1,25(OH)D vitamin D promotes NOS2 *expression in response to bacterial and viral PAMPs in primary bovine salivary gland fibroblasts*

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ORIGINAL ARTICLE

1,25(OH)D vitamin D promotes *NOS2* expression in response to bacterial and viral PAMPs in primary bovine salivary gland fibroblasts

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Abstract

Objectives The faecal-oral route is a predominant mode of infectious disease transmission and yet the immunology of the bovine oral cavity is poorly understood. The objectives of this study were to develop an in vitro cell model of bovine salivary gland cells and to characterize the role of vitamin D on the expression of innate immune genes induced by stimulation with bacterial and viral pathogen-associated molecular patterns (PAMPs).

Methods Submandibular glandular tissue was excised post-mortem, processed, cells isolated and cultured until confluency after which cells were incubated with the active form of vitamin D (1,25(OH)D) for 18 h before stimulation with lipopolysaccharide (LPS μg/ml), lipoteichoic acid (LTA μg/ml) or polyinosinic:polycytidylic acid (poly I:C-20 μg/ml) PAMPs for 6 h and immune gene expression was assessed by Quantitative Real-Time PCR (RT-qPCR).

Results RT-qPCR analysis of vimentin expression in cells derived from the bovine submandibular gland shows that cultured cells were fibroblast in origin. These cells significantly induce the pro-inflammatory cytokine *IL1B*, β-defensin and cathelicidin genes but these were not significantly altered in response to 1,25(OH)D. In contrast, 1,25(OH)D significantly up-regulates the expression of the *NOS2* gene encoding iNOS in bovine submandibular stromal cells compared to EtOH (vehicle) control and this is a maintained response to all three bacterial and viral ligands. We have developed a new in vitro model to allow detailed investigations of mechanisms to enhance oral immunity in cattle. We show that these cells are fibroblast in nature, immunologically competent and vitamin D responsive. Their vitamin D-mediated enhancement of NOS2 expression warrants further investigation in saliva as a potential mechanism to boost oral immunity against infectious agents.

Keywords Primary bovine fibroblast culture · 1,25(OH)D vitamin D · Submandibular gland · Innate immune response · iNOS · Antimicrobial peptides

Introduction

The bovine salivary gland can produce upward of 220 L of saliva per day, a fluid mixture of water, electrolytes and proteins; and the classical role of saliva in ruminant digestion is well known (McDougall 1948). What is less well characterised however, is the immune protein components of saliva, and their role in the protection of the oral cavity. Although some of these molecules are present in low concentrations, their effects are often additive and/or synergistic, resulting in an efficient molecular defence network of the oral cavity (Fabian et al. 2012). Previous studies in humans have revealed expression of antimicrobial peptides such as defensins and cathelicidins in parotid, mandibular and sublingual salivary glands and their subsequent secretion into saliva (Murakami et al. 2002) including HBD2 in response to LPS (Mathews et al. 1999). Now in light of their important role in the innate defenses against oral microorganisms epithelial antimicrobial peptides are regarded as guardians of the epithelial cavity (Hans and Madaan Hans 2014). However in livestock species, where faecal-oral passage of infectious agents gives rise to a number of agriculturally-relevant diseases, the identity and function of these proteins remain elusive.

One study has performed a proteomic analysis on bovine saliva identified a wide-range of proteins, including some with well-characterised immune functions (Ang et al. 2011) such as

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cathelicidins, lactoperoxidase and lactotransferrin. These and related findings in other species illustrate the informative value of biomolecules in saliva associated with health and disease, and hint at the potential utility of such molecules for improving disease diagnosis (Javaid et al. 2016). The expression of the acute phase protein C-reactive protein (CRP) in saliva has been used to discriminate healthy pigs from those with experimentally-induced inflammation (Gutierrez et al. 2009). The potential inducible role that these immune proteins may play in the protection of the oral cavity, and along the digestive tract holds also exciting potential for improving disease therapeutics.

Another element of host defence, inducible nitric oxide synthase (iNOS) produces nitric oxide (NO) which is a messenger molecule with diverse functions throughout the body. It is now recognised that iNOS is a central component of innate immunity and an effective antimicrobial agent present in the oral cavity (Xue et al. 2018). iNOS is encoded by the *NOS2* gene and has been shown to be an important component of mycobacterial killing in humans and in mice studies and this response is vitamin D modulated (Rockett et al. 1998; Waters et al. 2004). It remains unknown if this mechanism is conserved in cattle.

In vitro models for the mechanistic study of the immune response in the bovine oral cavity is limited by a lack of suitable cell lines. Where ex-vivo analyses have been performed, interesting insights have emerged regarding innate proteins that may play a role in mycobacterial infection including lactotransferrin and lactoperoxidase (Mallikarjunappa et al. 2019). Therefore, with the adaptation of a detailed protocol for the isolation, culture and characterisation of primary bovine endometrial cells (Kelly et al. 2020), the aim of this study was to develop an in vitro model of salivary stromal cells to assess the influence of vitamin D on the innate immune response to pathogen-associated molecular patterns (PAMPs) in the bovine oral cavity.

Methods

Tissue collection and cell culture

Samples of bovine submandibular gland tissue were obtained post-mortem from an abattoir and processed with the aid of a detailed protocol for the isolation, culture and characterisation of primary bovine endometrial cells (Kelly et al. 2020), recently published by our group. Nodules of the submandibular gland were excised from behind the lower jaw and dissected into sections of approximately 1 cm^3, using a scalpel. The sections were washed in sterile cell culture grade PBS (Gibco) supplemented with antibiotics (50 IU penicillin, 50 μg/mL streptomycin) (Gibco) and fungizone (2.5 g/mL amphotericin B) (Gibco). Isolated tissue was transported to the laboratory in transport medium which consisted of RPMI 1640 (Gibco) supplemented with antibiotics, fungizone and 10% FBS (Gibco) at 4 °C within 60 min of harvest. Upon arrival at the laboratory, the transport medium was removed using a dropper and samples were washed twice in pre-warmed (37 °C) PBS (Gibco) supplemented with 1% antibiotics and fungizone as previously described. The samples were emptied onto a petri dish and prepared by mincing the tissue into very fine pieces (<1mm^3) using a scalpel. Prepared tissue was digested in a 50 mL falcon tube containing 10 mL 0.25% Trypsin-EDTA (Gibco). Tissue samples were digested for 1.5 h at 37 °C in an orbital shaker set at 150 rpm. Cell solution was then filtered through a 70 μm nylon mesh cell strainer (VWR) and digestive action was halted with 10% heat-inactivated FBS to each sample. Cells were pelleted by centrifugation at 1200 x g for 10 min and the supernatant was discarded. Cells were re-suspended in 12 ml of culture medium prior to cell count using a haemocytometer and culture. Culture medium consisted of LHC-9 media (Gibco) supplemented with 10% FBS (Gibco), 5% ITS-X (Gibco), 50 IU penicillin, 50 μg/ml streptomycin (Gibco) and 2.5 g/ml amphotericin B (Sigma-Aldrich). Cells were counted using a haemocytometer with Trypan Blue (Sigma-Aldrich, Poole, UK) staining to distinguish live from dead cells. A 1:2 dilution was prepared with 10 μl of 0.4% Trypan Blue and 10 μl of original cell suspension for cell counting. Cells were then seeded at a density of 2×10^6 cells/ml and maintained in 24-well plates. Plates were stored in a 37 °C incubator in 5% CO_2. Media was changed after 24 h, with subsequent media changes every 2–3 days.

Cell characterisation and stimulation

Visual examination of cell morphology under the light microscope was used to check purity of cultures. Cell growth was evident after 7 days, with cells approaching near-confluency (70–80%) after approximately 3 weeks. Cells were characterised by means of RT-qPCR. Purity of the stromal cell populations was demonstrated by the expression of cell specific cytoskeletal proteins; keratin-18 for epithelial cells and vimentin for stromal cells,

Cells were pre-incubated in culture media with or without 100 nM 1,25(OH)D Vitamin D for 18 h prior to addition of media with or without LPS (1 μg/ml), LTA (1 μg/ml) or poly (I:C) (20 μg/ml), and additional Vitamin D was added to cells pre-incubated with Vitamin D. Triplicate cultures were performed and incubated for 6 h. The concentration of 1,25(OH)D was chosen based on previous stimulations with bovine cells (Nelson et al. 2010). After stimulation, supernatant was removed and stored at −20 °C. TRIzol reagent

(Invitrogen) was added to cells and stored at -20 °C for RNA extraction.

RNA extraction, cDNA synthesis and RT-qPCR

RNA was extracted from cells using a combination method of Trizol and the RNeasy Mini Kit (Qiagen). Briefly, chloroform was added to the cell pellet in Trizol and shaken vigorously. The solution was then centrifuged for 15 min, 12,000 x g at 4 °C. The RNA containing aqueous layer was transferred to a clean microfuge tube. Triplicates were pooled at this step. An equal amount of 70% ethanol was then added and mixed vigorously. This solution was transferred to an RNeasy kit column. The manufacturer's instructions were then followed from this point. RNA quantity was determined using ND-1000 NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA integrity was assessed using 2100 Bioanalyzer Instrument (Agilent).

10 µl of each RNA sample was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit (Thermo Scientific) using the following cycler conditions: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min and held at 4 °C. cDNA solutions were diluted 1:5 prior to qPCR analysis. cDNA samples were prepared on a MicroAmp™ Fast Optical 96-Well Reaction Plate (Applied Biosystems) with primers using Fast SYBR® Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. RT-qPCR was performed using the following primers:

IL1B forward- CCCTGCAGCTGGAGGAAGTA, *reverse*-CTTCGATTTGAGAAGTGCTGATGT; *iNOS forward*- GATCCAGTGGTCGAACCTGC, *reverse*-CAGTGATGGCCGACCTGATG; *LAP forward*-GACAGCATGAGGCTCCATC, *reverse*- CTCCTGCA GCATTTTACTTGGGCT; *TAP forward*-TCCTGGTC CTGTCTGCTTC, *reverse*- TCCTGGTCCTGTCTGCTTC; *CATHL5 forward*-GGAGAATGGGCTGCTGAAAG, *reverse*- CACAGCACAGGTGATGTCG; *CATHL6 forward*-TCAGTTCAATGAGCGGTCCT, *reverse*- TTGAAGTC ACACTGCTCTGC; *KRT-18 forward*- ATTTCAGT CTTGGCGACGCT, *reverse*-GCCTCAGTGCCTCA GAACTT; *VIM forward*- TGCGCTCAAAGGGACTAACG , *reverse*-TCGAGCGCCATCTTGACATT, *RPS15 forward*-GCAGCTTATGAGCAAGGTCGT, *reverse*- GCTCATCA GCAGATAGCGCTT; *GUSB forward*-ACCATCGC CATCAACAACAC, *reverse*- TCCCGCGTAGTTGA AGAAGT; *ACTB forward*- AGATGACCCAGATC ATGTTCGA, *reverse*-TGACCCCGTCACCGGAGTCC ATCACGAT.

Primer pairs for all genes were designed using publicly available bovine gene sequences and the Primer BLAST software to be intron spanning where possible. A no-template control and no reverse transcriptase control were included to check for non-specific amplification. Quantitative PCR was run on a 7500 Fast Real-Time PCR System (Applied Biosystems) using the following cycler conditions: 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s and a final amplicon dissociation step at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. GUSB was found to be the most stably expressed gene from a panel of three reference genes tested based on low M-values calculated using geNorm tool in the GenEx software v.5.2.7.44, and was subsequently used to generate normalized relative expression values.

Statistical analysis

GraphPad Prism 7 was used to carry out statistical analysis. Stimulation data was assessed for statistical significance using an ordinary one-way ANOVA for each individual treatment. A student's t test was utilized in the evaluation of all other data.

Results and discussion

Human fibroblasts have been shown to generate a robust response to LPS exposure under laboratory conditions (von Bernuth et al. 2008) and a renewed appreciation for their role in innate immunity is emerging (Bautista-Hernandez et al. 2017). In addition, a growing number of studies have used these cells to investigate the immune response to important pathogens, like *E. coli* in cattle (Green et al. 2011; Kandasamy et al. 2011). However, to date, salivary fibroblasts have not previously been isolated in cattle. The bovine salivary gland consists of the parotid, the sublingual and the submandibular regions. The submandibular region originates from the endoderm, and this has been shown to drive the secretion of mucus consisting of high concentrations of antimicrobial peptides in humans (Khurshid et al. 2016). These antimicrobial or host defence peptides are then secreted into saliva via the contractile action of myoepithelial cells which drain into the oral cavity. Our knowledge on the species-specific differences in oral cavity host defence remains scant, particularly in relation to livestock species. Our previous work identified differential gene expression of a number of immune-related genes in salivary glands from cattle infected with the bacterium, *Mycobacterium avium subsp. paratuberculosis* (Mallikarjunappa et al. 2019) and now we develop a novel model to investigate the response of these immune genes to vitamin D.

Characterization of bovine submandibular stromal cells

After post-mortem collection and cell culture, purity of the cell populations was demonstrated by the expression of cell-

specific cytoskeletal proteins. Keratin-18 for epithelial cells and vimentin for stromal cells. RT-qPCR demonstrated that vimentin was expressed in the cultured cells, indicating that cell populations were stromal in origin [Fig. 1]. Initially, stromal cells have been known to play a structural role in synthesizing and remodelling the extracellular matrix in tissues and repair of damage, but they are becoming broadly appreciated for their immunological roles, such as the expression of toll-like receptors (TLRs), anti-microbial peptides (AMPs), cytokines and growth factors to ultimately defend against antigens (Bautista-Hernandez et al. 2017).

Cells are responsive to immunological ligands and express genes encoding the inflammatory cytokine IL-1β and host defence peptides

We investigated the immune responses of primary bovine submandibular stromal cells to LPS, LTA and poly (I:C) by assessing differences in expression of genes encoding host defence peptides and the potent innate immune effector molecule iNOS. iNOS is encoded by the *NOS2* gene.

Three PAMPs were selected on the basis of their representation of infectious agents of relevance to cattle; LPS representing gram-negative bacteria such as *E. coli*; LTA representing gram-positive bacteria such as *Staphylococcus aureus* and poly (I:C) representing dsRNA viruses such as bovine viral diarrhea virus (BVDV). These PAMPs have been extensively investigated in other cells of epithelial origin and concentrations to induce responses have been established by our previous work (O'Brien et al. 2020) as well as others (Berghuis et al. 2014).

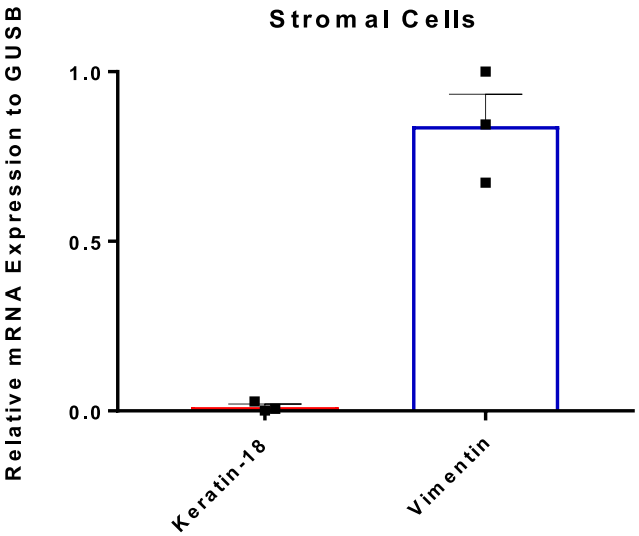

Fig. 1 Submandibular cell populations can be identified based on their differential expression of cytoskeletal proteins. RT-qPCR was used to measure relative vimentin and keratin-18 mRNA expression to *GUSB* in submandibular cell populations. Error bars represent the SEM of $n = 3$. Significance is identified between treatments where * = $P < 0.05$

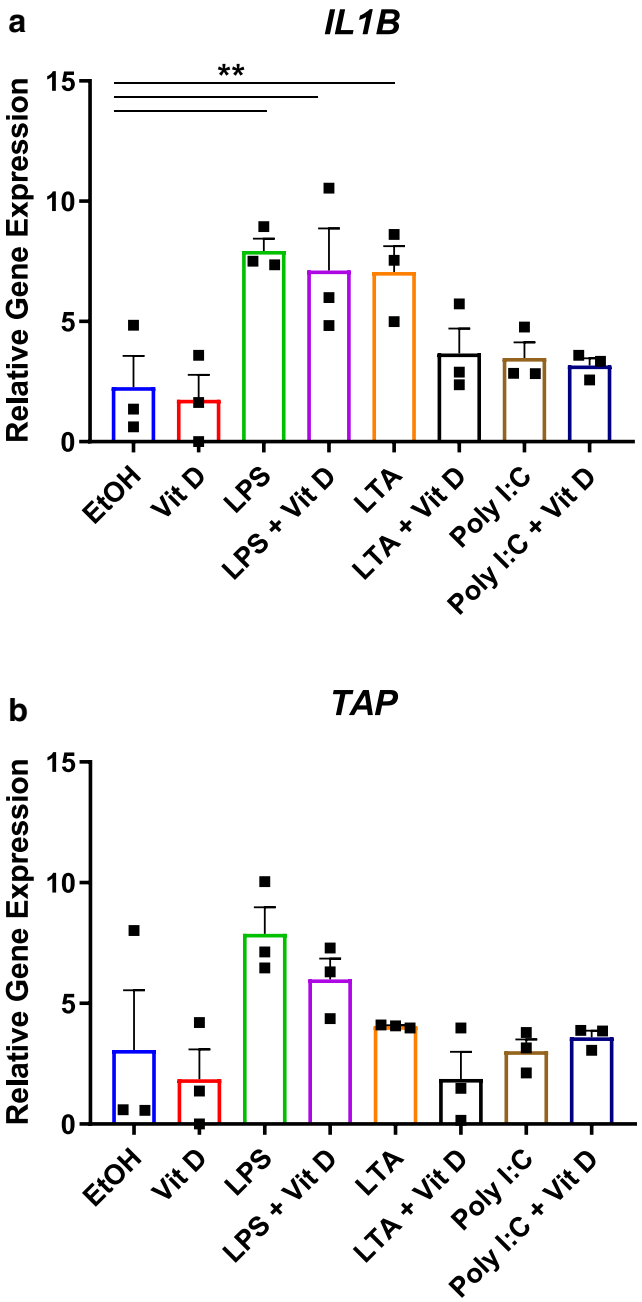

Fig. 2 Stromal cells isolated from the submandibular salivary gland express (**a**) *IL1B* and (**b**) *TAP* in response to gram-negative, gram-positive and viral PAMPs. Cells were isolated, cultured and stimulated for 6 h at 37 °C with LPS (10 μg/mL), LTA (10 μg/mL) and poly (I:C) (20 μg/mL). Error bars represent the SEM of n = 3. Significance is identified between treatments where ** = $P < 0.01$

Interleukin-1β (*IL1B*) gene expression was significantly elevated in response to LPS and LTA relative to control stimulated cells. However no significant effect of vitamin D was detected (Fig. 2a). Genes encoding tracheal anti-microbial peptide (TAP) – a member of the β-defensin gene family was also investigated by qPCR. Similarly, although TAP was induced in response to LPS and LTA,

there was no significant differential expression in response to 1,25(OH)D vitamin D (Fig. 2b). Despite their lack of differential expression in response to vitamin D, results show that salivary fibroblast cells are capable of mounting an immune response to these PAMPs, deeming them appropriate for future immunological studies.

1,25(OH)D vitamin D enhances the expression of iNOS in response to gram negative, gram positive and viral PAMPS

The *NOS2* gene, which encodes iNOS was significantly up-regulated in cells stimulated with all three PAMPs. *NOS2* gene expression was further synergistically enhanced with the addition of 1,25(OH)D vitamin D compared to the EtOH control (Fig. 3). Vitamin D is known to modulate the macrophage immune responses against mycobacteria in human studies (Rockett et al. 1998) and this is at least partially attributed to NOS production. A similar effect was also detected in bovine peripheral blood mononuclear cells from tuberculosis infected cattle in vitro (Waters et al. 2001). A more recent study specifically revealed that 1,25(OH)D vitamin D enhanced iNOS gene expression in bovine monocytes (Nelson et al. 2010). Here, we show that 1,25(OH)D vitamin D is also a potent activator of iNOS in bovine submandibular fibroblasts.

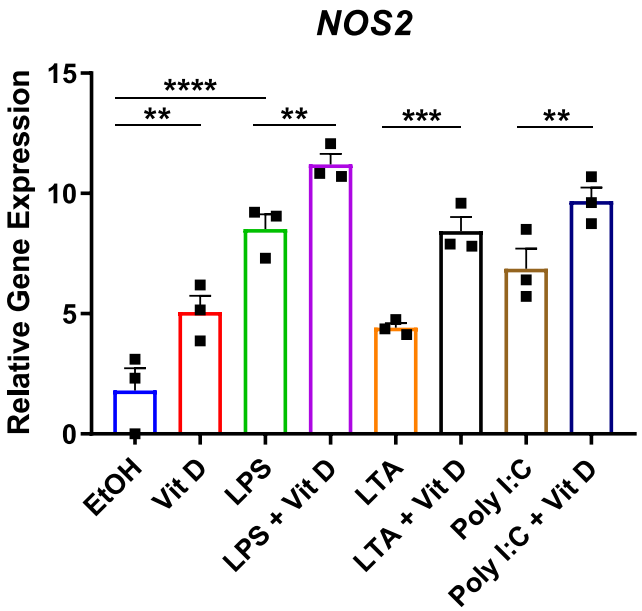

Fig. 3 Vitamin D modulates expression of *NOS2* gene encoding iNOS in stromal cells isolated from the submandibular salivary gland. Cells were isolated, cultured and pre-incubated for 18 h at 37 °C with culture medium with or without 100 nM Vitamin D. New culture media was then added with Vitamin D, prior to stimulation with LPS (10 μg/mL), LTA (10 μg/mL) and poly (I:C) (20 μg/mL). *NOS2* expression was measured by RT-qPCR. Error bars represent the SEM of n = 3. Significance is between treatments where ** = $P < 0.01$, *** = $P < 0.001$ and **** = $P < 0.0001$

Conclusion

We have developed a novel in vitro primary submandibular fibroblast cell model and document that this model is a useful tool to examine the bovine innate immune response to bacterial and viral stimulants in the salivary gland. For the first time, this data shows that bovine submandibular stromal cells hold a robust capacity to generate innate immune responses to bacterial cell wall and viral PAMPs. iNOS expression is also associated with antiviral protection in mouse fibroblasts (Mehta et al. 2012), and here we show that this is mediated by active vitamin D in the bovine oral cavity, which suggests that vitamin D could represent a potential mechanism to enhance immunity within the oral cavity. Future work will evaluate these responses to 1,25(OH)D vitamin D in vivo.

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Authors contributions Conceived the study: KM. Developed primary culture model: MB, MOB and CB. Performed experiments and interpreted data: MB, MOB and CB. Wrote the manuscript: MB, MOB and KM. All authors read and approved the final manuscript.

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Data availability The datasets analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Declarations All authors declare that they have no competing interests.

Ethics statement All procedures described were conducted post-mortem, and for which ethical approval and licences are not required.

Consent for publication Not applicable.

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