find me' or 'eat me' signals to microglia to migrate towards or phagocytize cellular debris [30]. In addition, P2Y receptors activated by ATP can trigger inflammation in a variety of diseases [31]. Inhibition of P2Y6 expression blocks the inflammatory response of Kupffer cells and ameliorates alcoholic steatohepatitis in mice [32]. In the present study, baicalin inhibited the *G. parasuis*–induced increase in P2Y6 expression in PAVECs. Based on the inhibitory effect of baicalin on P2Y6 expression and the function of this receptor in inflammation, the P2Y6 receptor could be considered a target for inflammatory responses treatment triggered by *G. parasuis*.

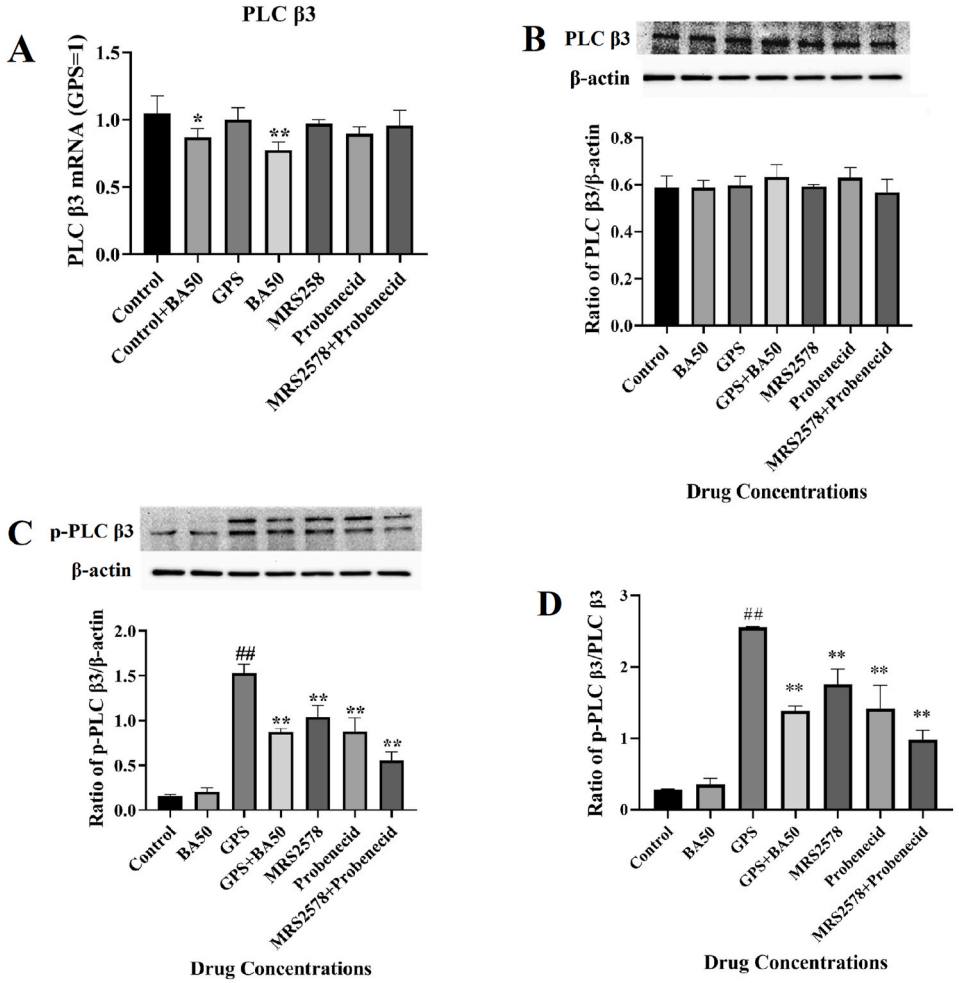

Fig. 5. The effects of baicalin on PLC β3 expression in *Glaesserella parasuis*–infected porcine aortic vascular endothelial cells (PAVECs). (A) PLC messenger RNA (mRNA) expression determined with reverse transcription–quantitative polymerase chain. **(B)** PLC β3 protein expression determine with Western blot. **(C)** Phosphorylated PLC β3 (*p*-PLC β3) expression determine with Western blot. **(D)** Ratio of *p*-PLC β3/PLC β3. Each experiment was repeated at least three times. BA, baicalin; GPS, *G. parasuis*; PLC β3, phospholipase C β3. $^{\#\#}p < 0.01$ versus control; $^{*}p < 0.05$; $^{**}p < 0.01$.

Previous research has shown that the P2Y6 receptor is specifically activated by UDP, leading to PLC-PKC signaling pathway activation [10]. This signaling pathway plays an important role in regulating inflammation. Studies have indicated that infection with group B streptococci activates the PLC-PKC signaling pathway in brain endothelial cells, contributing to macropinocytosis regulation [33]. Thrombospondin-2 promotes osteosarcoma metastasis via PLC/PKC pathway activation [34]. Octadecaneuropeptide can trigger differentiation of N2a cells through the PLC/PKC signaling pathway [35]. We found that *G. parasuis* promoted activation of the PLC/PKC signaling pathway, and baicalin inhibited this activation. Thus, we infer that baicalin might reduce PLC/PKC signaling to attenuate inflammation elicited by *G. parasuis*.

In the present study, baicalin inhibited *G. parasuis*–induced MLCK/MLC signaling pathway activation in PAVECs. In a previous study, the researchers reported that LPS increases human brain microvascular endothelial cell (HBEC) permeability and occludin expression, changes mediated by the MLC/MLCK signaling pathway [36]. Berberine improves intestinal epithelial tight junctions by downregulating the abnormal activation of tumour necrosis factor alpha (TNF-α)–NF-κB–MLCK pathway [37]. Total flavonoid extract from hawthorn attenuates TNF-α-induced intestinal epithelial barrier deficit through the MLCK-MLC signaling pathway [38]. In addition, *Lycium barbarum* polysaccharides ameliorate intestinal barrier dysfunction and inflammation through the MLCK-MLC signaling pathway in Caco-2 cells [39]. In a previous study, we showed that baicalin protects against vascular tight junction damage induced by *G. parasuis* by reducing activation of the PKC and MLCK/MLC signaling pathways in a piglet model [1]. Thus, we infer that baicalin inhibits the MLCK/MLC signaling pathway activation to relieve the vascular barrier damage induced by *G. parasuis*.

5. Conclusion

Taken together, our results demonstrated that *G. parasuis* could induce PANX-1 and P2Y7 expression; promote ATP, ADP, AMP,

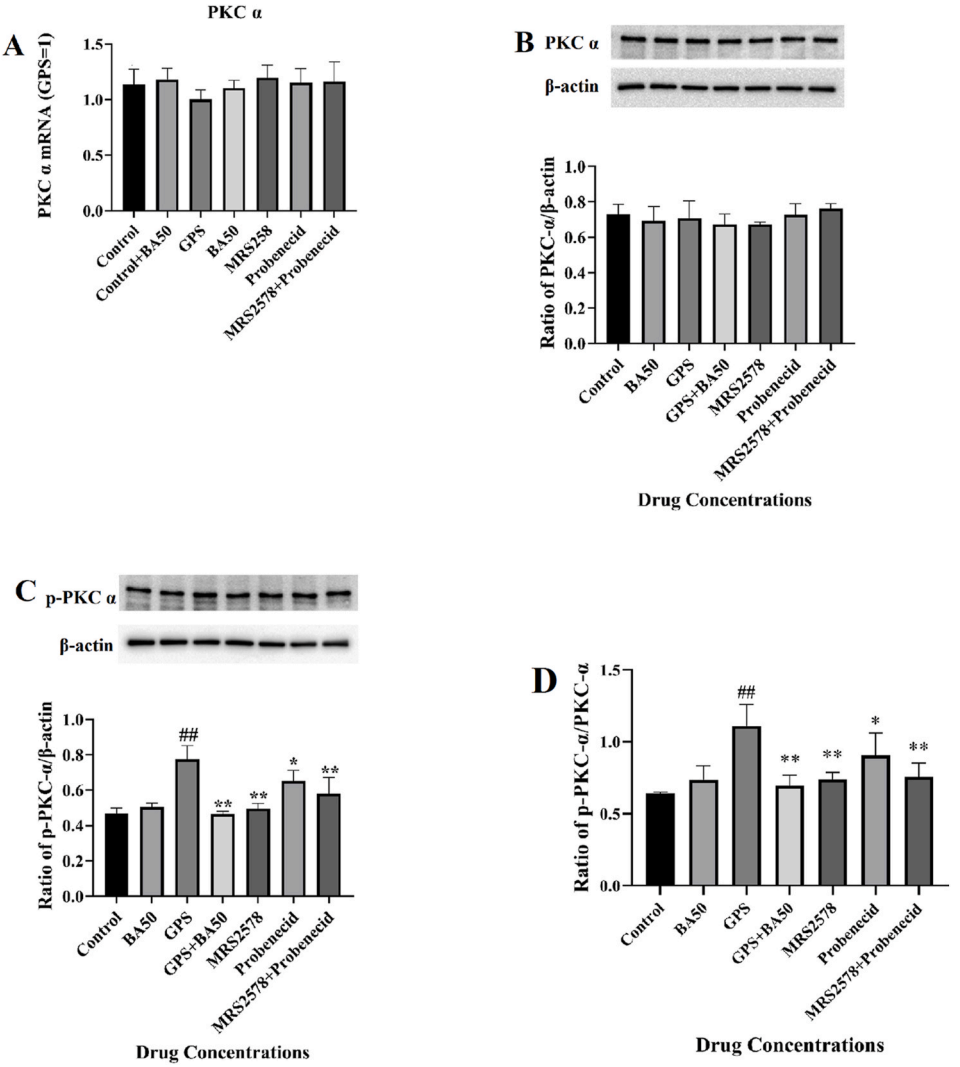

Fig. 6. The effects of baicalin on PKC expression in *Glaesserella parasuis*–infected porcine aortic vascular endothelial cells (PAVECs). (A) PKCα messenger RNA (mRNA) expression determined with reverse transcription–quantitative polymerase chain. **(B)** PKCα protein expression determined with Western blot. **(C)** Phosphorylated PKCα (*p*-PKCα) expression determined with Western blot. Each experiment was repeated at least three times. **(D)** Ratio of *p*-PKCα/PKCα. BA, baicalin; GPS: *G. parasuis*; PKCα: protein kinase C α. $^{\#\#}p < 0.01$ versus control; $^{*}p < 0.05$; $^{**}p < 0.01$.

UTP, UDP, and UMP release; and activate the PLC/PKC and MLCK/MLC signaling pathways in PAVECs. Baicalin could attenuate these *G. parasuis*–induced changes in PAVECs. PANX-1, P2Y7, and 'find me' signals such as ATP, ADP, AMP, UTP, UDP, and UMP might serve as targets for treatment of inflammation in pigs caused by *G. parasuis*.

Ethics statement

The animal experiments and euthanasia procedure were approved by the Animal Care and Use Committee of Wuhan Polytechnic University, Hubei Province, China (WPU202303001) and the Hubei Province Science and Technology Department of China (permit number SYXK[ER] 2010-0029).

Consent for publication

Not applicable.

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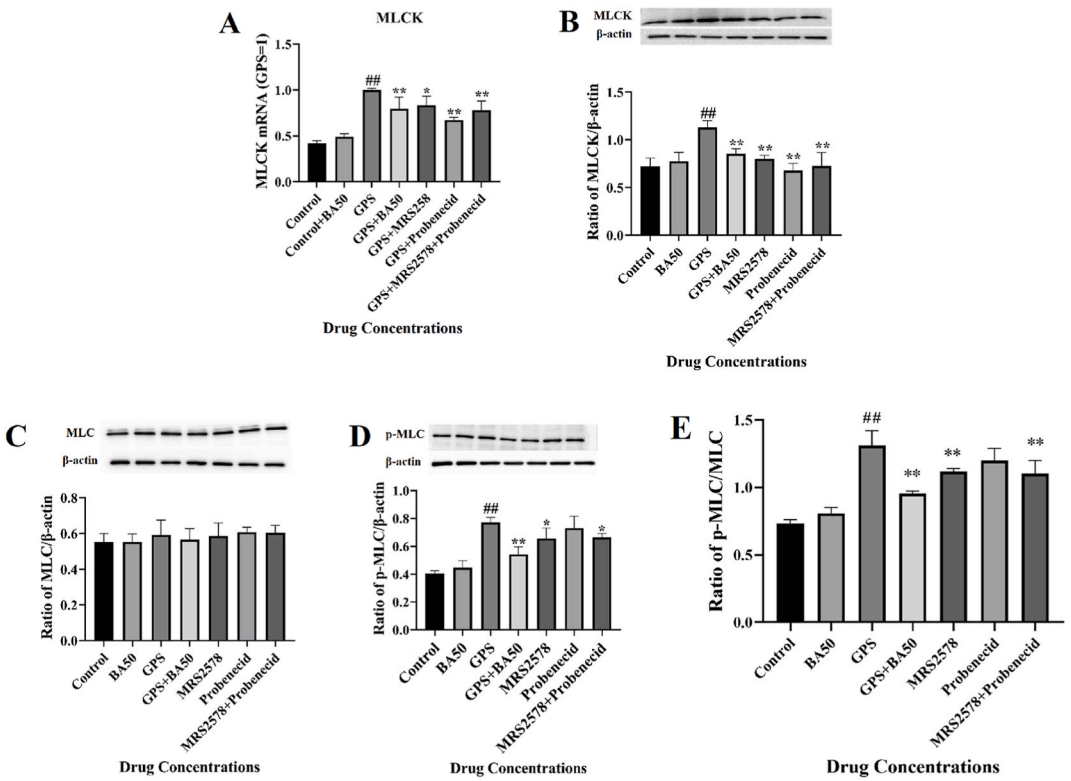

Fig. 7. The effects of baicalin on the MLCK-MLC signaling pathway in *G. parasuis*–infected porcine aortic vascular endothelial cells (PAVECs). (A) MLCK messenger RNA (mRNA) expression determined with reverse transcription–quantitative polymerase chain. **(B)** MLCK protein expression determined with Western blot. **(C)** MLC and **(D)** phosphorylated MLC (*p*-MLC) expression were determined with Western blot. **(E)** Ratio of *p*-MLC/MLC. Each experiment was repeated at least three times. BA, baicalin; GPS, *G. parasuis*; MLC, myosin light chain; MLCK, myosin light chain kinase. $^{\#\#}p < 0.01$ versus control; $^{*}p < 0.05$; $^{**}p < 0.01$.

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Data availability statement

Data included in article/supp. Material in article.

CRediT authorship contribution statement

Shulin Fu: Writing – review & editing, Writing – original draft, Formal analysis. **Xinyue Tian:** Formal analysis. **Chun Peng:** Formal analysis. **Dan Zhang:** Formal analysis, Data curation. **Linglu Zhou:** Formal analysis. **Yuzhen Yuan:** Formal analysis. **Jing He:** Formal analysis. **Ling Guo:** Funding acquisition, Formal analysis. **Yinsheng Qiu:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis. **Chun Ye:** Formal analysis. **Yu Liu:** Formal analysis. **Bingbing Zong:** Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23632.

Abbreviations

G parasuis, Glaesserella parasuis

RAGE	receptor of advanced glycation endproducts
MAPK	mitogen-activated protein kinase
HMGB1	high mobility group protein B1
UDP	uridine diphosphate
PKC	protein kinase C
PLC	phospholipase C
PANX-1	pannexin 1
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AMP	adenosine 3′-monophosphate
UTP	uridine triphosphate
UMP	uridine monophosphate
MLCK	myosin light chain kinase
MLC	myosin light chain
PAVECs	porcine aortic vascular endothelial cells

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