Induction of antimicrobial peptides in human cell lines representing epithelia and macrophages

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Abstract

In this project the effects of prolactin on the expression of various genes connected to innate immunity in THP-1 macrophages. In addition the effects of butyrate, Phenyl Butyric acid (PBA) and Vitamin D on the expression of genes connected to the Vitamin D receptor (VDR) pathway. There was no effect from the prolactin on the genes in question but interesting effects found in relation to the VDR pathway. Vitamin D does not induce *CAMP* expression in HT29 cells but greatly increases the expression of *Cyp24*. The effects of PBA and butyrate were also compared and are mildly increasing on *Cyp24* expression and do also increase *CAMP* expression.

Útdráttur

Í þessu verkefni voru áhrif prólaktíns á tjáningu gena tengdum náttúrulegu ónæmi skoðuð í THP-1 makrófögum. Að auki voru áhrif smjörsýru (butyrate), PBA og Vítamíns D á gen tengd VDR boðleiðinni skoðuð í HT29 frumum. Engin áhrif voru á tjáningu þeirra gena sem skoðuð voru með tilliti til prólaktíns en áhugaverðar niðurstöður komu í tengslum við VDR boðleiðinia. Vítamín D hefur engin áhrif á tjáningu *CAMP* í HT29 frumum en eykur hins vegar mikið tjáningu á *Cyp24*. Áhrif bútýrats og PBA voru einnig borin saman og í ljós kom að þau höfðu lítil en marktæk áhrif á tjáningu *Cyp24* og juku einnig tjáningu *CAMP*.

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Abbreviations

3D	Three dimensional
AMP	Antimicrobial peptide
CAMP	Cathelicidin antimicrobial peptide
cDNA	Complementary deoxyribonucleic acid
FBS	Fetal Bovine Serum
hCAP18	Human Cathelicidin antimicrobial protein
h.	Hours
min.	Minutes
PBA	Phenyl butyric acid
PBS	Phosphate Buffered Saline
PMA	Phorbol 12-Myristate 13-Acetate
RNA	Ribonucleic acid
VDR	Vitamin D Receptor
TLR2	Toll Like Receptor 2

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1 Introduction

1.1 The immune system and is roles

The immune system is a system that the body has to fight of infections. To fulfill its duty there are four main tasks it has to be able to do. Firstly it is immunological recognition; it has to recognize the pathogens to be able to take the necessary action. This task is carried out both by the white blood cells and the innate immunity. Secondly the system has to eliminate the threat of infection. The immune effector functions are carried out by both the white blood cells, proteins in the blood and antibodies produced by some lymphocytes. While it is important to fight off infections it is also very important to keep the immune response under control so that it does not do harm to the body. The third task of the immune system is immune regulation, the ability to regulate this response. Failure to regulate the system can cause allergies and autoimmune diseases. The fourth and last of the main tasks is the immunological memory, to be able to "memorize" pathogens and respond quickly to reoccurring infections. This task is fulfilled by the adaptive immune system. The immune system can be divided into the innate immune system and the adaptive immune system although these two "systems" are really two units of the same system (Murphy, 2012).

1.2 Innate immunity

The first lines of defense are physical and chemical barriers of epithelia like the skin for example. The innate immune system has developed to guard against infections and see that the microbes are unable to breech our barriers and multiply. The innate immune system consists of many leukocytes that secrete cytokines, ingest the pathogen or kill it directly. The innate immunity is a rapid, broad spectrum and powerful system in preventing infections. If the pathogens are however able to resist these defenses and develop into an infection the adaptive immune system comes in to play with a slower but more specific line of attack through the use of lymphocytes specific for the pathogen.

The cytokines that some leukocytes secrete as a part of innate immune response are of various kinds and have different functions. There are for example numerous cytokines that affect the cells around them, thus being able to conduct the battle. Another element of the

innate immune system are the antimicrobial peptides. These are peptides that are very toxic to bacteria and other microbes. They are vital in preventing pathogens from breaking through epithelial cell layers, entering the body.

1.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are, as the name suggests, short proteins that have an important role in fighting foreign microbes. They are an integral part of the innate immune system. These peptides are secreted by diverse cell types throughout the body, both conventional immune system cells and also by some epithelial cells.

These peptides are generally around 50 amino acid long and are characterized by cationic amphipathic properties it folded 3D structure.

The AMPs show antimicrobial properties against various types of microorganisms, including bacteria, fungi and viruses. The effects of the AMPs are non-specific and interestingly they work well against many drug resistant bacteria strains. AMPs have got a low propensity for developing resistance, possibly do to the direct way they work on their targets. Most AMPs directly work on the pathogens membrane, using their amphipathic nature and cationic properties to disrupt the pathogens lipid bilayer. Two of the most extensively researched categories of AMPs in mammals are cathelicidins and defensins(Lai & Gallo 2009; Lehrer & Lu 2012; Leonard *et al.,* 2012).

1.3.1 Cathelicidins and LL37

Cathelicidins have been described in both invertebrate and vertebrate species. Their name is based on a common, highly conserved N-terminus region, known as the cathelin domain. The protein, which is characterized by two disulfide bonds, has the ability to inhibit the protease cathepsin-L wherefrom the name is derived. In humans only one cathelicidin gene is known (*CAMP*) but in other species many different cathelicidins have been described, often more than one in the same species. *CAMP* codes for an inactive precursor protein of approximately 18 kDa, called hCAP18. The cathelicidins are only considered a gene family because of the well conserved and large domain mentioned above but other regions of the proteins encoded by the genes vary greatly. However the cathelicidin peptides show little similarity between them and are only considered a group because of the comparable structure of the precursor protein which is dominated by the large cathelin domain. In humans the precursor protein is processed to release a 37 amino acid long peptide from the

C terminus of the protein. The released product is the antimicrobial peptide known as LL37. The peptide starts with two leucine amino acids, explaining the name. The hCAP18 precursor product is stored in cells in high concentrations with the C-terminus end in an inactive state. The processing of the protein and cleaving of the LL37 from its precursor is essential for the activation of its antimicrobial function.

LL37 is produced both in granulocytes but also in epithelial cells all over the body, for example in the lungs, gut and skin. In the skin the peptide is processed beyond the LL37 point by serine proteases and secreted in various different forms with different antimicrobial functions. Overall the cathelicidin antimicrobial peptides are a highly diverse group of molecules with some similarities in their precursor (Lai & Gallo 2009).

Known inducers of *CAMP* are, amongst others, Vitamin D, Phenyl Butyric Acid (PBA) and butyrate. The *CAMP* gene is a direct target of the Vitamin D receptor (VDR) (Steinmann *et al.,* 2009; Termén *et al.,* 2008; Gombart 2009; Campbell *et al.,* 2012)

1.4 Prolactin

Prolactin is a 23 kDa hormone composed of 199 amino acids. It is produced by the anterior pituary gland. The best known role of this hormone is its influence on female mammal milk production postpartum. However many other functions have been described as the hormone affects the human homeostasis in various different ways. For example prolactin affects both the immune and the nervous system. Prolactin has been shown to have an important role in immunomodulation as a co-stimulating factor on T lymphocytes. It has also been reported that serum prolactin level is increased in psoriasis patients and that psoriasis symptoms are exaggerated during prolactinoma (a condition where serum prolactin levels are elevated) (Dilmé-Carreras *et al.,* 2011). Prolactin levels have also been reported to be abnormally high in other autoimmune diseases (Jara *et al.,* 2011). In addition to these noted effects a phosphorylated form of prolactin, S179D, has been showed to upregulate vitamin D receptor (VDR) expression which is interesting because cathelicidin expression is amplified by calcitrol through VDR (Díaz *et al.,* 2011) (Wu *et al.,* 2005).

1.5 Butyrate and PBA

Butyrate is a short chain fatty acid containing four carbon atoms. The only structural difference between butyrate and PBA is a phenyl group on the non-acidic end of the fat

chain. Both of these substrates work as a HDAC inhibitors (Steinmann *et al.,* 2009; Termén *et al.,* 2008). Butyrate is produced from fibers by the natural bacterial flora of the gut. Some short chain fatty acids, butyrate in particular, have been shown to upregulate LL37 expression. This upregulation is important for regulating the bacterial flora and also the immune system in the gut. Butyrate however is not a good drug because it smells terribly and thus is not suitable for consumption. PBA (Phenyl butyric acid) also upregulates LL37 expression. The effects of PBA and butyrate have not been systematically compared before. Both these substrates induce LL37 expression in synergy with 1,25-dihydroxyvitamin D_3. The pathway through which the substrates upregulate LL37 expression is currently unknown. (Lai & Gallo 2009; Steinmann *et al.,* 2009; Campbell *et al.,* 2012). There are several known receptors to which butyrate is a ligand, including GPR43 that is expressed throughout most of the immune system (Trompette *et al.,* 2014; Ulven 2012). However the receptors through which PBA work are not known but recent discoveries might shed some light on how PBA dictates its effect.

According to a recent study on PBA effects on LL37 expression in VA10 lung cells, PBA influences the LL37 expression through the VDR pathway. In the study siRNA was used to knock down the VDR expression in turn nullifying the PBA effects (N. Kulkarni, unpublished data).

This raises the question if butyrate also works through the VDR pathway. Additionally the question of why Vitamin D and PBA works in synergy rather than competitive manner comes to mind considering the substrates work through the same pathway.

2 Aims of this project

The first aim of this project was to study the effect prolactin had on innate immunity and check the change in LL37 expression it induced. To check this THP-1 cell line was used and differentiated to macrophages with PMA. The results in short were that prolactin had no effect on LL37 expression.

Since there was still time to proceed after the negative results of the first part of the project a second aim was set. This aim was to examine and compare the effects of Butyrate, PBA and Vitamin D in HT29 gut cell line. This was done to gather data for a examining if butyrate as well as PBA has effect through the VDR pathway. It has been reported before that Vitamin D does not seem to have effect on *CAMP* expression in HT29. The expression of *Cyp24* and *CAMP* were the main subjects because both of those genes are known to be upregulated through the VDR pathway.

3 Materials and methods

3.1 Cell culturing

A cell culture is where cells are grown in a strictly controlled environment. Infections and contamination are held limited by sterilization and all the cell work is thus done in as sterile environment as possible. The different cell types often require a different growing condition in respect of medium and confluency.

3.1.1 THP-1 cell culturing

The THP-1 cell line is a monocytic cell line derived from an acute leukemia patient. The cell line was grown on a RPMI medium, from Gibco, with a 10% Fetal Bovine Serum (FBS) from Gibco. The medium was changed every two or three days and split when they were getting too confluent (70-80%). To split the cells they were first spun down at 1200 rpm for 5 min. and the pellet then dissolved in 1xPBS (phosphate buffered saline) and then spun down again for cleaning purposes. The pellet formed in the later spinning was then dissolved in medium and diluted so that the culturing could continue.

3.1.2 Differentiation of the THP-1 cells

To differentiate the THP-1 monocytes into macrophages 25 ng/ml Phorbol 12-Myristate 13-Acetate (PMA) from Santa Cruz Biotechnologies was used on the monocytes that were in the concentration of $1x10^6$ cells/ml. The PMA was kept on for 24 h. Then the medium was changed and the cells were rested for another 24 h. before the experiments could start. The cells were grown on a six well plate and the amount of cells in each well was two million.

3.1.3 HT29 cell culturing

The HT29 cell line is a colorectal adenocarcinoma cell line originally derived from a tumor in 1964. When cultured the cells form an epithelia and stick together in clusters that is hard to break down for splitting. The cells were grown in a DMEM/F-12 media from Gibco, that was changed every two to three days. The cells were split whenever they were reached 70-80% confluency. To split the cells they have to be loosened by 10% trypsin from Gibco

for 20-25 min. and then 10% FBS is added. 3 ml of trypsin were used and 7 ml of FBS. Then the cells were spun down at 1200 rpm for 5 min. The pellet was then dissolved in 10 ml of PBS to clean the cells and spun down again. The pellet is then dissolved in about 50 ml of medium and shaken vigorously. This is done because the cells have a tendency to stick together. After that the cells were seeded again and the culturing could continue.
For the experiments the cells were counted and seeded on a six well plate. In each well 500.000 cells were seeded. The cells were then kept for 24 h. before the medium was changed and the experiments could start.

3.2 Substrates used and concentrations

3.2.1 Substrates used on the THP-1 macrophages

The THP-1 macrophages were used to check the effects of prolactin on the expression of a few genes, namely *CAMP*. The effect was examined both after different exposure time of the substrates on the cells and also different combinations and concentrations exposed for 24 hours.
In the first experiment the cells were exposed to 200 ng/ml prolactin from Sigma, 50 ng/ml S179D (phosphorylated prolactin) (a gift from Ameae Walker, University of California Riverside), 1 µg/ml PAM3CSK4 (TLR2 ligand) from Tocris and 10 nM Calcitriol (the active form of Vitamin D) from Tocris. This was done for 1, 3, 6, 24 and 48 hours.
In the second experiment the substrates were put in two different concentrations and then each concentration tried with added Vitamin D. PAM3CSK4 was put in 1 µg/ml and 5 µg/ml. S179D was put in 2 ng/ml and 50 ng/ml and prolactin was put in 20 ng/ml and 200 ng/ml. The Vitamin D concentration was the same as in experiment 1 (10 nM). Furthermore in one plate a combination of S179D (2 ng/ml) and prolactin (100 ng/ml and 200 ng/ml) were put on the cells and then the Vitamin D was also added to two wells.
After the experiments the RNA was retrieved and isolated from the cells using the NucleoSpin® RNA kit from Macherey-Nagel

3.2.2 Substrates used on the HT29 cells

The experiment conducted on the HT29 cells was checking the difference in expression of *CAMP*, *Cyp24*, *Cyp27* and *VDR* when certain substrates had been added to the medium. 500.000 cells were seeded in 2 ml of medium in each well. The substrates added were butyrate (1 mM and 3 mM), PBA (1 mM and 3 mM) and 100 nM Vitamin D. The cells

were cultured for 24 h. after seeding. Then the substrates were added and kept on the cells for 24 h. After that the cells were retrieved and the RNA was isolated, using the NucleoSpin® RNA kit.

3.3 Measuring the gene expression

To measure the gene expression of the cells, both the THP-1 and HT29 cells, quantitive real-time PCR was used.

First the RNA was isolated from the cells via the NucleoSpin® RNA kit. The RNA purity and concentration was then measured using Nanodrop technique. The concentrations measured were then used to normalize the concentrations of the cDNA that was then synthesized using the RevertAid® first strand cDNA synthesis kit from Thermo Scientific. After that the normalized cDNA was loaded on a 96 well plate along with SYBR green reagent and water. These plates were then run in the qRT PCR machine.

All the primers used (*CAMP, IL8, Cyp24, Cyp27, HPTR1, UBC* and *actB*) were from Integrated DNA technologies (IDT)

4 Results

4.1 The effects of prolactin on gene expression in THP-1 cells

Two experiments were conducted on the THP-1 macrophages, examining the effects prolactin had on them. In the first experiment the substrates, mentioned in the Materials and Methods chapter, were put on the cells for different periods of time, ranging from one to 48 hours. There was also one well on each plate that was used as a negative control were no substrates were put on the cells so they were growing normally in the medium.

In the second experiment different substrates were put on the cells together and the gene expression of the cells was measured after 24 h. exposure. The controls for this experiment were the negative control that got no substrate other than the media and the other control was only Vitamin D for all the different chemicals used on the cells in this experiment were also put together with Vitamin D.

In short the prolactin, the phosphorylated form of prolactin (S179D) and the combination of those two; had no effect on the gene expression of *CAMP* and *IL8*. There was upregulation of *CAMP* expression in the cells that got Vitamin D but no further induction on the cells that got both Vitamin D and the prolactin or S179D.

4.2 The effects of butyrate, PBA and Vitamin D on gene expression in HT29 cells

The aim of the experiment conducted on the HT29 cells was to compare the gene expression difference induced by butyrate, PBA and Vitamin D. The genes in question were *CAMP*, *Cyp24*, *Cyp27* and *VDR*. There was no evidence of *Cyp27* inductions after the first to experiments so it was decided to abandon further studies of that and focus on the other three genes instead.

CAMP expression was increased in the cells introduced to PBA and Butyrate but there was no effect on the cells that got Vitamin D. The most interesting part of these results was the difference between the butyrate and PBA induction. This difference shows a functional difference between these substrates not shown before and needs to be studied further (*Figure 1*).

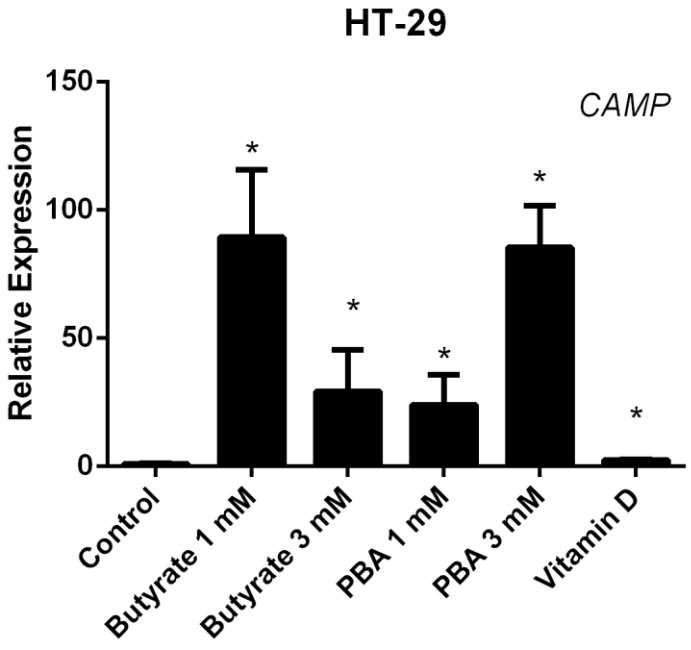

Figure 1. CAMP gene expression. The expression of the *CAMP* gene was increased in the cells after butyrate and PBA intoduction. Interestingly Vitamin D had no biologically significant effect although there was a small statistically significant increase in *CAMP* expression. It is also interesting that there was a stronger effect in the 1 mM butyrate medium than the 3 mM but the other way around in the PBA media. This shows an interesting difference between the two substrates that needs to be studied further. A 1way ANOVA test was used to determine statistical significance and the experiment was conducted three times, each run being independent.

The *Cyp24* gene expression is, like *CAMP*, known to be enhanced through the VDR pathway. Because of the interesting results mentioned in the introduction regarding the connection of PBA and this pathway in VA10 cells and the unusual lack of effect from Vitamin D on *CAMP* expression, it was interesting to look at another gene known to be expressed through the same pathway. Opposite to the *CAMP* expression there was a huge increase in expression of *Cyp24* after Vitamin D administration. There was also mild increase of expression in the cells in the butyrate and PBA medium. This would indicate that the VDR pathway is functional in the HT29 cells. It also suggests that even though butyrate and PBA could be working through the VDR pathway they do so in a different manner than Vitamin D (*Figure 2*).

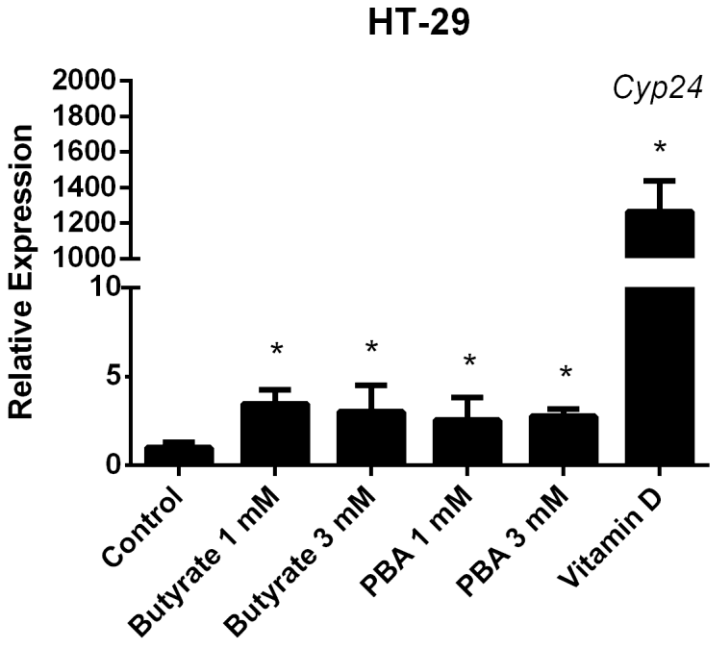

Figure 2. Cyp24 expression Although there is a significant increase in expression in the cells that got butyrate and PBA the effect of Vitamin D was around 300 times stronger than the other effects. This shows that the VDR pathway is intact and also that Vitamin D does have effect on the HT29 although it doesn't affect the *CAMP* expression. A 1way ANOVA test was used to determine statistical significance and the experiment was conducted three times, each run being independent.

Lastly the expression of VDR was examined. This was done to see if anything unusual was happening in respect of the VDR expression because of the unusual pattern in the *CAMP* expression. None of the substrates used were shown to have any significant effect on the VDR expression (*Figure 3*).

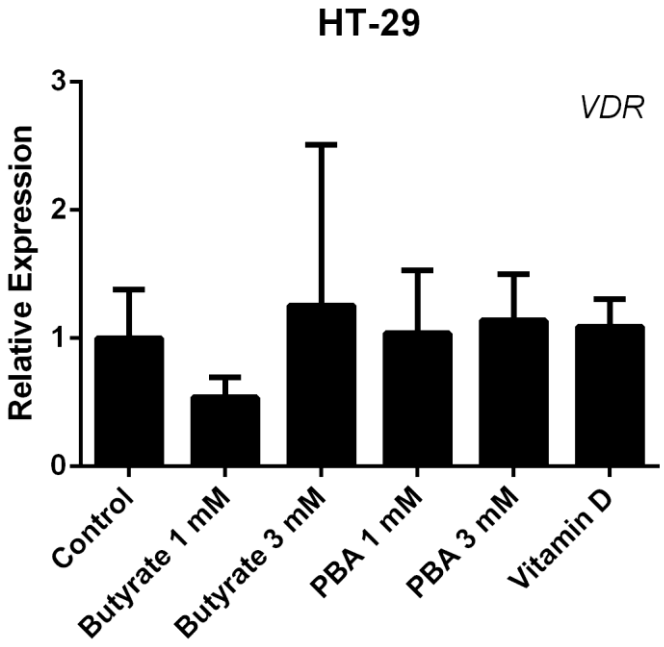

Figure 3 VDR expression As seen in this figure no significant difference in *VDR* expression. This shows that the effects show on *CAMP* and *Cyp24* expression was not due to difference in *VDR* expression. A 1way ANOVA test was used to determine statistical significance and the experiment was conducted three times, each run being independent.

5 Discussions

In this project the expression of various genes linked to innate immunity was examined in connection to prolactin, in the first part, and butyrate, PBA and Vitamin D in the second part. These experiments were conducted on THP-1 cells and HT29 cells, respectively.

The results of the prolactin experiments were negative; prolactin had no effect on the genes examined. These negative results lead nowhere and there was still time for more research so instead of stopping there the project was continued. In light of the new exciting discovery on the lab that PBA worked through the VDR pathway to induce LL37 expression HT29 culturing was started to examine and compare the effects of Butyrate, PBA and Vitamin D, on these cells. The results were that Vitamin D had no effect on LL37 expression but increased *Cyp24* expression immensely. However PBA and butyrate increased *CAMP* expression and also mildly induced *Cyp24* expression. These results are interesting because these genes are both expressed through the VDR pathway. In particular the effects of Vitamin D are interesting because it clearly binds to VDR but somehow doesn't induce *CAMP* expression. If PBA and butyrate work through the VDR pathway in HT29 the difference in the effects on *CAMP* are very interesting and need to be looked further into. Another interesting discovery was the difference seen in how PBA and butyrate induce *CAMP* expression. Butyrate showed stronger effect in inducing *CAMP* at 1 mM than at 3 mM but PBA worked the other way around. This shows that PBA and Butyrate work in a different way in inducing *CAMP* gene expression.

To look at if PBA and butyrate are working through VDR, a siRNA knockdown of VDR was tried but proved unsuccessful. Due to lack of time the siRNA experiment was not tried again in this project. However the next step in this research would be to see if the substrates are working through the VDR pathway and a good way to do that would be to try to knock the VDR expression down via siRNA.

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