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Nystatin Modulates Genes in Immunity and Wingless Signaling Pathways in Cow Blood

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Abstract

Nystatin is an antifungal agent isolated from bacteria found in the dairy cow environment. It disrupts small platforms in the cell membrane, composed of sphingolipids and cholesterol known as lipid rafts. Pathogen recognition receptors (PRR) may be embedded in these lipid rafts. This study was conducted to evaluate the *in vitro* effects of the lipid raft inhibitor Nystatin, on the expression of genes in the innate and adaptive immunity and wnt signaling pathway in cow peripheral blood. Blood collected from four adult female Holstein-Friesian cows (n=4) was treated with 100ng/mL of Nystatin *in vitro*. Samples treated with Phosphate Buffer Saline served as control. Total protein concentration and prostaglandin E2in plasma were determined. Total RNA was isolated from cells and was used for cDNA synthesis. The effect of Nystatin on the expression of 84 genes on the cow Wingless signaling pathway and human innate and adaptive immunity arrays were assessed in cow blood using real-time PCR. Fold change in transcript abundance was calculated using Livak's method. Nystatin was found to modulate transcription and translation of genes involved in homeostasis and immunity in cow blood. It also increased the concentration of total plasma protein and PGE2in cow blood and may thus have had a pro-inflammatory effect. This study provides evidence for the association between lipid raft inhibition and alterations in the wingless signaling pathway in ruminant blood. Furthermore, the results presented may inform antifungal drug design and use in cows.

Keywords: Nystatin, lipid raft, cow blood, wingless

1. Introduction

There are over 300 species of fungi that are recognized as real or potential pathogens responsible for mycoses in animals (Drouhet, 1998). In bovine dairy herds, inflammation of the mammary gland is one of the most important health problems. Bovine mycotic mastitis is reported to be responsible for 1—12% of all mastitis cases (Costa et al., 1998; Lagneau et al., 1996). Drugs such as Amphotericin B (Amp B) and Nystatin are used for the treatment of mycotic mastitis and other fungus related infections. However, Amp B has to be administered in higher doses and therefore is not favorable because of toxicity constraints (Cheng et al., 1982).

Nystatin was originally isolated from bacteria in the dairy cow environment and is now widely used as an prophylactic. It has been reported to be less toxic and superior to Amp B against experimental candidiasis in model animals (Massood et al., 2003). Nystatin has also been used widely to demonstrate the involvement of lipid rafts in biological processes (Smart et al., 2002). Lipid rafts are small platforms, composed of sphingolipids and cholesterol content of cell membranes. These assemblies are fluid but more ordered and tightly packed than the surrounding bilayer. They are evolutionarily conserved structures that play a role in a number of signaling processes involving receptors expressed by a variety of cell types (Brown & London, 2000). Nystatin disrupts lipid rafts by binding to cholesterol in the lipid raft thereby degrading its integrity through hole formations in cells.

Nystatin stimulates innate immunity by binding to Toll-like receptor 2 (TLR2) (Razonable et al., 2009). Toll-like receptors are pattern recognition receptors (PRRs) that recognize highly conserved structural motifs known as pathogen-associated molecular patterns (PAMPs). The PAMPs are involved in the mediation of innate immunity and the activation of adaptive immunity. TheTLR2-driven induction of anti-inflammatory cytokines is important in microbial recognition and control (Netea et al., 2004).

The wingless homolog gene family (Wnt), are a family of secreted glycoprotein signal transducers involved in embryonic development and cell polarity. They have also been identified as potential mediators of inflammation

(Sen, 2000). The Wnts affect diverse processes such as embryonic induction, generation of cell polarity and the specification of cell fate (Logan and Nusse, 2004). The Wnt signaling pathway is activated by the binding of Wnt to Frizzled and LRP5/6 co-receptors to affect transcription of target genes. A key role for Wnt signaling during adult homeostasis is the maintenance of stem cell pluripotency (Reya & Clevers, 2005). Both the Toll-like receptor (TLR) and Wnt signaling pathways play important roles in health and diseases (Logan,2004) and their interaction with receptors on the cell surface is the first step in transducing an extracellular signal into intracellular responses (Cong et al., 2004).

In this study, the objective was to evaluate the *in vitro* effects of the lipid raft inhibitor Nystatin, on the expression of genes in the innate and adaptive immunity and wnt signaling pathway in cow peripheral blood.

2. Materials and Methods

2.1 Animals

All protocols for the handling of the animals were approved by the Institute of Animal Care and Use Committee (IAUCUC). Clinically healthy adult female Holstein-Friesian cows in mid- lactation (n=4) from the North Carolina Agricultural and Technical State University dairy farm were used in the study. None of the animals used exhibited any evidence of disease prior to blood sampling.

2.2 Blood Sampling and Treatment of Blood with Nystatin in vitro

Fifty milliliters (50 ml) of whole blood was collected aseptically from the jugular vein of the animals into vacutainer tubes containing 5ml of the anti-coagulant acid citrate dextrose (Macdonald et al., 2006). The tubes were placed on ice immediately after collection and transported to the laboratory. Blood was processed within 2 hours of collection. One milliliter of blood (10^7 cells/ mL of viable cells) was exposed to 100 ng/mL of Nystatin (NYS) (Sigma St Louis, MO). Samples treated with PBS served as control. The cells were incubated at 37°C with 5% CO_2 (Adjei-Fremah et al., 2015), and 85% humidity for 30 minutes. All the reagents used were prepared with endotoxin-free phosphate buffered saline (PBS). Endotoxin assay was performed as described by Adjei-Fremah et al. (2016). All treatments were done in triplicates. At the end of the incubation, period cells were spun down at 1700 x g at 4°C for 5 minutes. Supernatants were collected and stored at -80°C to measure total protein concentration, prostaglandin E2 levels. Trizol was added to cell pellets and stored for RNA isolation.

2.3 Total Cell Count, Viable Cell and White Blood Cell Differential Counts

Cell Viability was assessed using the Trypan blue dye exclusion method on the TC20 cell counting instrument (Bio-rad) as previously described by Asiamah et al. (2016). Samples of whole blood were diluted 1: 100 in saline. Ten (10) µl of diluted whole blood and 10µl of Trypan blue were combined in a 1.5 ml test tube. The mixture was pipetted up and down for five times to mix the cells and the dye. Ten (10) µl of the mixture was loaded in one of two chambers of the counting slide. The slide was then inserted into the TC20 cell counter for automatic cell counting. Cell counting was done in duplicate and an average was taken. Cell viability was expressed as a percentage of [(total viable and non- viable cells/total cells)] on the TC 10 cell counter (Bio-Rad).

2.4 White Blood Cell Differential Counts

White Blood Cell Differential counts were conducted on whole blood treated with all ten treatments or maintained PBS control using Wright's staining procedure. A thin smear of blood was made on a glass slide and left to dry at room temperature overnight. The air-dried slide was dipped in Wright's stain for10 seconds. Excess stain was washed off the stained slide with deionized water. The slide was then air dried before reading under a light microscope (Carolina Biologicals). Smears were read under oil immersion for cell counts. The different cells were counted up to 100 for numerical representation of various cells present in the blood sample.

2.5 Evaluation of the Concentration of Total Plasma Protein

Total protein concentration in plasma from treated and control samples were measured using the Bicinchoninic acid assay (BCA) following the manufacturer's instructions (Thermo Scientific™ Pierce) as described by Worku et al. (2016). Each sample was done in triplicate.

2.6 Evaluation of Prostaglandin (PGE2) Secreted in Plasma

Prostaglandin (PGE2) concentration in plasma from treated and control groups were evaluated using a commercial Enzyme-linked immunosorbent assay (Cayman) following the manufacturer's instructions as described by Ekwemalor et al. (2016). Each sample was done in triplicates.

2.7 Isolation of RNA and cDNA Synthesis

Total RNA was isolated using Trizol (Sigma). The quality and quantity of the RNA was measured with the Nanodrop spectrophotometer. Total RNA was pipetted into an RNA 6000 Nano LabChip® (Agilent) and RNA integrity was determined using Agilent® Bioanalyzer. Manufacturer's protocol was followed. Complimentary DNA (cDNA) synthesis was performed with 500ng/µl RNA using the RT2 first strand synthesis kit per manufacturer's protocol (Qiagen). Each sample was done in triplicates.

2.8 Real-Time PCR

The Cow WNT Signaling Pathway RT² Profiler PCR (Qiagen) was used in profiling the expression of 84 genes related to WNT-mediated signal transduction. The Human Innate & Adaptive Immune Responses RT² Profiler™ PCR Array (Qiagen) was used in profiling the expression of 84 genes involved in the host response to bacterial infection and sepsis.

Gene expression results were analyzed using the Livaks method (Livak and Schmittgen, 2001). Housekeeping gene GAPDH and samples treated with PBS were used to determine the ΔΔCt.Where ΔCt= (Target genes$_{treat}$-GAPDH$_{treat}$) - ΔCt (Target genes $_{PBS}$- GAPDH$_{PBS}$).

Fold change = $2^{(-\Delta\Delta Ct)}$)

2.9 Statistical Analysis

Means of White blood cell differential cell counts, Total cell count, concentrations of total plasma protein and PGE2 ELISA of treatment groups were compared with one-way Analysis of variance (ANOVA). SAS 9.2 statistical package was used. Results were considered statistically significant at ($P \leq 0.05$). Data for RNA concentration and purity are represented as means.

3. Results

3.1 Total Cell Count, Viable Cell and White Blood Cell Differential Counts

The average cell concentration for cows before treatment was 1.01 x 10^6 cells/ml(Initial viable cells were considered to be 100%).Cell viability was not affected by treatment at $p>0.05$ compared to PBS (Nystatin =98% and PBS=97%).White blood cell (WBC) differential count was not affected by Nystatin at $p>0.05$ (Figure 1).

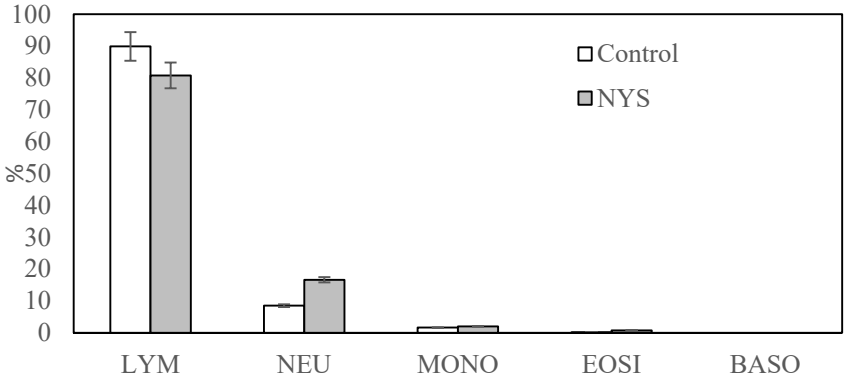

Figure 1. White blood cells differential counts in Nystatin-treated samples compared to control. NYS-Nystatin, LYM-Lymphocytes, NEU-Neutrophils, MONO-Monocytes, EOSI-Eosinophils and BASO-Basophils

3.2 Concentrations and Purity of Total RNA

The concentration and purity of RNA extracted from whole blood was measured to evaluate the treatment effect on mRNA transcription. Concentration and purity levels were not affected by treatment. There were no significant differences in RNA concentrations between the Nystatin treated samples and control samples ($p>0.05$). All RNA samples averaged 1.7(Figure 2).

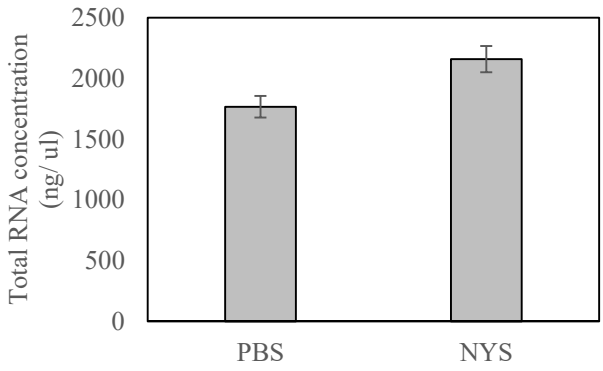

Figure 2. Total RNA concentration in Nystatin treated and PBS (control) samples

3.3 Total Plasma Protein Concentration

All cow blood samples incubated with Nystatin had significantly higher protein concentrations compared to the samples incubated with PBS (control) ($p < 0.05$) (Figure 3).

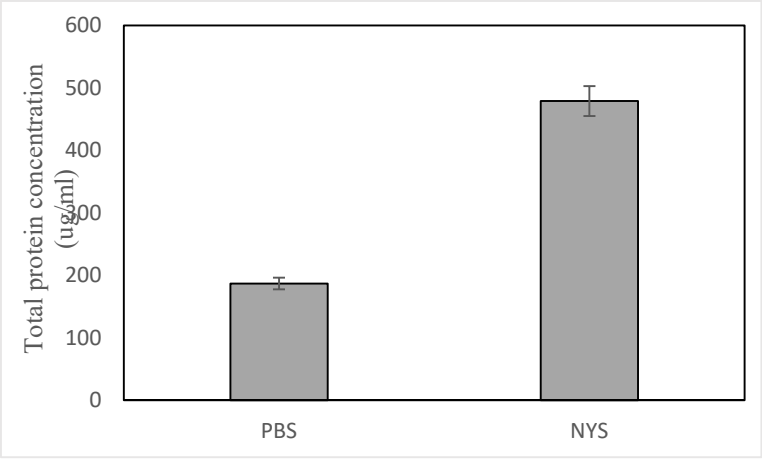

Figure 3. Total protein concentration in Nystatin treated and control group

3.4 Prostaglandin Levels (PGE2)

The results show that prostaglandin levels in blood samples incubated with Nystatin were significantly higher than the ones incubated with PBS (control) ($p < 0.05$) as presented in (Figure 4).

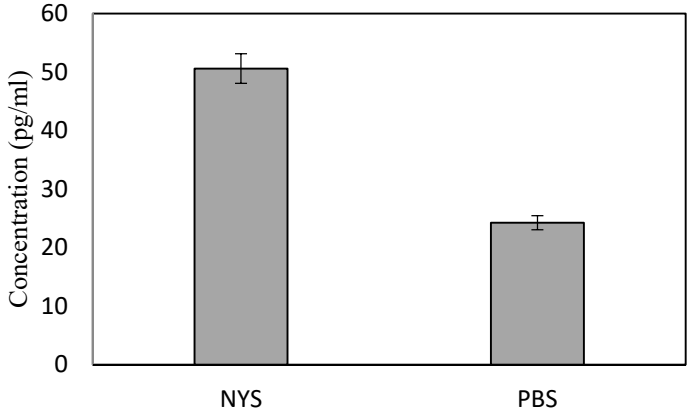

Figure 4. Levels of prostaglandin E2 concentration in blood plasma in NYS-treated and PBS-control group

3.5 Wnt Signaling Genes in Cow Blood

All 84 genes were expressed on the Wnt signaling pathway following Nystatin treatment. Seven of the expressed genes were upregulated (fold change ≥ 2) (Table 1).

Table 1. A list of Up-regulated genes on the Wnt array

Gene name	Fold change	Gene function
DAB2	6.4*	Tumor suppressor
FBXW11	2.0*	WNT Signaling Negative regulation
FZD3	*2.1	Wnt/ Canonical pathway (specific function unknown)
YWHAZ	5.6*	Tyrosine 3-monooxygenase/tryptophan 5- monooxygenase activation protein
WNT9A	3.1*	Cell fate regulator /patterning during embryogenesis
WISP1	2.5*	Wnt-1 Inducible Signaling Pathway/ mediates diverse developmental processes
TBP	2.5*	TATA-box binding factor /coactivator/promoter /modify general transcription factors
		TATA-box binding factor /coactivator/promoter /modify general transcription factors

*= significant, Fold changes ≥ 2 is considered significant.

3.6 Human Innate and Adaptive Pathway Genes in Cow Blood

Genes involved in both innate and adaptive immunity were expressed. Seven (7) out of the 51 new genes that (fold change ≥ 2) after treatment include receptors that respond to both bacteria and viruses (CD 40 and TLR 7, MX1 respectively) (Table 2) (Figure 5). Two genes (APCS and IFNA1) were down-regulated (Table 3).

Table 2. Up-regulated genes in the human innate and adaptive PCR array

Gene name	Fold changes	Gene function
MAPK1	81*	Cell signaling/ innate immunity
MAPK8	31*	Cell signaling/innate immunity
TLR 7	27*	Receptor/defense response to viruses
IL 10	3.7*	Down-regulator /cytokine innate immunity
CD 40	6*	Receptor/defense response to bacteria
MX 1	7.1*	Antiviral response
TBX 21	2.0*	Transcription factor

*= significant, Fold changes ≥2 is considered significant.

Table 3. Down-regulated genes on the human innate and adaptive array

Gene name	Gene function
APCS	Tumor suppressor /innate immunity
IFNA1	Antiviral activity/Adaptive immunity(cytokine)

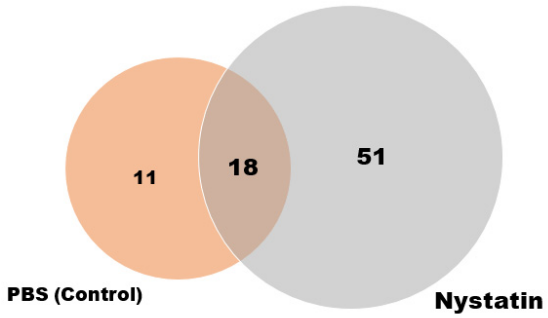

Figure 5 Venn diagram of number of genes expressed in both control and treated samples in Human Innate &Adaptive array

4. Discussion

In this study, we demonstrate that Nystatin modulates both the wnt signaling and the human innate and adaptive immunity pathways in cow blood. Nystatin is an antifungal compound that has been used widely to demonstrate the involvement of lipid rafts in biological processes (Smart et al., 2002). Nystatin disrupts lipid rafts by binding to cholesterol within the plasma membrane it. Lipid rafts are small platforms within the cell membrane, composed of sphingolipids and cholesterol content. It was first discovered in the environment of dairy cows. Scientific research has shown that Nystatin possesses proinflammatory properties (Razonable, 2009). This makes it a potential therapeutic for certain inflammatory diseases in cow e.g. Mastitis.

This study showed that treatment of cow blood with Nystatin modulates wingless signaling pathway. Nystatin activated and modulated the expression of genes associated with Wnt /Ca^{2+} pathway, and inhibited the expression of beta-catenin pathway associated genes. Wnt/Ca^{2+} pathway have been shown to antagonize the beta-catenin pathway (De, 2011). The Wnt/Ca^{2+} signaling pathway regulates cell movement, proliferation, and migration, along with modulation of cell behavior and structural change in a variety of organisms (Angers et al., 2009; Mikel et al., 2006). In this study, cell viability and white blood cell counts were not affected by treatment. Although there was no effect on blood count, cells were live and activated. Sen et al. (2010) in previous studies found no effect of Nystatin on cells at 0.6 μg/ml. In this study, it is evident that 100ng/ml of Nystatin was not enough to have a significant effect on cell counts and viability. Concurrently, genes that were upregulated on both the wnt signaling pathway were mostly involved in cell survival and proliferation inhibition. These include YWHAZ which has attracted interest because of its elevated expression associated with a variety of cancers (Matta et al., 2007). Recently, it is understood that YWHAZ has critical anti- apoptotic functions (Neal et al., 2009). Another gene of interest that increased in fold change was DAB 2. Studies have shown that the over-expression of DAB2 significantly inhibits cell proliferation in cultured lung cancer cells in vitro (Karam et al., 2007).

Nystatin also had effect on activation and expression of immune response genes. Razonable et al. (2009) showed that Nystatin induces the secretion of IL-1, IL-8, and TNF-alpha in TLR2-expressing but not in TLR2-deficient cells. Our results support their findings, that Nystatin is a potential pro-inflammatory agent that uses the TLR-dependent pathway since all TLRs were activated in our experiment (Wiegand et al., 1988; Razonable et al., 2009). Interestingly, TLR 7, which is a receptor for virus detection was upregulated on the innate and adaptive pathway.

Nystatin was also found to increase total protein and prostaglandin E levels in cow. This could be due to the binding of the lipid raft inhibitor to cholesterol in the lipid raft thereby degrading its integrity of the cell through hole formations. Cellular components are then released into the blood stream which then triggers inflammatory response that may lead to increase in prostaglandin formation (Wong et al., 2009). Nystatin increases, e.g., the intracellular concentration of Ca^{2+} (Wiegand et al., 1988). Any cell stimulation is accompanied by an increase in intracellular concentration of calcium. Although prostaglandin released in high amounts may cause tissue damage and other related inflammation problems, it stimulates and regulates a wide array of immune functions, thus playing an important role in the host defense system.

In both Wingless and immune signaling pathways, MAP Kinase activities were activated. These kinases are a chain of proteins in the cell that communicates signals from a receptor on the surface of the cell to the DNA in the nucleus of the cell. Since the receptors in both signaling pathways were upregulated, we can say that MAPK should also be up-regulated in order to transmit signals to the nucleus to ensure homeostasis. Increasing activity of MAPKs and their involvement in the regulation of the synthesis of inflammation mediators at the transcription level, make them potential targets for novel anti-inflammatory therapeutics. This could be a point of cross-talk for both Wnt and TLR pathways. Both wnt and human innate and adaptive pathways rely on membrane-embedded receptors for proper signaling and activation. This study highlights the significance of opportunistic microorganism to animal health. However, *in vivo* studies in cows are needed to assess the significance of our *in vitro* findings. Nevertheless, the present study provides new insights into the pharmacological mechanisms of action of Nystatin.

This study demonstrated that in cow blood, Nystatin affects genes involved in both wingless signaling pathway and human innate and adaptive pathway. TLR and Wnt-dependent process may serve as the molecular basis for the proinflammatory properties of Nystatin. It is evident in this study that the lipid raft inhibitor Nystatin, served as an agonist for TLR, whose consequent activation mediates effective antimicrobial properties. An appreciation of the role of TLR and WNT in pharmacologic immunotoxicology highlights their potential as novel therapeutic targets.

5. Conclusion

In this study, PRR associated lipid raft inhibition by Nystatin resulted in modulation of gene activation in cow blood. Exposure of cow blood to Nystatin modulated transcription and translation of genes involved in homeostasis

and immunity. This study provides evidence for an association between lipid raft inhibition and alterations in the wingless signaling pathway in ruminant blood. The expression of wingless homolog pathway genes in cow blood in response to Nystatin and other PAMPs needs to be explored since it may be important in homeostasis and inflammation. Furthermore, the results presented may inform antifungal drug design and use in cows.

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