Characterization of porcine NLRP3 inflammasome activation and its upstream mechanism

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Abstract Inflammasomes, which are intracellular sensors of endogenous or exogenous danger signals, activate caspase-1, resulting in interleukin (IL)-1β maturation. Although most studies on inflammasomes have been performed in human and/or mouse-derived macrophages, porcine inflammasome activation has not been elucidated even though pigs are considered one of the best animal models for translational and preclinical investigations. In this study, we optimized detection of porcine IL-1β secretion, which is the most well established indicator of inflammasome activation, and compared inflammasome activation between miniature and domestic pigs as well as between porcine and murine macrophages. In our results, anti-sera against murine IL-1β had higher affinity to porcine IL-1β than anti-sera against human IL-1β, even though the amino acid sequence of porcine IL-1β was more similar to that of human IL-1β. In addition, there was no significant difference in inflammasome activation between miniature and domestic pigs. Furthermore, well established inflammasome triggers (ATP, nigericin, and crystals) in humans and mice had similar effects on porcine NLRP3 inflammasome activation. We further elucidated the upstream signaling pathway of porcine inflammasome activation using pharmacological inhibitors. Similar to the mechanisms of inflammasome activation in humans and mice, potassium efflux and reactive oxygen species generation were confirmed as key pathways in porcine inflammasome activation. Thus, inflammasome activation in pigs is not different from that in humans or mice.

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Introduction

Inflammation of the innate immune system as the first line of host defense against pathogenic microorganisms is an acute response to infection and tissue damage (Schroder et al. 2010). Membrane-associated Toll-like receptors (TLRs), transmembrane c-type lectin receptors (CLRs), cytosolic nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs), retinoic acid-inducible gene (RIG)-like helicase receptors, and absent in melanoma (AIM)-like receptors comprise the five main pattern recognition receptor families. TLR activation triggers different signaling cascades, resulting in NF-κB activation as well as synthesis of various pro-inflammatory mediators and cytokines (Medzhitov 2001). Of the pro-inflammatory cytokines, interleukin (IL)-1β is an important inflammatory mediator that is generated at sites of injury or immunological challenge, coordinating diverse programs such as cellular recruitment to sites of infection or injury as well as regulation of sleep, appetite, and body temperature (Dinarello et al. 2010). Expression of the inactive pro-form of IL-1β is induced by pro-inflammatory stimuli, whereas its maturation and release are controlled by inflammasomes. Inflammasomes are multi-protein complexes that operate as platforms for the activation of caspase-1, and they consist of one of several NLR and PYHIN proteins, including NLRP1, NLRP3, NLRC4, and AIM2 (Rathinam et al. 2012). Inflammasomes are sensors of endogenous or exogenous pathogen-associated or damage-associated molecular patterns, which govern cleavage of effector pro-inflammatory cytokines such as pro-IL-1β. Inflammasomes have been found to regulate other important aspects of

inflammation and tissue repair such as pyroptosis, a form of cell death (Lamkanfi and Dixit 2011; Lamkanfi 2011).

Since most inflammasome data have been obtained using human or mouse myeloid cells, porcine inflammasome activation remains poorly described. Pigs are considered one of the major animal species used in translational research and are established as an alternative to dogs and monkeys as the non-rodent of choice in preclinical research (Swindle et al. 2012). Numerous technical procedures for using pigs in translational and preclinical research have been developed, and many reports on the anatomy, physiology, and pathology of pigs are also available (Lloyd-Jones et al. 2010; Swindle et al. 1988). Additionally, pigs are preferentially considered as a model for surgical training, including interventional catheter techniques, complex trauma procedures, and endoscopic procedures. Lastly, pigs are the most commonly used preclinical animal model for testing medical devices and techniques, as the US Food and Drug Administration has accepted data from pigs (Swindle et al. 2012). Collectively, this provides our rationale for studying inflammasome activation in a porcine model. Hence, we optimized and compared IL-1β secretion resulting from inflammasome activation in porcine peripheral blood macrophages with that in murine macrophages. In addition, we further elucidated the upstream pathway of porcine inflammasome activation using pharmacological inhibitors.

Materials and methods

Preparation of porcine monocytes and murine macrophages

Unless otherwise indicated, all materials for cell culture were obtained from PAA Laboratories (GE Healthcare Bio-Sciences Co., NJ, USA).

Porcine peripheral blood monocytes (PBMCs) were isolated using Lymphocyte Separation Medium (LSM) from freshly drawn peripheral venous blood obtained from miniature or domestic pigs. The blood of miniature pigs were collected before the previously reported (Kwak et al. 2013). Briefly, EDTA (1.5 mg per ml of blood; Sigma-Aldrich Co., MO, USA) treated blood (10 ml) was diluted with equal parts of PBS and carefully poured 10 ml of LSM on the bottom of a centrifuge tube (SPL Life Science Co., Gyeonggi-do, Republic of Korea). The tube was centrifuged at 500×g at room temperature for 30 min to create a blood-LSM interphase. The mononuclear cell layer was collected into new tube and diluted with 3 volumes of PBS. The tube was centrifuged at 500×g at room temperature for 10 min. To discard RBC contamination, the cell pellet was further treated with RBC lysis buffer (iNtRON Biotechnology, Seongnam-si, Korea).

For preparing murine bone marrow-derived macrophages (BMDMs), C57BL/6 mice (6- to 8-weeks-old) were purchased from Narabio Co. (Seoul, Republic of Korea). BMDMs were obtained by differentiating bone marrow progenitors from the tibia and femur using L929-cell conditioned medium (LCCM) as a source of granulocyte/macrophage colony-stimulating factor (Englen et al. 1995; Ahn et al. 2013; Kim et al. 2014). The progenitors were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS), 30 % LCCM, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were seeded in non-tissue culture-treated Petri dishes (SPL Life Science Co.) and incubated at 37 °C in a 5 % CO_2 atmosphere for 7 days.

Inflammasome activation or inhibition

PBMCs (2.0×10^6 cells per well) or BMDMs (1.0×10^6 cells per well) were plated in 12-well plates (SPL Life Science Co.) and primed with 10 μg/ml of lipopolysaccharide (LPS; Cat. No. L4130, Sigma-Aldrich Co.) in RPMI 1640 containing 10 % FBS and antibiotics for 3 h. After LPS priming, BMDMs were subjected to the following activation step for 1 or 3 h. For NLRP3 inflammasome activation, medium was replaced with RPMI 1640 medium supplemented with ATP for 1 h (InvivoGen, CA, USA), nigericin for 1 h (NG; Tocris Bioscience, Bristol, UK), calcium pyrophosphate dihydrate for 1 h (CPPD; InvivoGen), or aluminum potassium sulfate for 3 h (Alum; 200 μg/ml; Daejeung Chemicals & Materials Co., Gyeonggi-do, Republic of Korea). To determine the inhibitory effect of glibenclamide (Santa Cruz Biotechnology, CA, USA), KCl (Biosesang, Seoul, Republic of Korea), Z-VAD-FMK (R&D Systems, MN, USA), or diphenyleneiodonium (DPI; Tocris Bioscience) on NLRP3 inflammasome activation, chemicals were co-treated with ATP (2 mM) for 1 h. Cellular supernatant (Sup) and lysate (Lys) were collected for further analysis.

Western blot analysis

Sup and Lys samples were separated by SDS-PAGE (10 or 16 %) and then blotted onto a polyvinylidene difluoride membrane (Pall Co., NY, USA). Immunoblots were probed overnight at 4 °C with anti-human IL-1β antibody (AF-201-NA, R&D Systems), anti-mouse IL-1β antibody (AF-401-NA, R&D Systems), or anti-actin antibody (sc-1615, Santa Cruz Biotechnology). The membranes were further probed with HRP-conjugated 2nd anti-sera (sc-2020, Santa Cruz Biotechnology) and visualized using Power-Opti ECL™ solution (BioNote Co., Gyeonggi-do, Republic of Korea) and a cooled CCD camera system (AE-9150 EZ-Capture II, ATTO technology, Tokyo, Japan).

Results

Optimization to detect porcine IL-1β maturation

Classical detection methods such as ELISA may induce false positive results since all available anti-sera for detection of IL-

1β cannot distinguish the mature form (p17) from pro-IL-1β (p35). Therefore, researchers studying inflammasome activation prefer Western blotting, which can differentiate between the two IL-1β forms based on size. To select the proper anti-sera for porcine IL-1β detection, we tested two commonly used anti-sera against human or mouse IL-1β. As shown in Fig. 1a and b, antibody against murine IL-1β was better than antibody against human IL-1β at recognizing porcine pro-IL-1β. However, the amino acid sequence of porcine pro-IL-1β was more similar to that of human pro-IL-1β than to that of mouse pro-IL-1β when we analyzed sequence identity and similarity between human (GeneBank ID: NP_000567) and porcine IL-1β (NP_999220) or mouse (NP_032387) and porcine IL-1β using the EMBL-EBI web-tool (http://www.ebi.ac.uk/Tools). The identity and similarity between human and porcine IL-1β were 60 % (162/270) and 75.3 % (203/270), respectively, whereas the identity and similarity between mouse and porcine IL-1β were 59.9 % (163/272) and 73.9 % (201/272), respectively. Porcine pro-IL-1β differed from the human and mouse forms upon comparison of the pro-IL-1β amino acid sequences of all three species in the phylogenic tree (Fig. 1c). Multiple sequence alignment is presented in (Fig. 1d). Taken together, the amino acid sequence of porcine pro-IL-1β was similar with that of human type, and mouse IL-1β antibody showed much higher affinity to porcine IL-1β compared to human antibody.

Characterization and comparison of porcine inflammasome activation between miniature and domestic pigs

We next investigated whether or not the selected anti-sera recognize the mature form of porcine IL-1β (p17) in the cellular supernatant (Sup). In addition, we compared NLRP3 inflammasome activation between miniature and domestic pigs. Porcine PBMCs and murine BMDMs were primed with LPS, resulting in upregulation of pro-IL-1β via TLR4 signaling (Palsson-McDermott and O'Neill 2004), and then treated with ATP to induce NLRP3 inflammasome activation (Mariathasan et al. 2006). As shown in Fig. 2, mouse IL-1β anti-sera successfully recognized porcine IL-1β (P17, mature form) following ATP-mediated NLRP3 inflammasome activation. Moreover, the pattern of NLRP3 inflammasome activation between miniature and domestic pig was not different.

Effect of ATP and nigericin on porcine NLRP3 inflammasome activation

To further elucidate NLRP3 inflammasome activation, one of the most well characterized inflammasomes (Wen et al. 2012; Strowig et al. 2012), we treated ATP or nigericin to porcine PBMCs. Both activators mediate NLRP3 inflammasome activation through induction of potassium efflux (Munoz-Planillo et al.

Fig. 1 Detection of IL-1β secretion in porcine macrophages. a Human or porcine PBMCs, or mouse BMDMs, were primed with LPS (10 μg/ml) for 3 h to induce pro-IL-1β, and the cellular lysate was immunoblotted with the indicated anti-sera. b Mouse BMDMs or porcine PBMCs were primed with/without LPS for 3 h, and the expression of pro-IL-1β in the lysate were detected by anti-mouse IL-1β serum. c Phylogenic tree of NLRP3 amino acid sequences. The unrooted tree was built using the neighbor-joining method based on alignment of NLRP3 amino acid sequences. Number indicates the divergence time. d Alignment of amino acid sequences of human, porcine, and murine pro-IL-1β. Numbers indicate amino acid positions. Identical amino acid residues are marked with a *box shading* code. Gap was introduced to optimize alignment
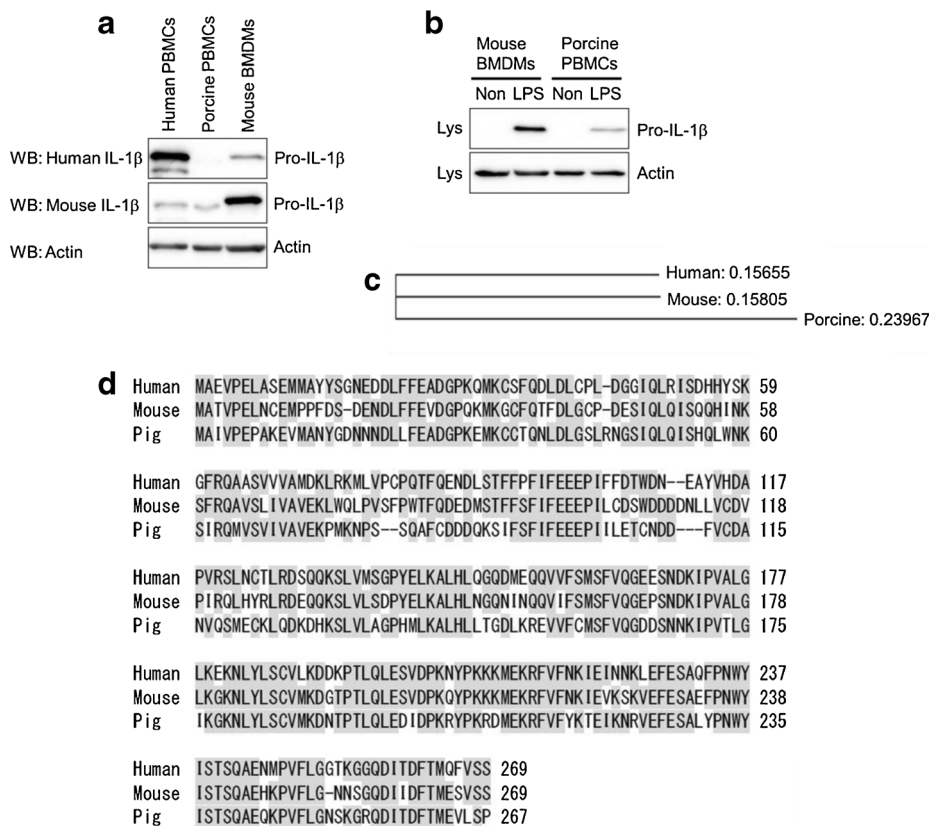
Fig. 2 Characterization and comparison of NLRP3 inflammasome between miniature and domestic pigs. PBMCs were isolated from miniature or domestic pigs. PBMCs and mouse BMDMs were primed with LPS (10 μg/ml) for 3 h and then treated with ATP for 1 h. Cellular supernatants (*Sup*) and lysate (*Lys*) were analyzed for IL-1β (p17), pro-IL-1β, and actin expression as indicated. All immunoblotting experiments were repeated independently at least three times
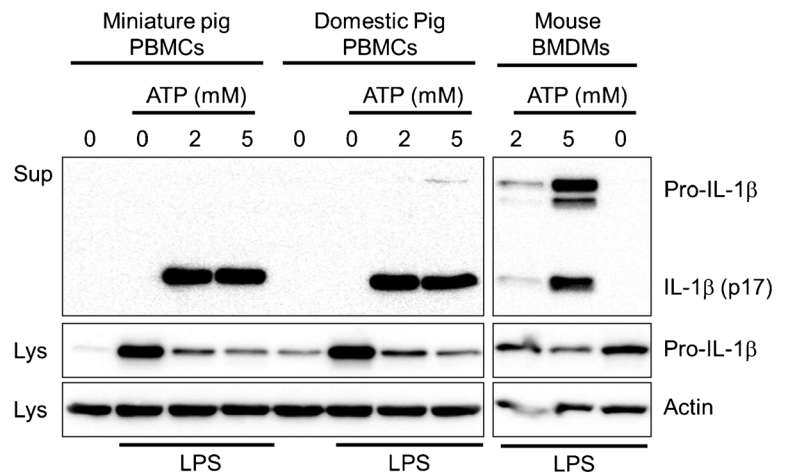
2013). To determine the dose-dependent effect of ATP on porcine NLRP3 inflammasome activation, we treated serial dosages of ATP to porcine PBMCs or murine BMDMS and then measured IL-1β secretion as an indicator of inflammasome activation (Fig. 3a). Porcine PBMCs highly secreted IL-1β in response to 1 mM ATP, whereas PBMCs treated with 4 mM ATP completely consumed pro-IL-1β in the cellular lysate. On the other hand, mouse BMDMs secreted IL-1β after 2.5 mM ATP treatment, whereas BMDMs treated with 10 mM ATP consumed pro-IL-1β in the lysate. In addition, we applied various doses of nigericin to porcine PBMCs and mouse BMDMs after LPS priming, followed by comparison of IL-1β secretion between species (Fig. 3b). Porcine PBMCs produced mature IL-1β in response to a very low concentration of nigericin

(around 0.31 μM), and secretion became saturated at around 10 μM nigericin. However, mouse BMDMs only secreted IL-1β in response to greater than 40 μM nigericin treatment. Similar to ATP, porcine NLRP3 inflammasome activation was more sensitive to nigericin treatment compared to other species. Thus, ATP- or nigericin-mediated NLRP3 inflammasome activation in porcine PBMCs was more sensitive compared to that in mouse BMDMs, although the number of porcine PBMCs was twice that of BMDMs.

Effect of crystals on porcine NLRP3 inflammasome activation

The NLRP3 inflammasome is also activated by crystalline particles such as amyloid fibrils, aluminum adjuvant (alum), silica, asbestos, monosodium uric acids, calcium

Fig. 3 Effect of ATP and nigericin on porcine NLRP3 inflammasome activation. Mouse BMDMs or porcine PBMCs were primed with LPS (10 μg/ml) for 3 h and then treated with ATP (**a**) or nigericin (**b**) for 1 h. Cellular supernatants (*Sup*) and lysate (*Lys*) were analyzed for IL-1β (p17), pro-IL-1β, and actin expression as indicated. All experiments were repeated independently at least three times
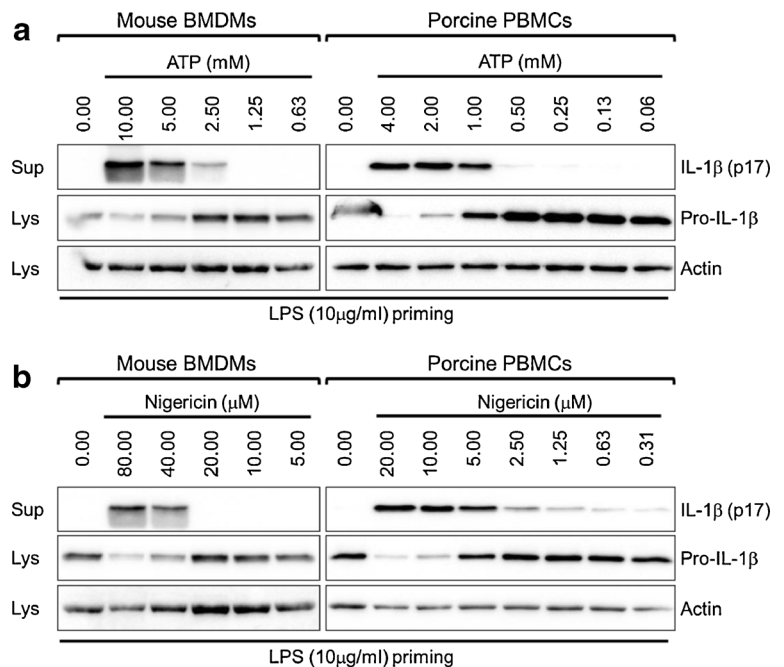
pyrophosphate dehydrate (CPPD), and nanomaterials (Tschopp and Schroder 2010). We determined and compared the effects of CPPD and alum, two well known crystals, on NLRP3 inflammasome activation between porcine PBMCs and mouse BMDMs. As seen in Fig. 4a, an increasing dosage of CPPD, which is the etiological agent of the joint acute inflammatory disease pseudogout (Martinon et al. 2006), was applied to LPS-primed PBMCs or BMDMs, followed by measurement of IL-1β secretion. Similar to ATP or nigericin, CPPD-mediated IL-1β secretion in porcine PBMCs was predominant compared to that in mouse BMDMs. Specifically, greater than 200 μg/ml of CPPD was required to trigger IL-1β secretion in mouse BMDMs while less than 10 μg/ml of CPPD induced IL-1β secretion in porcine PBMCs. Alum, which is a commonly used vaccine adjuvant known to activate caspase-1 as well as induce IL-1β and IL-18 secretion (Li et al. 2008), triggered IL-1β maturation via NLRP3 inflammasome activation in both porcine and mouse cells (Fig. 4b). In contrast to CPPD, the sensitivity of porcine PBMCs to alum was much lower than that of mouse BMDMs.

Potassium efflux and ROS generation are common triggers of porcine NLRP3 inflammasome activation

To further confirm the upstream molecular pathway of porcine NLRP3 inflammasome activation, we applied several inhibitors of NLRP3 inflammasome activation. Firstly, we co-treated glibenclamide or high KCl solution, which are inhibitors of potassium efflux (Laliberte et al. 1999; Perregaux and Gabel 1994), with ATP to mouse BMDMs, resulting in dose-dependent attenuation of IL-1β secretion (Fig. 5a). High doses of glibenclamide (150 μM) and KCl (50 mM) were further treated to LPS-primed porcine PBMCs, resulting in ATP-mediated IL-1β secretion (Fig. 5b). Similar to mouse BMDMs, glibenclamide and KCl inhibited ATP-induced porcine NLRP3 inflammasome activation. Moreover, we investigated the role of reactive oxygen species (ROS) in porcine inflammasome activation. For this, we tested the effect of an ROS scavenger, diphenyleneiodonium (DPI), on inflammasome activation in response to ATP treatment. NLRP3 inflammasome activation in mouse cells was attenuated by DPI, as evidenced by reduced ATP-mediated IL-1β secretion (Fig. 5a). Similar inhibitory effects for DPI were observed in porcine cells (Fig. 5b). As a control, we treated Z-VAD-FMK, a caspase inhibitor, to ATP-treated murine and porcine macrophages and observed complete abrogation of IL-1β secretion in both cell types. Taken together, potassium efflux and ROS generation are common triggers of porcine NLRP3 inflammasome activation.

Discussion

In the current study, we characterized the porcine NLRP3 inflammasome using well established triggers and inhibitors in human and mouse macrophages. That is, the activation pattern of porcine NLRP3 inflammasome was not significantly different from that of mouse NLRP3 inflammasome, whereas sensitivities to each trigger did vary. In addition, the

Fig. 4 Effect of crystals on porcine NLRP3 inflammasome activation. Mouse BMDMs or porcine PBMCs were primed with LPS (10 μg/ml) for 3 h and further treated with CPPD for 1 h (a) or Alum for 3 h (d). Cellular supernatants (*Sup*) and lysate (*Lys*) were analyzed for IL-1β (p17), pro-IL-1β, and actin expression as indicated. All experiments were repeated independently at least three times
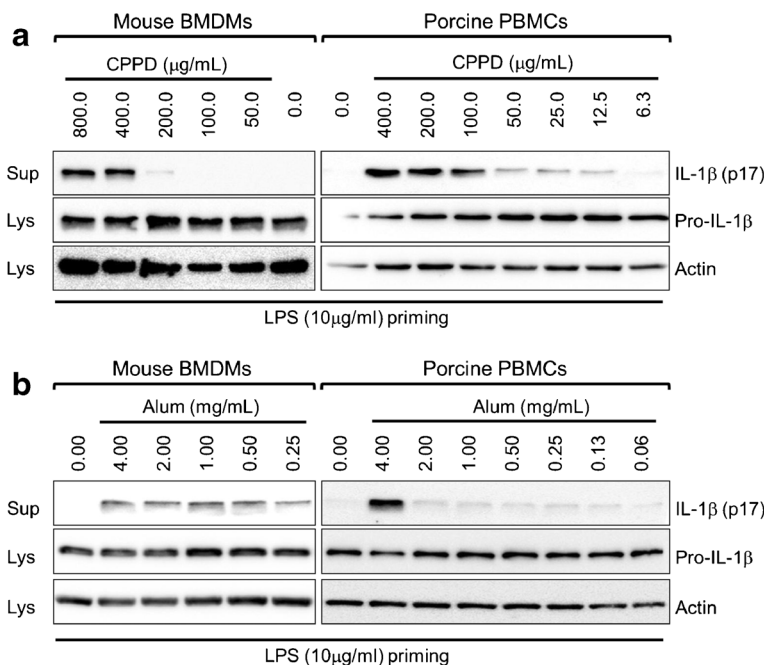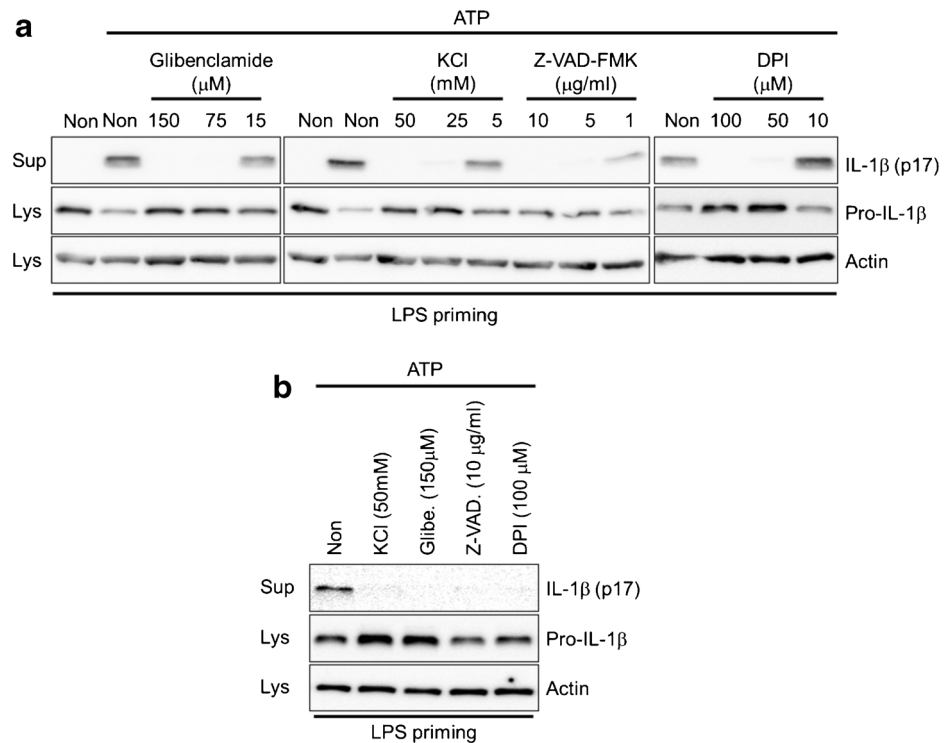
Fig. 5 Potassium efflux and ROS generation induce porcine NLRP3 inflammasome activation. **a** Murine BMDMs were primed with LPS for 3 h and then treated with ATP (2 mM) and the indicated chemicals for 1 h. Cellular supernatants (*Sup*) and lysate (*Lys*) were analyzed for IL-1β (p17), pro-IL-1β, and actin expression as indicated. **b** Porcine PBMCs were primed with LPS for 3 h and then treated with ATP (2 mM) and the indicated chemicals for 1 h. Sup and Lys were immunoblotted using the indicated anti-sera. *Glibe*, glibenclamide; *Z-VAD*, Z-VAD-FMK. All experiments were repeated independently at least three times

upstream mechanism of porcine NLRP3 inflammasome activation was shown to be associated with potassium efflux and ROS generation, which is similar to human and mouse NLRP3 inflammasome activation. Taken together, the porcine NLRP3 inflammasome is functionally similar to its human and murine counterparts. To assess inflammasome activation, we performed immunoblotting to detect IL-1β secretion in the cellular supernatant since both the pro- and active forms of IL-1β, pro-IL-1β and IL-1β (p17), are released by macrophages and ELIZA cannot distinguish between them. Therefore, most inflammasome studies chose to measure IL-1β secretion in the supernatant (Martinon and Tschopp 2004; Mariathasan et al. 2006; Martinon et al. 2006; Hornung et al. 2009; Zhao et al. 2011; Zhou et al. 2011; Lee et al. 2012).

The NLRP3 inflammasome is one of the most well characterized inflammasomes due to its role as a key component in infectious and metabolic diseases (Wen et al. 2012; Strowig et al. 2012). In the current study, we determined NLRP3 inflammasome activation in porcine PBMCs using several NLRP3 activators, which are well known in human and murine models (Pelegrin and Surprenant 2006; Mariathasan et al. 2006; Hornung et al. 2008). Firstly, we selected ATP to induce porcine NLRP3 inflammasome activation. ATP is a well known NLRP3 inflammasome activator (Mariathasan et al. 2006; Locovei et al. 2007) and triggers opening of the non-selective cation channel of purinergic P2X7 receptor (Pelegrin and Surprenant 2006). Activation of P2X7 receptor results in potassium efflux, which is necessary for post-translational maturation of IL-1β (Qu et al. 2007). In the

present study, ATP-mediated NLRP3 inflammasome activation in porcine PBMCs was more sensitive than that in mouse BMDMs, although the number of porcine PBMCs was twice that of BMDMs. Another well known NLRP3 inflammasome activator is nigericin, a microbial toxin derived from *Streptomyces hygroscopicus* that acts as a potassium ionophore. Release of IL-1β in response to nigericin has been demonstrated to be NLRP3-dependent (Mariathasan et al. 2006). Similar to ATP, nigericin induces a net decrease in intracellular potassium levels (Perregaux and Gabel 1994), and it requires pannexin-1 signaling to induce caspase-1 maturation as well as IL-1β processing and release (Pelegrin and Surprenant 2006). Similar to ATP, the porcine NLRP3 inflammasome was more sensitively activated by nigericin.

Crystal-induced phagosomal destabilization as well as cytosolic release of lysosomal cathepsin drive NLRP3 inflammasome activation (Hornung et al. 2008). Phagocytosis of crystalline and particulate molecules may cause damage to the lysosomal membrane, which consequently leads to leakage of lysosomal cathepsins into the cytosol. Cathepsin B-mediated processing of cytosolic factors is suggested to act upstream of NLRP3 activation by silica, alum, and amyloid-β fibrils (Hornung et al. 2008; Halle et al. 2008). Cytosolic release of cathepsin B has also been implicated in caspase-1 activation by nigericin (Hentze et al. 2003), which suggests a unifying mechanism for NLRP3 activation by both particulate and non-particulate stimuli. However, the previous observation that NLRP3

inflammasome activation is not affected in cathepsin B-deficient macrophages exposed to malaria hemozoin, uric acid crystals, silica, and alum suggests redundancy with other cathepsins or the presence of other pathways leading to NLRP3 activation (Dostert et al. 2009; Tschopp and Schroder 2010).

It was recently shown that intracellular potassium depletion acting directly upstream of the NLRP3 inflammasome is necessary and sufficient for activation (Munoz-Planillo et al. 2013). To modulate intracellular potassium efflux, we selected glibenclamide or high KCl solution. Glibenclamide is an ATP-sensitive potassium channel inhibitor that blocks maturation of caspase-1 and pro-IL-1β through attenuation of potassium ion efflux (Laliberte et al. 1999). In addition, a high concentration of extracellular potassium ion blocks potassium efflux mediated by NLRP3 inflammasome activators (Perregaux and Gabel 1994). In the current study, ATP-mediated IL-1β secretion in both porcine PBMCs and murine BMDMs was attenuated by glibenclamide and KCl, implying that potassium efflux occurs upstream of porcine NLRP3 inflammasome activation. ROS generation especially from mitochondria is another well characterized stimulus of inflammasome activation in both human and murine macrophages (Tschopp and Schroder 2010; Zhou et al. 2011). To clarify the role of ROS in porcine inflammasome activation, we tested the effect of DPI, an ROS scavenger, on inflammasome activation in response to ATP. Porcine NLRP3 inflammasome activation was attenuated by DPI, indicating that ROS generation induces porcine NLRP3 inflammasome activation.

Characterization of the porcine inflammasome has received much attention due to the economic importance of swine as a livestock as well as its common use as a human disease model (Hochrein and Wagner 2004). Porcine NLRP3 showed higher amino acid similarity with human NLRP3 than to its mouse counterpart, suggesting that pigs are useful as an experimental model for exploring the human immune system (Tohno et al. 2011). Consistent with this notion, the molecular basis of human diseases, such as involvement of the NLRP3 inflammasome in atherosclerosis, has been progressively studied in porcine models (Li et al. 2013). In addition, porcine inflammasome-mediated IL-1β maturation has been associated with porcine reproductive and respiratory syndrome (PRRS), an acute infectious disease threatening swine production worldwide (Zhang et al. 2013). PRRS is characterized by late-term reproductive failure in pregnant sows as well as respiratory disorder in piglets and young pigs (Lunney et al. 2010). Thus, knowledge of porcine NLRP3 inflammasome activation may provide insights into the pathogenic mechanisms of exogenous and endogenous disorders.

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