Elemental iron does repress transferrin, haemopexin and haemoglobin receptor expression in *Haemophilus influenzae*

Abdulaziz A. Hasan\(^a\), Julie Holland\(^a,*\), Ann Smith\(^b\), Paul Williams\(^a\)

\(^a\) Department of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK
\(^b\) School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO 64110, USA

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Abstract

The iron repressible nature of *Haemophilus influenzae* transferrin binding proteins suggests a regulatory role for elemental iron in their expression. The existence of a *Haemophilus* ferric uptake repressor (Fur) binding motif identified in the promoter region of both *tbpA* and *tbpB* further supports this hypothesis. However, a recent study using brain heart infusion growth medium suggested that transferrin binding protein synthesis in *H. influenzae* was haem- rather than iron-regulated. The present study re-investigates this observation and using a chemically defined medium, we demonstrate that elemental iron haem or protoporphyrin IX can each regulate *Haemophilus influenzae* transferrin, haemopexin and haemoglobin receptor expression.

**Keywords:** Haemophilus influenzae; Transferrin; Iron; Protoporphyrin IX; Regulation; Transferrin-binding proteins

1. Introduction

The receptor-mediated binding of ferri-transferrin by *Haemophilus influenzae* and its subsequent utilisation as an iron source involves two iron-repressible outer membrane proteins, Tbp1 and Tbp2 [1–3]. Although the genes coding for Tbp1 and Tbp2, termed *tbpA* and *tbpB* respectively, have been cloned and sequenced [4], relatively few studies have addressed their regulation. Fresh clinical *H. influenzae* type b isolates constitutively produce Tbps and Tbp synthesis only becomes iron-repressible after prolonged in vitro passage on iron-rich laboratory media [5]. In laboratory adapted strains, receptors for both haem-haemopexin [6] and transferrin [3] are induced following growth in brain heart infusion (BHI) broth rendered iron-restricted by the addition of a non-utilisable iron chelator such as desferrioxamine or ethylenediamine-di-o-hydroxyphenylacetic acid (EDDA). These data support a role for the iron-dependent repression of *tbp* expression. Furthermore, the existence of a *H. influenzae* ferric uptake repressor (Fur) binding motif located within the promoter/operator region of *tbpA* further supports this contention [4]. However, Morton et al. [7] reasoned that BHI broth supplemented with protoporphyrin IX (PPIX) and a non-utilisable iron chelator was in fact haem- rather than iron-restricted. They therefore concluded that Tbp synthesis in *H. influenzae* is haem- rather than iron-regulated. To support this contention they reported that whole *H. influenzae* cells grown in haem-depleted BHI bound transferrin and that this binding could be abolished by
supplementing the medium with haem but not elemental iron [7]. However, BHI is an iron-replete medium and it is clear that, on a molar basis, sufficient haem to satisfy the requirements of *H. influenzae* for the porphyrin macrocycle does not provide sufficient iron [8].

To evaluate the respective roles of iron, haem and PPIX in controlling both Tbp, haemoglobin and haemopexin receptor expression and to avoid the problems associated with the use of a non-utilisable iron chelator, we have developed a chemically defined iron-depleted growth medium. Using this medium, we demonstrate that either iron, haem or PPIX, can each regulate synthesis of the transferrin, haemopexin and haemoglobin receptors of *H. influenzae*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

*H. influenzae* type b strain 760705 has been described previously [5]. Bacteria were stored at −70°C in BHI broth containing 20% (v/v) glycerol and routinely cultured on chocolate blood agar plates. All cultures were grown in liquid medium at 37°C in an atmosphere containing 5% CO₂ and growth was monitored at 605 nm. For growth in haem-deficient, iron-replete BHI (hdBHI), bacteria were cultured in BHI supplemented with 10 µg/ml β-NAD and 0.1 µg/ml haem. For some experiments, hdBHI was also supplemented with FeCl₃ (at concentrations ranging from 100 µM to 10 mM). For growth in haem-replete, iron-replete BHI (sBHI), bacteria were cultured in BHI supplemented with 10 µg/ml β-NAD and 10 µg/ml haem.
2.2. Design of an iron-depleted growth medium capable of supporting the growth of *H. influenzae*

Chemically defined RPMI 1640 medium (Sigma) was modified to support the growth of *H. influenzae* as follows: RPMI was supplemented with 6.6 μg/ml β-NAD, 0.5 μg/ml PPIX, 8 μg/ml uracil, 3.3 μg/ml hypoxanthine, 55 μg/ml L-cysteine, 99.8 μg/ml L-alanine, 0.07 mM CaCl₂·2H₂O, and 0.7 mM MgSO₄·7H₂O and the final pH was adjusted to 7.5. Iron-depletion was achieved by treating this modified medium with Chelex 100 by gently mixing 6% (w/v) Chelex-100 resin (Sigma) for 24 h prior to sterilization by filtration. This iron-depleted growth medium is termed NRPMI. The iron content of BHI, RPMI and modified RPMI media was determined using a spectrophotometric iron determination kit (Sigma).

2.3. Solid-phase dot enzyme assays

The binding of transferrin, haemopexin and haemoglobin to whole *H. influenzae* cells was evaluated using the dot enzyme assays described before [3,6]. Following 10 h growth in the appropriate medium, bacteria were pelleted, washed and resuspended in 10 mM Tris-HCl (pH 7.4) to OD₆₀₀ 1.0, and 5 μl samples spotted onto nitrocellulose membranes. For transferrin-binding assays, dot blots were probed with a human transferrin-horseradish-peroxidase conjugate (50 ng/ml) and developed with 25 μg/ml of 4-chloro-1-naphthol and 0.01% (v/v) H₂O₂ in 50 mM Tris-HCl (pH7.4). Binding to the haemopexin and haemoglobin receptors was assayed using the respective protein biotinylated as described previously [6]. For the detection of bound biotinylated proteins, nitrocellulose membranes were incubated with a streptavidin-HRP conjugate (2 μg/ml) for 2 h prior to development with 4-chloro-1-naphthol as described above. Dot blots were quantified using a Shimadzu CS-9000 flying spot scanning densitometer.

2.4. Affinity purification and electrophoresis of Tbps

Tbps were isolated as described before [5].Briefly, bacterial cells were grown overnight in the appropriate medium and after disruption by sonication, cell envelopes were collected and incubated with 50 μg biotinylated human transferrin. After incubation for 60 min at 37°C, the cell envelopes were pelleted and resuspended in 1 ml of buffer containing 2% (w/v) sodium N-lauroyl sarcosinate (Sarkosyl) and 1 mM EDTA. Following 30 min incubation, insoluble material was removed by centrifugation and 50 μl of streptavidin-agarose beads (Sigma) was added to supernatant. Following 60 min incubation at room temperature the beads were pelleted, washed and the Tbps solubilized by treatment of the beads with SDS-PAGE sample buffer at 37°C for 30 min. After centrifugation, the supernatant was subjected to
Fig. 4. Solid phase dot enzyme assays showing the binding of HRP-human transferrin conjugate to whole cells of *H. influenzae* type b strain 760705 grown in NRPMI with increasing concentrations of haem (A), haemoglobin (B) and PPIX (C). Values (arbitrary units) are means ± standard deviations.
FIG. 5. Solid phase dot enzyme assays showing the binding of haemopexin and haemoglobin to whole cells of *H. influenzae* type b strain 760705 grown in NRPMI with increasing concentrations of FeCl₃ (A), haem (B), haemoglobin (C) and PPIX (D). Values (arbitrary units) are means±standard deviations.
Fig. 5 (continued).
SDS-PAGE and stained with Coomassie blue or electrophoretically transferred to nitrocellulose. Western blots were probed with transferrin-horse radish peroxidase conjugate and developed as in Section 2.3 above.

3. Results and discussion

3.1. Growth and transferrin binding of H. influenzae in NRPMI

Treatment of NRPMI with Chelex 100 reduces its iron content to below detectable levels (1×10⁻⁶ M) making this a suitable medium to study the effect on Tbp synthesis of increasing the concentration of exogenous iron. Fig. 1 shows the growth characteristics of H. influenzae 760705 in NRPMI compared with BHI. The addition of FeCl₃ to NRPMI enhanced growth of the organism in a concentration-dependent manner up to 10 μM. At higher iron concentrations (up to 100 μM) no further stimulation of growth was noted (Fig. 1). Fig. 2 reveals that transferrin binding was greatest in bacteria grown in NRPMI with no added iron, and that increasing the concentration of iron reduced transferrin binding such that it is abolished in NRPMI medium supplemented with 10 μM FeCl₃. Furthermore, Western blotting of affinity purified Tbps confirmed that both Tbp1 and Tbp2 are induced in NRPMI but not in NRPMI supplemented with 10 μM FeCl₃ (data not shown).

These data demonstrate that simple modification of a commercially available defined medium (RPMI) facilitates the study of iron deprivation in H. influenzae since NRPMI can be employed directly to investigate the influence of medium elemental iron content on iron-regulated receptor expression.

3.2. Transferrin binding after growth in BHI

A comparison was made between the ability of 760705 to bind transferrin after growth in NRPMI or sBHI or hdBHI (Fig. 3). Transferrin binding is clearly abolished after growth in either NRPMI plus 10 μM FeCl₃ or in the haem- and iron-replete medium, sBHI (which contains 18 μM Fe³⁺). As reported by Morton et al. [7], transferrin binding to whole cells is apparent after growth in hdBHI and this binding is repressed by supplementing with haem but not with elemental iron. However, Morton et al. [7] did not quantify their data and Fig. 3 clearly shows that after growth in hdBHI, transferrin binding is less than 20% of that observed for bacteria grown in NRPMI.

Despite the abolition of transferrin binding in NRPMI supplemented with 10 μM FeCl₃, additional exogenous iron was unable to repress transferrin binding in cells grown in hdBHI. However, it is not immediately apparent why haem but not iron is capable of repressing transferrin receptor expression in hdBHI. Since BHI is complex and undefined, exogenously added iron may be rendered unavailable through interaction with medium components. The total iron content in BHI therefore may not reflect the true amount of iron available to the growing bacterial cell.

3.3. Regulation of transferrin binding by haem, haemoglobin, and PPIX

We have established that elemental iron, supplied as FeCl₃, is capable of downregulating transferrin receptor expression. To determine whether haem, haem supplied as haemoglobin, or PPIX could also repress receptor expression, H. influenzae was first cultured in NRPMI for 6 h to deplete the internal iron and PPIX pools and then inoculated into fresh NRPMI containing β-NAD and a range of concentrations of either haem, haemoglobin or PPIX (from 0 to 10 μM). Fig. 4 reveals that transferrin binding is downregulated in bacteria grown with around 10 μM haem, haemoglobin or PPIX.

The observation that increasing the PPIX content of the growth medium influenced transferrin binding was surprising. To determine whether PPIX was directly affecting transferrin receptor expression or merely competing with transferrin for a bacterial surface binding site, we affinity purified the Tbps. The levels of both Tbp1 and Tbp2 markedly diminish as the concentration of PPIX is increased such that no Tbps are affinity purified from cells grown in NRPMI containing around 10 μM PPIX (data not shown). Although these results were unexpected, growth of H. influenzae in BHI containing PPIX rather than haem has previously been shown to
lead to alterations in the outer membrane protein profiles and the appearance of novel but as yet uncharacterised polypeptides [9,10].

3.4. Regulation of haemopexin and haemoglobin receptors binding by iron, haem, haemoglobin and PPIX

To determine whether iron, haem, haemoglobin or PPIX repressed the expression of the haemopexin and haemoglobin receptors of *H. influenzae* 760705, the same experiments described in Sections 3.2 and 3.3 were repeated using the respective biotin-labelled ligand. Fig. 5 confirms that increasing the concentrations of FeCl₃, haem, haemoglobin or PPIX up to approximately 10 μM abolish both haemopexin and haemoglobin receptor expression.

4. Conclusions

Using a chemically defined, iron-depleted medium, we have clearly demonstrated that the regulation of receptors for transferrin, haemoglobin and haemopexin binding in *H. influenzae* is dependent on the available concentrations of either iron, haem or PPIX. It is probable that each of these receptors is regulated at the genetic level by the Fur repressor protein [4,11]. In *E. coli*, Fur has been reported to require Fe²⁺ as a co-repressor [12]. More recently Smith et al. [13] have obtained evidence to suggest that haem can displace iron from the *E. coli* Fur protein. It is therefore conceivable that in *H. influenzae*, iron, haem or PPIX can all function as the Fur co-repressor. Further work is currently under way to determine whether this is indeed the case.

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References