Salmonella Typhi sense host neuroendocrine stress hormones and release the toxin haemolysin E

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***Salmonella enterica* serovar Typhi (*S. typhi*) causes typhoid fever. We show that exposure of *S. typhi* to neuroendocrine stress hormones results in haemolysis, which is associated with the release of haemolysin E in membrane vesicles. This effect is attributed to increased expression of the small RNA *micA* and RNA chaperone Hfq, with concomitant downregulation of outer membrane protein A. Deletion of *micA* or the two-component signal-transduction system, CpxAR, abolishes the phenotype. The hormone response is inhibited by the β -blocker propranolol. We provide mechanistic insights into the basis of neuroendocrine hormone-mediated haemolysis by *S. typhi*, increasing our understanding of inter-kingdom signalling.**

Keywords: *Salmonella*; hormones; haemolysin; *micA*; *cpx*

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INTRODUCTION

Bacterial pathogens use molecular sensors to detect and facilitate adaptation to changes in their environment. Mechanisms that allow interpretation of host signalling systems such as neuroendocrine stress hormones might enable them to adapt to and survive within different host compartments (Pacheco & Sperandio, 2009). The neuroendocrine hormone norepinephrine is abundant in the gut, whereas epinephrine is found mostly in the bloodstream (Furness, 2000). Remarkably, macrophages synthesize and respond to epinephrine and norepinephrine on exposure to bacterial lipopolysaccharide (Flierl *et al*, 2007, 2009).

Bacteria communicate by producing autoinducer molecules and sensing their concentration in a process called quorum sensing (Williams, 2007; Pacheco & Sperandio, 2009). In *Escherichia coli*, autoinducer 3 functions synergistically with epinephrine and norepinephrine to regulate motility and virulence through QseBC and QseEF (Rasko *et al*, 2008; Pacheco & Sperandio, 2009). In *Salmonella enterica* serovar Typhimurium, epinephrine modulates resistance to oxidative stress and host-produced antimicrobial peptides through a mechanism involving BasSR (Karavolos *et al*, 2008).

Salmonella enterica serovar Typhi (*S. typhi*) is a human pathogen that causes typhoid fever (Huang & DuPont, 2005). In *S. typhi* and *S. paratyphi*, *hlyE* (also known as *clyA* or *sheA*) encodes a haemolysin pore-forming toxin, which might contribute to virulence and the development of systemic infections (Fuentes *et al*, 2008; von Rhein *et al*, 2009). HlyE accumulates in the bacterial periplasm, from where it is packaged into membrane vesicles and released from the cell (Wai *et al*, 2003).

We observe that exposure of *S. typhi* to neuroendocrine hormones resulted in increased haemolytic activity. A proteomics-based dissection of the haemolytic phenotype identified a significant reduction in levels of outer membrane protein A (OmpA). This was attributed to increased levels of the small RNA (sRNA) chaperone protein Hfq and the sRNA *micA* repressing *ompA* expression. These effects could be reversed by the addition of the β -adrenergic blocker propranolol. The haemolytic response was specific to membrane vesicles, and was not observed in an *S. typhi* strain lacking the sRNA, *micA*. Finally, the neuroendocrine hormone-mediated haemolysis required the CpxAR two-component signal-transduction system and was independent of the *E. coli* O157:H7 bacterial adrenergic receptor orthologue QseBC (Rasko *et al*, 2008).

RESULTS

Host hormones increase haemolysis by *S. typhi*

Although *S. typhi* is not haemolytic on blood agar plates, exposure of *S. typhi* to neuroendocrine hormones resulted in zones of clearance, indicating the release of HlyE or another haemolytic

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Fig 1 | Neuroendocrine hormones induce haemolysis by *S. typhi*. (A) Neuroendocrine hormones induced zones of clearance (arrows) indicating lysis of red blood cells in blood plates that had been inoculated with *S. typhi*. The addition of epinephrine to blood plates that had been inoculated with a strain lacking *hlyE* ($\Delta hlyE$) or addition of water (H₂O) to plates that had been inoculated with the *S. typhi* parent strain failed to induce haemolysis. (B) Epinephrine (50 μ M) caused haemolysis, which was significantly reversed by the addition of propranolol and by overexpression of OmpA from an arabinose-inducible promoter (E + (pBAD-A) + ARA). (C) Haemolytic activity in filtered supernatant from *S. typhi* exposed to epinephrine (50 μ M) was enriched in the fraction containing membrane vesicles and was not detected in the ultracentrifuged supernatant (150SN) or filtered supernatant obtained from a strain lacking *hlyE* (SN-*hlyE*). Vesicles were visualized using negative staining and electron microscopy. Scale bar, 100 nm. (D) Membrane vesicle preparations were examined quantitatively by protein content. Asterisks indicate significance ($P \leq 0.05$) by the Student's *t*-test. ARA, arabinose; E, epinephrine; MV, membrane vesicle; NE, norepinephrine; OmpA, outer membrane protein A; PO, propranolol; SN, supernatant.

factor (Fig 1A). In a liquid assay, neuroendocrine hormone exposure boosted haemolysis by approximately 40% (Fig 1B). Propranolol significantly inhibited the haemolytic effect (Fig 1B). BRD948hlyE failed to produce detectable haemolysis (Fig 1A).

The haemolytic activity was contained in membrane vesicles (Fig 1C, photograph) and not within ultracentrifuged supernatants, ruling out leakage into the supernatant (Fig 1C, 150SN). Furthermore, exposure of *S. typhi* BRD948 to neuroendocrine hormones increased levels of membrane vesicles in supernatants independently of HlyE (Fig 1D).

Increased haemolysis is due to reduced OmpA expression

To further elucidate the mechanistic basis of the neuroendocrine hormone-mediated haemolysis, we determined the effect of epinephrine and norepinephrine on the *S. typhi* proteome. Levels of the mature form of OmpA were significantly reduced (approximately 2.2-fold) on exposure of *S. typhi* to neuroendocrine hormones (Fig 2A). The addition of β -adrenergic blocker propranolol before neuroendocrine hormone treatment abolished the reduction in OmpA levels (Fig 2A). OmpA has a key role in regulating osmotic homeostasis by adjusting the permeability and integrity of the outer membrane (Wang, 2002). Overexpression of OmpA reversed epinephrine-induced haemo-

lysis by *S. typhi* (Fig 1B). Similar results were obtained by using norepinephrine (data not shown).

Exposure of *S. typhi* BRD948(pOmpA) to epinephrine significantly reduced expression of the *ompA* promoter-reporter (Fig 2C) and tagged OmpA levels (Fig 2D). OmpA protein levels, but not transcription levels, were reversible by the addition of propranolol, highlighting post-transcriptional regulation through a blockable signalling pathway (Fig 2C,D). There were no measurable effects on *ompA* transcription or protein levels from blocker alone (data not shown). Transcription of *hlyE* was unaffected by exposure to neuroendocrine hormones (data not shown).

Host hormones affect levels of the sRNA *micA*

The sRNA *micA* is an antisense regulator of *ompA* mRNA translation through binding to the *ompA* mRNA leader (Udekwa et al, 2005). Neuroendocrine hormones significantly increased *micA* promoter expression (Fig 3A) and *micA* transcript levels (three–fourfold; Fig 3B) in a response that is blockable by propranolol. Exposure of cells to propranolol alone had no effect on expression of the *micA* promoter or on levels of *micA* transcript (data not shown).

The significant increase in the *ompA* translational repressor sRNA *micA* (Fig 3A,B) is in agreement with the observed reduction in OmpA levels and implies that a *micA*-dependent

Fig 2 | Neuroendocrine hormones reduce levels of outer membrane protein A. (A) A 30 min exposure of *S. typhi* to neuroendocrine hormones or a hormone/blocker combination led to significant (analysis of variance test $P \leq 0.05$) alterations in the levels of the mature form of outer membrane protein A, as determined by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight analysis. (B) The *ompA* gene under the control of its native promoter (P) was designed to incorporate a carboxy-terminal StrepTag (S) and cloned on pMK1lux luminescence expression vector resulting in pMK1luxO. (C) Transcriptional or (D) protein levels were determined by spectrophotometry or western blot analysis, respectively, as described in the Methods section. Both neuroendocrine hormones had an identical phenotypic profile; only epinephrine data are shown. Densitometry measurements showing levels relative to control are displayed below the western blot (average of three independent experiments). Asterisks indicate significance ($P \leq 0.05$) by the Student's *t*-test. Experiments were repeated at least three times. Standard error bars are shown. E, epinephrine; NE, norepinephrine; OmpA, outer membrane protein A; PO, propranolol.

signalling route might be part of the neuroendocrine hormone-mediated haemolytic cascade in *S. typhi*. Notably, haemolysis was abolished in a *micA* deletion strain (BRD948*micA*; Fig 3C). Overexpression of the *micA* transcript in BRD948*micA* + using arabinose induction fully restored haemolysis in the BRD948*micA* (Fig 3D).

Hfq protein levels are elevated by host hormones

The small bacterial protein Hfq is an important factor that contributes to *Salmonella* virulence (Sittka et al, 2007). Interestingly, *micA* interacts with Hfq to mediate translational regulation of the *ompA* mRNA (Udekwi et al, 2005). We observed a significant (approximately 60%) increase in the levels of Hfq in cells exposed to neuroendocrine hormones (Fig 3E). This agrees with the reduction in OmpA protein levels in *S. typhi* treated with neuroendocrine hormones and the Hfq-mediated direct translational repression of OmpA protein levels in *E. coli* (Vytvytska et al, 2000).

CpxAR is required for hormone-mediated haemolysis

In *E. coli* O157:H7, neuroendocrine hormones are sensed by the QseBC and QseEF two-component systems (Rasko et al, 2008).

A defined *qseBC* deletion had no effect on the ability of *S. typhi* to generate neuroendocrine hormone-mediated haemolysis (data not shown). An *in silico* search for *qseBC* orthologues in *S. typhi* identified the *cpxA* sensor kinase, which shares approximately 29% of its amino-acid sequence identity with the QseC sensor kinase. The *cpxAR* two-component signal-transduction system is involved in the stress responses elicited on exposure to diverse envelope stresses and is important for the pathogenicity of *Salmonella in vivo* (Humphreys et al, 2004).

In *S. typhi* lacking the sensory (*cpxA*) and response regulatory (*cpxR*) components of the *cpx* system (BRD948c), neuroendocrine hormones failed to induce haemolysis (Fig 4A). Furthermore, there were no concomitant increases in the levels of either the Hfq protein (Fig 4B) or the *micA* transcript (Fig 4C,D). Complementation of the *cpx* system *in trans* from a single-copy plasmid (BRD948c *cpx+*) fully restored the haemolytic phenotype on exposure to the neuroendocrine hormone (Fig 4E,F).

DISCUSSION

The ability of pathogens to sense and respond to their environment is crucial to their capacity to cause disease. Pathogenicity

Fig 3 | Neuroendocrine hormones affect sRNA *micA* and Hfq. (A) Expression and (B) transcript levels of the sRNA *micA* were significantly increased by the neuroendocrine hormones in a reversible manner. (C) A *S. typhi* strain lacking the sRNA *micA* (BRD948micA) is non-haemolytic upon neuroendocrine hormone exposure (only epinephrine shown). (D) *In trans* overexpression of *micA* in BRD948micA + by arabinose (ARA) induction restored haemolysis in *S. typhi* to levels similar to those observed on the addition of adrenaline (BRD948; E). The addition of glucose (GLU) represses the arabinose promoter in pBAD33. (E) Protein levels of the sRNA chaperone protein Hfq increased significantly on exposure of *S. typhi* to neuroendocrine hormones (only epinephrine shown). Densitometry measurements showing levels relative to control are displayed below the western blot. Asterisk indicates significance ($P \leq 0.05$) by the Student's *t*-test. Experiments were repeated at least three times. Standard error bars are shown. E, epinephrine; NE, norepinephrine; PO, propranolol; sRNA, small RNA.

Island 18 (SPI18) contains a pore-forming haemolysin, *hlyE*, which is important for *S. typhi* virulence (Fuentes *et al*, 2008). *S. typhi* causes a systemic infection involving extracellular and intracellular phases within macrophages. Macrophages produce neuroendocrine hormones such as epinephrine and norepinephrine, which they subsequently release into the bloodstream (Flierl *et al*, 2007, 2009).

We have determined the *S. typhi* response to the host neuroendocrine hormones epinephrine and norepinephrine, leading to haemolysis through the release of membrane vesicles containing haemolysin HlyE. In *S. typhi*, *OmpA* is crucial for maintaining envelope integrity and preventing haemolysis through membrane vesicle secretion (Fuentes *et al*, 2008). We propose that the increase in haemolysis is due to the neuroendocrine hormone-mediated reduction in *OmpA* transcript and protein levels with the subsequent release of haemolysin in membrane vesicles. Indeed, increased expression of *ompA* *in trans* reversed the haemolytic effect of the addition of neuroendocrine hormones. The observed reduction in *ompA* transcription levels (Fig 2C) might indicate that alternative regulatory pathways are functioning at the transcriptional level, independently of the sRNA-mediated post-transcriptional downregulation.

Examination of the regulatory pathway controlling the production of *OmpA* revealed increased levels of the regulatory sRNA *micA*, as well as the RNA chaperone protein Hfq. The increase in Hfq levels might, therefore, represent an additional neuroendocrine hormone-mediated action to regulate *OmpA* protein levels directly, by binding to *ompA* mRNA, or indirectly, by increasing *micA* sRNA stability. The absence of neuroendocrine-mediated haemolysis in *S. typhi* lacking the sRNA *micA* (BRD948micA) supports this hypothesis. Furthermore, *in trans* overexpression of *micA* restored haemolysis in BRD948micA, highlighting its role in the regulatory cascade.

Intriguingly, the inhibition of neuroendocrine hormone-mediated haemolysis by the adrenergic β -blocker propranolol provides evidence to support the involvement of a putative new bacterial adrenergic receptor. Previous reports have suggested a role for the QseBC system in *S. typhimurium* motility and virulence (Bearson & Bearson, 2008; Rasko *et al*, 2008). However, more recently, transcriptional profiling of QseBC in *Salmonella* revealed significant differences to the previous observations, for example, regarding motility (Merighi *et al*, 2009). We have shown that, in *S. typhimurium*, QseBC does not mediate the adrenergic signalling cascade, leading to increased sensitivity to the

Fig 4 | Role of the two-component signal-transduction system *cpxAR* in *S. typhi*-mediated haemolysis. (A) Deletion of both sensor and response regulator components of the *cpx* system in BRD948c eliminates neuroendocrine hormone-mediated haemolysis by *S. typhi*. (B) Western blotting with corresponding densitometry (average of three independent experiments) indicates that levels of the sRNA chaperone protein Hfq increase on exposure of BRD948 to neuroendocrine hormones, but fail to do so in the *cpx* mutant BRD948c. There is no significant change in (C) expression or (D) transcript levels of the sRNA *micA* in BRD948c upon neuroendocrine hormone exposure, compared with the changes observed in the parent. Complementation by *cpxAR* on a single-copy plasmid in BRD948c *cpx* + fully restored neuroendocrine hormone-mediated haemolysis in a (E) liquid or (F) blood plate assay, compared with the control BRD948c pNMD. Both neuroendocrine hormones had an identical profile; only epinephrine data are shown. Asterisk indicates significant difference ($P \leq 0.05$) by the Student's *t*-test. Experiments were repeated at least three times. Standard error bars are shown. E, epinephrine; sRNA, small RNA.

antimicrobial peptide LL37 (Spencer *et al*, 2010). Furthermore, in *S. typhimurium*, it has been shown that QseBC is not required for norepinephrine-enhanced enteritis or for intestinal colonization in calves (Pullinger *et al*, 2010). In *S. typhi*, neuroendocrine hormone-mediated haemolysis is independent of the known *E. coli* O157:H7 adrenergic receptor QseBC and is mediated by the CpxAR two-component system. Mechanistically, it seems that CpxAR might contribute—either directly or indirectly—to the sensing of neuroendocrine hormones, resulting in increased levels of Hfq and the sRNA *micA*. Subsequently,

micA suppresses *ompA* translation (Udekwi *et al*, 2005), leading to the release of HlyE within membrane vesicles (supplementary Fig S1 online).

Neuroendocrine hormone-mediated release of HlyE might help host-cell invasion and pathogen survival. Thus, host neuroendocrine hormones can provide important environmental cues for *Salmonella* to navigate its way through the infectious cycle. These observations provide important insights into host-pathogen crosstalk, illustrating the way in which *Salmonella* can intercept host communication signals to its advantage.

METHODS

Bacterial strains, plasmids and growth conditions. *S. typhi* Ty2 was grown at 37 °C in 15 ml Luria broth with shaking (200 r.p.m.) or on Luria agar plates. Epinephrine and norepinephrine were used at a final concentration of 50 µM. The β-adrenergic blocker propranolol was used at a final concentration of 500 µM. *S. typhi* BRD948 (Chatfield *et al*, 1992) was used for expression analysis experiments. BRD948 was grown at 37 °C in Luria broth or agar plates supplemented with aromatic amino-acids (Chatfield *et al*, 1992). Ampicillin was used at a concentration of 50 µg ml⁻¹ and chloramphenicol at 25 µg ml⁻¹. Chromosomal gene deletion strains were constructed using λ-red (Datsenko & Wanner, 2000; <http://falkow.stanford.edu/whatwedo/wanner>). A list of strains, plasmids and primers can be found in supplementary Table S1 online.

***S. typhi* proteomics.** Bacteria were grown to an OD₆₀₀ of 1.0 and exposed to the neuroendocrine hormone or controls for 30 min, and collected and processed for two-dimensional electrophoresis. Detailed protocols are available in the supplementary information online.

Expression analysis. Expression from promoter-*lux* transcriptional expression vectors was evaluated by growing *S. typhi* BRD948 carrying the specific expression vector in 15 ml of Luria broth at 37 °C, 200 r.p.m. Transcript levels were evaluated by quantitative reverse transcription-PCR analysis using the Qiagen QuantiTect SYBR Green system and a Roche Lightcycler 480. Protocols are described in detail in the supplementary information online.

Membrane vesicle manipulation and electron microscopy. For membrane vesicle collection, cells were removed by centrifugation (20 min, 4,000 g) and the culture supernatant was filter-sterilized (0.22 µm). The sterile supernatant was subjected to ultracentrifugation at 150,000 g for 3.5 h (4 °C). The pellet (membrane vesicle) was resuspended in sterile water (1:1000 of starting culture volume) and, together with the ultracentrifugation supernatant (150SN), was stored at -80 °C. Membrane vesicle preparations were quantified by the protein content, as described previously (Mashburn-Warren *et al*, 2008, 2009). Detailed protocols for electron microscopy are available in the supplementary information online.

Haemolysis. To assess the role of neuroendocrine hormones in haemolysis, 100 µl of cells from a 16 h Luria broth culture (37 °C, 200 r.p.m.) and 5 ml of defibrinated sheep blood were added to 100 ml (46 °C—equilibrated) melted Luria broth agar. The agar was mixed and immediately poured into sterile Petri dishes (10 ml in each dish). To test the direct effect of neuroendocrine hormones and blocker on red-blood-cell lysis, a control plate without bacteria was also prepared and tested. Neuroendocrine hormones and hormone/blocker combinations were spotted (5 µl, 10 mM) on to sterile filter-paper discs placed on the agar. Incubation was continued at 37 °C for 24 h.

For the liquid haemolysis assay, 10 µl of cells from a 16 h Luria broth culture (37 °C, 200 r.p.m.), 20 µl of defibrinated sheep blood and the appropriate test compound (epinephrine, 50 µM; norepinephrine, 50 µM; propranolol, 500 µM; arabinose, 1 mM; dextrin, 30 mM) were added to a total volume of 1 ml and incubated at 37 °C for 8 h. The cells were spun down briefly and the OD₄₁₀ of the resulting supernatant was analysed using a Tecan Infinite200 spectrophotometer. Ampicillin (25 µg ml⁻¹) was used during complementation experiments involving pNMD220 derivatives.

For arabinose-inducible *micA* complementation, BRD948*micA*⁺ was preinduced for 16 h (37 °C, 200 r.p.m. arabinose, 1 mM, chloramphenicol, 20 µg ml⁻¹). Cells were washed in one volume of sterile dH₂O and 10 µl was added to the assay containing 20 µl defibrinated sheep blood, 100 µM arabinose and 20 µg ml⁻¹ chloramphenicol in a total volume of 1 ml. Assays were incubated for 8 h and OD₄₁₀ read as before.

HlyE-mediated haemolytic activity in filtered supernatants or membrane vesicle suspensions was assessed by adding 100 µl of test substance to 900 µl of a 5% (v/v) suspension of defibrinated sheep blood and incubated at 37 °C for 8 h. A membrane vesicle suspension corresponding to the vesicle content of 100 µl of filtered supernatant and the equivalent ultracentrifuged supernatant (150SN) were used for testing. The OD₄₁₀ was determined as described above.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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