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Short communication

Functional expression of the damage-associated molecular pattern receptor P2X7 on canine kidney epithelial cells

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ABSTRACT

Epithelial cells are important in inflammation and immunity. In this study, we examined if Madin–Darby canine kidney (MDCK) epithelial cells express functional P2X7 receptors, which bind the damage-associated molecular pattern extracellular adenosine 5′-triphosphate (ATP). Reverse transcription (RT)-PCR and immunoblotting revealed the expression of P2X7 in MDCK cells. A flow cytometric assay demonstrated that ATP or 2′(3′)-*O*-(4-benzoylbenzoyl)ATP induced ethidium^{+} uptake into MDCK cells, and that this process was impaired by the P2X7 antagonists KN-62 and A438079. RT-PCR also demonstrated the presence of Toll-like receptor 4, NALP3, caspase-1, interleukin-1β and interleukin-18 in MDCK cells, as well as in positive control LPS-primed canine monocytes. In conclusion, the MDCK epithelial cell line expresses functional P2X7, as well as Toll-like receptor 4 and molecules associated with the NALP3 inflammasome. This cell line may help elucidate the role of these molecules in kidney epithelial cells and renal disorders in dogs and humans.

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1. Introduction

Damage-associated molecular patterns (DAMPs) play important roles in inflammation and immunity by functioning as signals of cell damage, stress or death during infection, injury or disease (Kono and Rock, 2008; Chen and Nuñez, 2010). One of the best-characterized DAMP is extracellular adenosine 5′-triphosphate (ATP) which mediates its effects through the activation of purinergic receptors particularly P2X7, a trimeric ligand-gated cation channel (Bours et al., 2011; Wiley et al., 2011). Activation of P2X7 by extracellular ATP or the most potent P2X7 agonist 2′(3′)-*O*-(4-benzoylbenzoyl)ATP (BzATP) causes the flux of Ca^{2+}, Na^{+} and K^{+}, as well as the uptake of organic cations such as ethidium^{+} (Jarvis and Khakh, 2009). Furthermore, P2X7 activation induces various downstream events including the NALP3 inflammasome/caspase-1-dependent maturation of IL-1β and IL-18, and their subsequent release from various myeloid cell types (Di Virgilio, 2007). This event, at least in monocytes, requires the prior activation of cells with the Toll-like receptor 4 (TLR4) ligand, LPS, a well-characterized pathogen-associated molecular pattern that induces the up-regulation and assembly of the NALP3

Abbreviations: ATP, adenosine 5′-triphosphate; BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl)ATP; DAMP, damage-associated molecular pattern; MDCK, Madin–Darby canine kidney; MFI, mean fluorescence intensity; PAMP, pathogen-associated molecular pattern; RT, reverse transcription; TLR4, Toll-like receptor 4.

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inflammasome, as well as the synthesis of IL-1β and IL-18 (Mehta et al., 2001; Bauernfeind et al., 2009).

The role of P2X7 in inflammation and immunity is largely attributed to its expression on myeloid and lymphoid cells, but there is emerging evidence that P2X7 on epithelial cells is also involved in these responses. P2X7 activation prevents chlamydial infection in cervical epithelial cells (Darville et al., 2007), while kidney epithelial P2X7 is involved in inflammation and renal injury (Goncalves et al., 2006; Taylor et al., 2009). Moreover, P2X7 activation induces caspase-1-dependent IL-1β release from intestinal epithelial cells, implicating a role for the NALP3 inflammasome in this process (Cesaro et al., 2010). As a result of these and other observations, P2X7 is attracting considerable interest as a therapeutic target in kidney and other disorders (Arulkumaran et al., 2011). The role of P2X7 on epithelial cells from the kidney and other tissues however remains poorly understood.

The presence of functional P2X7 on human and rodent cell types is well established, but little is known about P2X7 in other mammalian species. We have previously demonstrated that peripheral blood monocytes, lymphocytes and erythrocytes from English Springer Spaniels and other breeds express functional P2X7 (Sluyter et al., 2007; Shemon et al., 2008; Stevenson et al., 2009; Jalilian et al., 2012), however similar studies in other canine cell types are lacking. Given the importance of P2X7 in human health and disease (Sluyter and Stokes, 2011), new knowledge about this receptor in the dog is necessary to establish and understand the role of P2X7 in canine heath and disease.

Therefore, using molecular, immunochemical and pharmacological approaches we investigated whether the Madin–Darby canine kidney (MDCK) epithelial cell line, originally derived from a Cocker Spaniel, expresses functional P2X7 receptors. Moreover, we investigated whether this cell line also expresses TLR4 and molecules associated with the NALP3 inflammasome.

2. Materials and methods

2.1. Materials

RPMI-1640 medium, L-glutamine and ExoSAP-IT were from Invitrogen (Grand Island, NJ). FCS was from Bovogen Biologicals (East Keilor, Australia). Ficoll-Paque™ PLUS was from GE Healthcare Biosciences (Uppsala, Sweden). Ethidium bromide was from Amresco (Solon, OH). BigDye Terminator v3.1 was from Applied Biosystems (Carlsbad, CA). ATP, BzATP, LPS (*Escherichia coli* serotype 055:B5) and nigericin were from Sigma Chemical Co. (St. Louis, MO). KN-62 and A438079 were from Alexis Biochemicals (Lausen, Switzerland) and Tocris Bioscience (Ellisville, MO), respectively.

2.2. Cells

MDCK (European Collection of Cell Cultures, Porton Down, UK) and RAW264.7 (American Type Culture Collection, Rockville, MD) cells were maintained in complete culture medium (RPMI-1640 medium containing 2 mM L-glutamine and 10% FCS) at 37 °C/5% CO_2. For PBMC isolation, peripheral blood was collected into VACUETTE® lithium heparin tubes (Greiner Bio-One, Frickenheisen, Germany) from either pedigree or crossed breed dogs with informed, signed consent of pet owners, and with the approval of the University of Wollongong Ethics Committee (Wollongong, Australia). PBMCs were then isolated from buffy coats using Ficoll-Paque™ density centrifugation. PBMCs in complete culture medium were then incubated for 2 h at 37 °C/5% CO_2, the non-adherent cells were removed by gently washing twice with PBS, and the plastic-adherent cells incubated for a further 4 h in complete culture medium containing 0.1 μg/ml LPS.

2.3. Detection of mRNA and protein

Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Equal amounts of RNA for each cell type were amplified by reverse transcription (RT)-PCR using the MyTaq™ One-Step RT-PCR Kit (Bioline, Sydney Australia) according to the manufacturer's instructions using primer pairs (GeneWorks, Hindmarsh, Australia) specific for various canine mRNA transcripts (Table 1). The identity of each transcript was confirmed by sequencing of Exo-SAP-IT purified amplicons using the above primer pairs with BigDye Terminator and an Applied Biosystems 3130xl Genetic Analyzer.

Equal amounts of protein for whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotting was performed using a rabbit anti-mouse P2X7 polyclonal Ab (Alomone Labs, Jerusalem, Israel) as described (Constantinescu et al., 2010). Mean fluorescence intensity (MFI) of cell-surface TLR4 expression was detected using PE-conjugated murine anti-human TLR4 (clone HTA125) and IgG_{2a} isotype mAbs (both eBioscience, San Diego, CA) and flow cytometry as described (Gadeock et al., 2010).

2.4. Ethidium+ uptake

Nucleotide-induced ethidium+ uptake into cells suspended in either sucrose (280 mM sucrose, 5 mM KCl, 10 mM N-methyl-D-glucamine, 10 mM glucose, 0.1% BSA, 10 mM HEPES, pH 7.4) or NaCl (145 mM NaCl, 5 mM KCl, 5 mM glucose, 0.1% BSA, 10 mM HEPES, pH 7.4) medium was determined using a fixed-time flow cytometric assay as described (Gadeock et al., 2012).

2.5. IL-1β release

MDCK cells were incubated overnight in 24-well plates (2×10^5 cells/0.5 ml/well) at 37 °C/5% CO_2, and then in the absence or presence of LPS (as indicated) for 24 h. Alternatively, MDCK cells were incubated overnight in 24-well plates (1×10^5 cells/well) at 37 °C/5% CO_2 and then in the absence or presence of 10 μg/ml LPS for 4 or 24 h. These cells were then pre-incubated in RPMI-1640 medium (containing 0.1% bovine serum albumin) or in sucrose medium (containing 2 mM Ca^{2+} and 1 mM Mg^{2+}) (0.3 ml/well) at 37 °C/5% CO_2 for 15 min, followed by 60 min incubation

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