Dual stimulation with bacterial and viral components increases the expression of hepcidin in human monocytes

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Running header: The role of hepcidin during bacterial infection

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Abstract

Hepcidin belongs to the antimicrobial peptide (AMP) family and is the key regulator of iron metabolism. It modulates iron homeostasis by binding to, and degrading the iron exporter molecule ferroportin thus inhibiting cellular iron efflux. Many antimicrobial peptides have a dual function; some are able to act directly as an antimicrobial agent as well as having an immunoregulatory role in the host. Toll-like receptors (TLRs) bind to components of microbes, activate cellular signal transduction pathways and stimulate innate immune responses. The effect of viral and bacterial TLR co-stimulation of THP-1 derived monocytes showed that 24 hours after exposure to TLR 9 and TLR3 agonists in combination, hepcidin expression was significantly increased (10 fold) when compared to the untreated control. This combination of TLR ligands mimics simultaneous bacterial and viral infections thus suggesting a potential key role for hepcidin in combined infections.

Additionally, by using a chequerboard assay, we have shown that hepcidin has an antagonistic (FICI >4) effect with therapeutic levels of the antibiotics rifampicin and tetracycline against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Streptococcus pyogenes*. This finding has important implications for future treatment regimens especially in an era of increasing antimicrobial resistance.

Introduction

Iron is required by nearly all life forms because it is involved in many cellular and metabolic processes. Pathogens employ a variety of means to acquire iron from their hosts during infection, while the host attempts to withhold it from pathogens to impede microbial growth. Systemic iron homeostasis in humans is controlled by the 25 amino acid peptide hormone hepcidin, produced primarily by hepatocytes. Hepcidin was first documented in 2001 (Park *et al*, 2001) and it has since been described as the principal regulator of iron homeostasis. Hepcidin binds to the only known mammalian iron exporter molecule, ferroportin, and this binding causes the internalization and degradation of the hepcidin-ferroportin complex (Weinstein *et al*, 2002; and Rodriguez et al 2014). Consequently, cellular iron efflux is inhibited which restricts iron availability to invading pathogens. Ferroportin is highly expressed on duodenal enterocytes and macrophages; therefore, when hepcidin levels are raised such as in time of infection or inflammation, both dietary iron uptake and recycling of iron from senescent red blood cells by macrophages are reduced. Hepcidin levels that are persistently elevated in conditions such as chronic infection or inflammation, can lead to iron redistribution into the reticuloendothelial system, causing anaemia due to the reduced supply of iron to the bone marrow for erythropoiesis (Nicholas *et al*, 2001; Roetto *et al*, 2003).

Hepcidin itself belongs to the family of antimicrobial peptides (AMP). Other members of this family, such as defensins, function as important effectors of innate immunity and modulate adaptive immune responses at nano-molar concentrations (Harder *et al*, 2007). Many AMPs exhibit direct antimicrobial action against bacteria, fungi, parasites and viruses and are therefore regarded as 'natural antibiotics' (Brown and Hancock, 2006). Previous studies have shown that hepcidin has direct antibacterial activity *in vitro* at high

concentrations, but the role of hepcidin as an AMP or its potential ability to influence the immune response has not been fully elucidated (Andrews 2004).

A limited number of studies have reported the effect of Toll-like receptors (TLRs) on hepcidin expression but little information is known about the effect on hepcidin expression by combined bacterial and viral stimulation. TLRs bind to components of microbes and activate cellular signal transduction pathways which initiate specific innate immune responses. Each member of the TLR family recognises specific pathogen associated molecular patterns (PAMPSs) which are effective alone or in combination with other TLRs (Tang *et al*, 2012). Contemporary evidence suggests that there is crosstalk between TLRs engaging multiple agonists, resulting in either the suppression or synergism of particular immune responses (Aresnault *et al*, 2013). Accordingly, this study explored the effect of exogenous TLR agonists on hepcidin expression in human monocytes in combination, and aimed to further explore the role of hepcidin during the infection process.

Materials and methods

Bacterial strains

Staphylococcus aureus (EMRSA-15), *Streptococcus pyogenes* MGAS6180 and *Pseudomonas aeruginosa* ATCC9027 (NCIMB 8626) were used throughout this study. *S. aureus* and *P. aeruginosa* were cultured aerobically in nutrient broth (NB; Oxoid) at 37°C; *S. pyogenes* was cultured under the same conditions in tryptone soya broth (TSB; Oxoid).

Hepcidin

Hepcidin (Peptinova, Osaka) was reconstituted and stored according to manufacturer's instructions to give a stock concentration of 100µM. Dilutions were prepared with NB to obtain the appropriate concentration for each experiment.

Determining minimum inhibitory concentrations (MIC)

The MIC of rifampicin, tetracycline and hepcidin were determined using the microbroth dilution method in 94-well microtitre plates (MTP; Oxoid - Cambridge, UK). Concentrations of each antimicrobial were prepared as follows: rifampicin and tetracycline 0-0.003809µg ml^{-1} and 0.125µg ml^{-1} as a doubling dilution in NB; hepcidin 0-100µM in 25µM increments in NB. Each well of the MTP was inoculated with approximately 1x10^5 cfu/mL of bacteria and were incubated at 37°C for 24 h. Assays were performed in triplicate on at least three separate occasions for each antimicrobial agent under test. Absorbency readings used a Spectrostar Nano spectrophotometer (BMG Labtech) at *A*650.

Chequerboard assay

Chequerboard assays were prepared using sterile 96-well MTPs, broadly as described above. Serial dilutions of each antibiotic and hepcidin were prepared in NB (Oxoid, Basingstoke, UK). The combinations were tested (in triplicate) against each microorganism as follows: i) *S. aureus* with rifampicin (concentration range 0-0.0625µg ml^{-1}) plus hepcidin (at 0-100µM), ii) *S. pyogenes* with rifampicin (concentration range 0-0.0625µg ml^{-1}) plus hepcidin (at 0-100µM), and iii) *P. aeruginosa* with tetracycline (concentration range 0-2µg/ml) plus hepcidin (0-100µM). These concentration ranges were selected using the initial MIC data. Interactions

between hepcidin and antibiotics were determined as synergistic, antagonistic or additive using FICI values (≤0.5– synergy; >0.5 to ≤4– additivity and >4 – antagonism (Odds, 2003).

Cell culture

The human monocytic cell line THP-1, obtained from American Type Culture Collection (TIB-202, ATCC, Manassas, VA, USA). THP-1 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with Glutimax (Invitrogen; Paisley, UK). Media was supplemented with 10% (v/v) heat-inactivated Foetal Calf Serum (FCS) (Biosera, East Sussex, UK), 1% (v/v) non-essential amino acids and 1mM sodium pyruvate (all supplements were purchased from Gibco BRL, Paisley, UK). THP-1 cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 for optimal growth and proliferation.

TLR Ligands

Agonists for TLR3, polyinosinic-polycytidylic acid (Poly I:C) and TLR9 (CpG ODN M362) were purchased from Invitrogen (San Diego, CA). THP-1 cells were stimulated for 3hrs, 6hrs and 24hrs with poly I:C (25µg ml^{-1}), CpG (5 µg ml^{-1}) or a combination of poly I:C and CpG.

Measurement of Hepcidin Gene Expression

RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol, and hepcidin expression was determined using quantitative reverse transcription PCR (qRT-PCR) on an AB7600 real-time system (Applied Biosystems). First strand cDNA was amplified and detected using SYBR Green PCR Mastermix (Applied Biosystems). Results are expressed as a ratio of the gene of interest (hepcidin) to the housekeeping gene *gusβ*. The following primer

sequences were used: *hep-F* 5'-GCACTGAGCTCCCAGATCTG-3', *hep-R* 5'-GCAGGGCAGGTAGGTTCTAC-3'; *gusβ-F* 5'-TCTGTATTCATTGGAGGTGC -3 and *gusβ-R* 5'-AAGGTTTCCCATTGATGAGG -3'.

Statistical Analysis

Statistical analyses were performed using Graphpad Prism 5 (Graphpad Software Inc, California USA). Data are expressed as means +/- standard error of the mean for each group. Differences between groups were determined using Students t-test for multiple comparisons by one way ANOVA, followed by Bonferroni's post hoc analysis as appropriate. Results were deemed significant when P<0.05.

Results

Co- stimulation of THP-1 cells with CpG and Poly I:C induces hepcidin expression

Poly I:C is a synthetic analog of double-stranded RNA (dsRNA), a molecular pattern associated with viral infection. Poly(I:C) is recognized by TLR3 which induces activation of NF-kB and the production of cytokines. CpG ODNs are synthetic oligonucleotides that contain unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs). These CpG motifs are present in bacterial DNA and are recognized by Toll-like receptor 9 (TLR9) which induces strong immunostimulatory effects. At 3h and 6h post stimulation, no significant increase in hepcidin gene expression was observed in either the TLR ligands alone, or in combination p = 0.5738 (3hrs) and 0.1619 (6hrs) respectively (Figure 1). However, at 24h a significant 10 fold increase in hepcidin gene expression was observed, indicating that CpG and Poly I:C act in combination

to induce hepcidin expression in THP-1 cells in a time dependent manner. This is evidenced at the 24hr time point where p < 0.0001 as shown on Figure 1.

Hepcidin inhibits the growth of both Gram positive and Gram negative pathogens; but is antagonistic in combination with systemic antibiotics

Hepcidin expression was significantly upregulated in the presence of combined microbial and viral components, and given that dual infections are regarded as highly refractory to antimicrobial therapy, we investigated the effect of exogenous hepcidin on the antibacterial activity of two commonly utilized antibiotics, namely rifampicin and tetracycline. We firstly determined their MIC concentrations for each of three pathogens as described in materials and methods.

The MICs of hepcidin against *S. aureus*, *S. pyogenes* and *P. aeruginosa* were found to be 50uM for each pathogen The MIC of rifampicin and tetracycline were determined at 0.01563 µg ml^{-1} and 0.25µg ml^{-1} respectively for the organisms under test (Table 1). Hepcidin was tested in combination with antibiotics using chequerboard assay at a concentration range of 1-100µM in 25µM increments. It was noted that in combination with hepcidin, the MIC for each antibiotic was higher than for the antibiotic alone. FICI index was calculated for each combination and established that hepcidin was antagonistic (FICI >4) in combination with either rifampicin or tetracycline (Table 2).

Discussion

There is increasing interest in the role of antimicrobial peptides as a means addressing the problem of antimicrobial resistance and the decline of new classes of antibiotics. It is also becoming increasingly apparent that many antimicrobial peptides have a dual function and can act directly in an antimicrobial capacity, but also possessing a regulatory function in the host. This study investigates the effect of viral and bacterial co-stimulation on hepcidin expression. Over a period of 24h and following exposure to a combination of CpG (bacterial ligand) and Poly I:C (viral ligand), hepcidin expression was significantly increased, compared to the untreated control; moreover hepcidin expression was not observed when each ligand was applied individually. This combination of TLR ligands mimics simultaneous bacterial and viral infections thus supporting a possible role of hepcidin in combined infections that has not been previously reported. Concurrent bacterial and viral infection is known to increase sensitivity of immune cells to microbial components, in some cases resulting in lethal synergy, if bacterial infection occurs at the peak of viral infection.

Infections caused by antimicrobial resistant bacteria often fail to respond to conventional treatment, resulting in prolonged illness, greater risk of death and elevated costs (Anderson and Hughes, 2010). A growing proportion of infections are caused by antibiotic resistant bacteria and the continued emergence of new resistance mechanisms in response to the latest generation of antimicrobials has begun to render these treatments increasingly ineffective. (Pray, 2008). Our studies reiterate the direct antimicrobial effect that hepcidin has against both Gram positive and Gram negative bacteria (Park *et al*, 2001). Concentrations of hepcidin required to inhibit bacterial growth were higher than those found physiologically, and we hypothesise that during infection a localised spike of hepcidin from immune cells such as monocytes (as reported here), might be sufficient to impede pathogen multiplication until other defence mechanisms are activated. Due to a lack of appropriate

model, we were not able to determine a concentration of hepcidin that might impede viral replication.

During an established infection, antimicrobial interventions are required to reduce the bacterial load to a level that can be dealt through host response. Therefore we further investigated whether combinations of hepcidin and two commonly used, broad spectrum antimicrobials resulted in increased efficacy and reduced MICs against *P. aeruginosa, S. pyogenes* and *S. aureus*, all of which are capable of invasive and life-threatening infection. Contrary to our expectations, when these antimicrobials were administered in combination with hepcidin, antagonism occurred, resulting in increased MICs for both tetracycline and rifampicin against each of the microorganisms studied.   This antagonism would suggest that clinical doses of antimicrobials, determined *in vitro* might be ineffective *in vivo* potentially resulting in exposure of pathogens to concentrations of an antimicrobial that are in fact sub-inhibitory, which could drive antimicrobial resistance.

This *in vitro* model of infection employed the human monocyte cell type, which originate from progenitors in the bone marrow and traffic via the bloodstream to peripheral tissues.   During both homeostasis and inflammation, circulating monocytes leave the bloodstream and migrate into tissues where, following conditioning by local growth factors, pro-inflammatory cytokines and microbial products, they differentiate into macrophage or dendritic cell populations. Recruitment of monocytes is essential for effective control and clearance of viral, bacterial, fungal and protozoal infections, but recruited monocytes also contribute to the pathogenesis of inflammatory and degenerative diseases.

Our data supports a paradigm in which hepcidin expression is induced following concurrent viral and bacterial infection; it is possible that such stimulation is the result of

hyper-sensitisation of cells to the dual ligands, as described above. The increased expression of hepcidin could have a local, direct bactericidal effect as well as restricting iron to impede further bacterial growth. Simultaneously cytokines such as IL-6 that mediate the inflammatory immune response would be up-regulated in response to hepcidin accumulation.

The resulting cytokine storm could have the potential to result in apoptosis of virally infected cells, but if unchecked might result in a shift from moderate to severe disease or sepsis as a consequence of lethal synergy. We are currently undertaking further studies to clarify this model for the role of hepcidin during infection. Further examination of the myeloid cell contribution to hepcidin biology and iron homeostasis could provide a considerable new insight into mammalian iron homeostasis and innate immunity.

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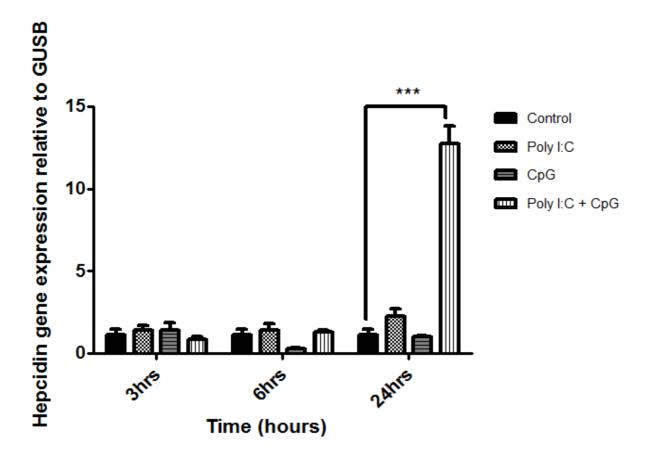
Figure 1. Hepcidin gene expression in THP-1 cells in the presence of the TLR ligands Poly I:C

(TLR 3) and CpG ODN M362 alone and in combination. Results are expressed as a mean +/-

S.E.M of at least three independent experiments performed in triplicate. *** indicates a

statistically significant difference p< 0.001 from control.

Table 1. Sensitivity of *S. aureus*, *S. pyogenes* and *P. aeruginosa* to rifampicin and tetracycline

using the microbroth dilution method

	Mean MIC µg ml^{-1} (+/-SEM)		
	Rifampicin	**Tetracycline**	**Hepcidin (µM)**
S. aureus	0.01563 (+/- 0.10)	-	50
S. pyogenes	0.01563 (+/- 0.02)	-	50
P. aeruginosa	-	0.25 (+/- 0.009)	50

SEM = Standard error of the mean

Table 2. Sensitivity of *S. aureus*, *S. pyogenes* and *P. aeruginosa* rifampicin (µg ml⁻1) and tetracycline (µg ml⁻1), in combination with hepcidin (50µM) using the microbroth dilution method.

	Antimicrobial interaction			
	Rifampicin + Hepcidin	**Tetracycline + Hepcidin**	**FICI**	**Outcome**
S. aureus	0.0625 (+/- 0.02)	-	>4	**Antagonism**
S. pyogenes	0.0625 (+/- 0.12)	-	>4	**Antagonism**
P. aeruginosa	-	2 (+/- 0.01)	>4	**Antagonism**

SEM = Standard error of the mean