

Elucidation of the mechanism by which catecholamine stress hormones liberate iron from the innate immune defense proteins transferrin and lactoferrin

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Running title: Transferrin/lactoferrin-catecholamine interactions

Key words: transferrin and lactoferrin, catecholamine stress-hormones, iron-removal, EPR, bacteria

Abstract

The ability of catecholamine stress hormones and inotropes to stimulate growth of infectious bacteria is now well established. A major element of the growth induction process has been shown to involve the catecholamines binding to the high affinity ferric iron binding proteins transferrin and lactoferrin, which then enables bacterial acquisition of normally inaccessible sequestered host iron. The nature of the mechanism(s) by which the stress hormones perturb iron binding of these key innate immune defense proteins has not been fully elucidated. The present study employed EPR spectroscopy and chemical iron binding analyses to demonstrate that catecholamine stress hormones form direct complexes with the ferric iron within transferrin and lactoferrin. Moreover, these complexes were shown to result in the reduction of Fe(III) to Fe(II) and the loss of protein-complexed iron. Use of bacterial ferric iron uptake mutants further showed that both the Fe(II) and Fe(III) released from the Tf could be directly used as bacterial nutrient sources. We also analyzed the transferrin-catecholamine interactions in human serum and found that therapeutically relevant concentrations of stress hormones and inotropes could directly affect the iron binding of serum-transferrin, such that the normally highly bacteriostatic tissue fluid became significantly more supportive of growth of bacteria. The relevance of these catecholamine-transferrin/lactoferrin interactions to the infectious disease process is considered.

INTRODUCTION

Iron is a key nutritional element required for the growth of almost all bacteria (15, 22); therefore its sequestration by the mammalian ferric iron-binding proteins (principally transferrin, Tf, in serum, and lactoferrin, Lf, in mucosal secretions) represents a primary non-specific host-defence mechanism against microbial infection. Tf has one of the highest metal binding affinities recorded, a binding constant for ferric iron of 10^{23} M (16). The principal physiological role of serum Tf is Fe transport through the circulating blood and its release to Fe-dependent cells; its concentration in serum is usually about 35 μ M (16). Importantly, serum-Tf is not iron-replete with about 70% of it existing in the apo-form (16). Work from our laboratories has shown that the “fight or flight” catecholamine stress hormones epinephrine (Epi), norepinephrine (NE) and dopamine (Dop), and the widely used structurally similar inotropes (heart and kidney therapeutic drugs) isoprenaline and dobutamine, are all able to form complexes with Tf and Lf (7, 8, 10, 21). This complex formation is important microbiologically as it reduces the Fe-binding capability of these key innate immune defence proteins to an almost insignificant level, and renders them vulnerable to Fe theft by bacterial pathogens that would be unable to access this normally highly secure iron. We and others have shown that these catecholamines are all able to support greater than million-fold increases in bacterial growth by providing iron from Tf (1, 7-8, 10-11, 21). Significantly, in terms of their ability to deliver Tf/Lf-complexed iron to bacteria, certain pharmacologically inactive catechol-containing metabolites were also found to be similar in potency and effect to the parent catecholamine molecule (8).

The interaction between catecholamines, Tf and Lf can reduce the bacteriostatic nature of blood and serum and mucosal secretions to the extent that they become a highly supportive bacterial culture medium (7-8, 10-11, 21). This ability of stress hormones to mediate bacterial acquisition of the Tf/Lf-iron has been shown to have important clinical implications, for example, they have been proposed to have roles in sepsis due to the formation of staphylococcal biofilms in intravenous lines

(18) and in the development of stress-related intra-abdominal sepsis by Gram-negative bacteria (8). Although we and others have identified some of the molecular components which bacteria use to acquire iron from these stress hormone-Tf/Lf complexes (1, 4, 7, 9, 25), the precise mechanism(s) by which the catecholamines themselves modulate Tf- and Lf- iron-binding remain to be determined. Elucidation of the mechanism by which stress elaborated hormones enable bacterial pathogen access to host sequestered iron is therefore important both scientifically and clinically. Because the iron within Tf and Lf is in a high spin Fe(III) oxidation state (16), and therefore paramagnetic, electron paramagnetic resonance (EPR) spectrometry is an ideal tool to study the dynamics of the interaction between the catecholamines and the Tf and Lf. The present study utilises EPR spectrometry, biochemical and microbiological approaches to elucidate the mechanism by which catecholamine stress hormones and inotropes liberate Tf and Lf-complexed Fe.

MATERIALS AND METHODS

Reagents. Human serum transferrin, lactoferrin, ferrous ammonium sulfate, ferric nitrate, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4- triazine (ferrozine), and the catecholamines NE, Epi and Dop, dobutamine and isoprenaline were purchased from Sigma Chemical Co. (Poole, Dorset, UK). [^{55}Fe] FeCl_3 (IES, specific activity 5 mCi/mg Fe), was obtained from Amersham Life Science, UK.

Analysis of Tf/Lf-catecholamine interactions. Tf and Lf samples for EPR analysis were prepared by mixing 6 mg/ml of iron saturated Tf or Lf (60 μM concentration) buffered in 50 mM Tris-HCl, pH 7.5 with the concentrations of catecholamines indicated in individual experiments, or with an equivalent volume of water in the case of the control sample. Serum-Tf was analyzed in serum prepared freshly from the blood of healthy donors. The Tf/Lf-catecholamine and serum-Tf-catecholamine mixtures once prepared were either incubated at 37°C for the time indicated in the text, or frozen and analyzed immediately. samples were frozen in 0.40 ml volumes in liquid nitrogen

or helium and analyzed using either a JEOL-RE1X EPR or a Bruker EMX-500 Spectrometer. EPR spectrometer analysis parameters were: centre field: 150 mT sweep width: 80 mT, field modulation: 1.0 mT, microwave power: 10 mW, time scan: 14 min with the reaction conducted at a temperature of 77K. Analysis of Tf iron removal was also carried out using electrophoresis on urea polyacrylamide gels containing 6 M urea, as described previously (7). Ferrous iron release from Tf/Lf was monitored by complexation with the ferrous iron specific dye ferrozine (0.4 mM) and measured spectrophotometrically at 560 nm over a 24 hour period using a Varioskan spectrophotometer (Thermo, UK).

Bacterial growth and iron uptake analyses. Demonstration of the role of ferrous iron uptake systems in the mechanism of bacterial iron assimilation from Tf was carried out using enterobactin siderophore synthesis and uptake mutants (*entA* and *tonB*) of *Escherichia coli* O157:H7 (9). To test the ability of the *E. coli* O157:H7 strains to acquire iron from Tf, 5 ml of sterile SAPI medium (7, 9) buffered with 50 mM Tris-HCl, pH 7.5, was supplemented with 100 μ M NE or an equivalent volume of water. Filter-sterilized [^{55}Fe]-Tf was prepared as described in (7) and added at 2×10^5 cpm ml^{-1} (equivalent to a concentration of 10 $\mu\text{g ml}^{-1}$ Tf), either directly into the medium (in contact with the bacteria) or enclosed within 1 cm diameter dialysis membrane (4 kDa cut-off). Exponential growing bacteria were added directly to uptake assay mixtures at 2×10^8 CFU ml^{-1} , and incubated at 37°C in a 5% CO_2 atmosphere for 4 h, during which time there was essentially no additional growth. Cultures were then harvested, washed in PBS and assayed for cell numbers and [^{55}Fe] incorporation as described previously (7, 9).

To analyze the growth of bacteria in human serum after the addition of therapeutically relevant concentrations of inotropes, 10^2 CFU ml^{-1} *Staphylococcus epidermidis* was added, and the mixture incubated at 37°C in a humidified, static 5 % CO_2 incubator for 18 hrs; final cell numbers were determined by mixing, serial dilution of the cultures and plating on luria agar.

Chemical structures. The chemical complexes shown in Figure 5 were created using the ChemDraw Ultra 10 Chem Office drawing package (Cambridge Software, UK).

Statistical analyses. All experiments were performed in at least duplicate, and on at least 3 separate occasions. Where appropriate, statistical analysis was performed using an unpaired t-test in which a two-tailed P value was calculated (Instat program, GraphPad Software, San Diego, CA, USA). Statistical significance was defined as a P value of less than 0.05.

RESULTS

EPR analysis of Tf-catecholamine interactions. NE was chosen as our principal test catecholamine because of its widespread distribution in the human body and its involvement in the mammalian stress response, and because the majority of previous reports which describe the interaction of stress hormones with Tf and Lf have focused on this catecholamine (11). The EPR spectrum of iron-replete Tf in the presence of increasing NE concentrations is shown in Figure 1A. Tf alone has a di-peak EPR signal, with *g* value of 4.3 (150 mT field strength), which is universally accepted as the signature spectrum of iron-replete holo-Tf and corresponds to the presence of bound high-spin Fe(III) iron (3, 14). Addition of the catecholamine NE to Tf resulted in a rapid and distinct transformation of the Tf EPR signal (Figure 1A), with the characteristic di-peak Tf profile being replaced by a new single peak EPR signal. Figure 1A shows that the disappearance of the characteristic iron-replete Tf-iron signal in the presence of the catecholamine was concentration dependent, and the higher the molar ratio of NE:Tf, the greater the loss of the Tf-iron signal.

The emergence of a new EPR signal when Tf was incubated with NE indicates that modification of the Tf-Fe complex must have occurred; this could be due either to the release of iron from the Tf, a change in the valency of the iron, or modification of the Tf protein (7). That this new signal's emergence was associated with a physical loss of iron from the Tf was confirmed using urea PAGE analysis of the Tf-NE complex (Figure 1B); this data shows that in the presence of the

catecholamine most of the initially iron replete Tf was converted into the mono-ferric or apo-form of the protein. Since it has been demonstrated that NE can complex inorganic iron (III) salts (5, 12), we also determined the EPR spectra of NE-Fe(III) and NE- Fe(II) mixtures. We used the Fe salts at 120 μ M, a total iron concentration similar to that used in the Tf-NE experiments in Figure 1A. Figure 1C shows that NE-iron mixtures form an EPR-detectable signal with similar *g* value characteristics to the new signal produced when Tf was incubated with NE, suggesting complex formation has occurred (iron salts or NE alone are EPR silent, data not shown). Addition of an iron(II) sink in the form of ferrozine reduced the signal intensity of the Fe(III)-NE signal; this effect was possibly due to the ferrozine complexation protecting the reduced Fe from spontaneous re-oxidation (air was not excluded from our experiments). It is also notable that the signal intensity of the product formed from the NE-Fe(II) iron salt interaction was much less intense than that of the NE-Fe(III) indicating greater abundance for the NE-Fe(III) complex, and showing that at the physiological pH of our experiments NE has a greater affinity for ferric iron, a finding in agreement with other studies (5, 12).

The question then arose as to how NE was modifying the Fe(III) within the Tf protein to cause its removal. Insight into the likely oxidation state of the Fe liberated from Tf can be seen in Figure 1D, which shows a urea gel of the iron binding status of NE-treated Tf in the presence of ferrozine, an Fe(II)-specific dye. Comparison of Figure 1D with Figure 1B reveals that the presence of the Fe(II) sink results in a much more rapid and extensive removal of Tf-iron by the catecholamine. This observation is confirmed in Figure 1E, which shows that the production of Fe(II) from the NE-treated Tf in Figure 1D correlated with the catecholamine-mediated loss of iron from the protein; importantly the iron (II) sink used, ferrozine, alone had no effect on Tf-iron binding (data not shown). Although Tf has, at 10^{-22} M, one of the highest binding affinities for iron in the biological world, this affinity is specific for ferric iron not ferrous (16), which may explain the loss of iron observed.

Previous work from our laboratories has shown that in addition to NE, a number of other catecholamines and structurally related molecules are also capable of liberating Tf-complexed iron and providing it to bacteria for growth (1, 7-8, 10, 18, 21). We therefore undertook analyses similar to those shown in Figure 1 for Epi and Dop (Figure 2). Epi (Figures 2A-D) clearly liberates Tf-complexed iron by a mechanism similar to that demonstrated for NE, specifically, by iron reduction. In contrast, while we have clearly shown that Dop can remove Tf-iron (8, 10, 21), a similar analytical approach to that used for NE and Epi produced a somewhat different spectrum of data. Figure 2E-H shows that the EPR spectrum of Tf in the presence of Dop underwent the characteristic change indicative of catecholamine-iron binding, however higher concentrations of dopamine and longer incubation (24 hours) as well as the presence of a ferrous iron sink (ferrozine) were required to achieve a clear EPR signal change (Figure 2E). Incubating Dop with Tf and ferrozine did not result in the expected Fe(II)-ferrozine complex formation, and the mixture became a dark brown colour which also showed absorption at 560 nm (Figure 2F); further investigations showed this was due to the oxidation of the dopamine (data not shown). Urea gel electrophoresis, however, showed that there was still Dop-induced loss of iron from Tf which was enhanced in the presence of ferrozine, though the overall iron loss from Tf (compare Figures 2G-H) was less than that observed with the other catecholamines (Figure 1B-D and Figure 2B-C). This difference in catecholamine potency may have its explanation in the chemical nature of the anion of the catecholamine-salts. NE and Epi were purchased as bitartrate salts (the clinically used formulations) whereas dopamine is obtainable only as a hydrochloride. Fortuitously, Epi is available both as bitartrate and hydrochloride salt, and therefore we were able to carry out the analyses shown in Figure 2B-D using Epi hydrochloride. The results obtained were very similar to those shown for Dop (data not shown), and revealed that while catecholamine-mediated Fe(III) reduction of Tf-iron was occurring, the chloride anion interfered with this process, probably by competing for the ferric iron pool (by forming FeCl_3) and so reducing the available iron for the catecholamine complex formation.

Additional analyses of the type shown in Figures 1 and 2 were also performed using the synthetic catecholamine inotropic agents isoprenaline and dobutamine, which we had previously shown were also provide to bacteria Tf and Lf-complexed iron (8, 21). Both catecholamines produced results that indicated their mechanism of iron removal from Tf involved ferric iron reduction (data not shown).

Lf-catecholamine interactions. Previous studies (7, 8) have shown that NE and other catecholamines can also facilitate removal of iron from Lf and supply it for bacterial growth. Therefore, we tested whether the mechanism demonstrated for Tf, involving catecholamine-mediated ferric iron reduction, could be applicable to Lf as well. Figure 3A shows the change in EPR spectrum of NE-treated Lf, which parallels the profile of the EPR changes observed with Tf (Figure 1A). Lactoferrin resolves very poorly on urea gels (7), therefore we were not able to directly demonstrate Fe loss from Lf using urea gel electrophoresis. However, Figure 3B illustrates the time course of ferrozine complexation of NE-released Lf-iron and shows a clear time dependent increase in ferrozine-Fe(II) production. This indicates that the mechanism by which NE liberated Lf-iron is similar to that shown for Tf and involves reduction of the Lf-associated Fe(III). What was significantly different about NE-Lf interactions from those shown with Tf was the time taken for the catecholamine to remove the Lf-complexed iron; as monitored by EPR spectrometry, iron removal (and signal change) typically required up to 72 hrs, while for Tf this process typically required less than 2 hrs.

Bacterial uptake of Tf-iron in the presence of catecholamines utilizes both ferrous and ferric uptake systems. Multiple reports have shown a positive correlation between growth stimulation by NE (and other catecholamines) and bacterial acquisition of iron from Tf (1, 7-9, 18, 21). Additionally, we and other groups have demonstrated that for bacteria such as *Bordetella*, *E. coli*,

and *Salmonella* siderophore-based ferric iron acquisition systems are central elements in the mechanism of this growth induction process (1, 7, 9, 25). Our initial studies suggested that the role of the siderophore is to bind and internalise the catecholamine-released Tf iron (9).

Table 1 shows the influence of NE on bacterial uptake of [^{55}Fe] from [^{55}Fe]-Tf on wildtype *E. coli* and ferric iron acquisition mutants (deletions in *entA* and *tonB*) (9) when in direct contact with the ^{55}Fe -Tf, or with the [^{55}Fe]-Tf sequestered away in dialysis membrane (but still accessible to any low molecular weight iron-chelating solutes released by the bacteria). Overall, whether in direct or indirect contact, the siderophore synthesis and uptake mutants assimilated from the [^{55}Fe]-Tf much less [^{55}Fe] than their wildtype parent, thereby demonstrating for *E. coli* the importance of a complete ferric iron acquisition system in uptake of Tf-iron. For wildtype bacteria, NE significantly increased uptake of [^{55}Fe] in both contact and non-contact conditions ($P < 0.001$). In contrast, for the ferric iron acquisition mutants the influence of the catecholamine was dependent on the proximity of the bacteria to the [^{55}Fe]-Tf. When they were sequestered within the dialysis tubing, not only did the mutants acquire less iron than wildtype, but the presence of NE could significantly reduce [^{55}Fe] incorporation ($p < 0.001$). In contrast, when the siderophore mutants (which are wildtype for ferrous iron uptake) were in close physical association with the [^{55}Fe]-Tf the catecholamine significantly enhanced [^{55}Fe] incorporation ($p < 0.001$). Since we have clearly shown in the current report that catecholamines can reduce Tf-iron, this enhanced provision of [^{55}Fe] by NE in ferric iron uptake mutants indicates that bacterial ferrous iron acquisition systems are also providing an additional route of entry of Tf-sequestered iron into the bacterial cell.

The possible clinical significance of the catecholamine-Tf interaction. The experiments described so far have used high concentrations of catecholamines to elucidate the mechanism by which stress hormones and inotropic agents liberate Tf and Lf-iron. A question important to human healthcare is whether at therapeutic catecholamine concentrations (μM) these widely utilized drugs, which may be

prescribed to up to half of intensive care patients (23) might affect the integrity of Tf iron binding to the extent that they reduce the bacteriostatic nature of blood. We therefore isolated fresh serum from whole human blood, added concentrations of NE and Dop attainable in clinical settings (up to 10 μ M) (26), and assessed changes in both the EPR spectrum of the serum Tf-Fe, and the ability of the same serum to restrict bacterial growth. As can be seen (Figure 4A), after 24 hrs treatment the clinically attainable concentration of inotropes consistently caused a reduction in the intensity of the serum-Tf EPR signal. We also analyzed the ability of this serum to modulate the growth of infectious bacteria (in this case a 18 hr incubation with a 10^2 CFU/ml inoculum of *Staphylococcus epidermidis*, a skin bacterium known to cause nosocomial infections (6, 18, 21). We found that compared with the growth levels of control cultures, NE and Dop-treatment of the serum typically increased the growth of the bacteria a factor of between 3 and 7-fold (in the case of Figure 4B the reduction was 4 and 3.5-fold, respectively) ($P<0.01$). This indicates that clinically relevant doses of inotropic agents can compromise the iron-binding integrity of Tf, and by so doing, potentially make serum less bacteriostatic and thereby render blood more supportive of the proliferation of infectious bacteria.

DISCUSSION

Iron is an essential nutrient for the growth of the majority of bacteria and, especially in the case of pathogenic species, accessing host-iron normally sequestered by Tf and Lf can be a decisive factor in determining the outcome of an infection (15, 22). Previously, we and others have shown that treatment of Tf or Lf with the catecholamine stress hormones NE, Epi and Dop or the inotropic agents isoprenaline and dobutamine resulted in the release of iron from the Tf or Lf iron-protein complex, which then became accessible for bacterial uptake and growth (1, 7-9, 10, 21). In the current study we demonstrate that the mechanism by which this host iron accession process occurs is via direct catecholamine binding of Tf/Lf-complexed iron, with the resultant reduction of the Tf/Lf-coordinated Fe(III) to Fe(II), an iron valency for which these iron sequestering proteins have much

reduced binding affinity (16). Although it has been shown that bacterial siderophore-based Fe(III) uptake systems are integral elements in the catecholamine growth induction process (1, 4, 7, 9) in the current report we also demonstrate that catecholamines can deliver host-sequestered iron to bacteria through Fe(II) as well as Fe(III) uptake systems.

Our EPR spectroscopy findings that NE, Epi and Dop could complex Tf-iron and alter the EPR signal from Tf are in general agreement with the work of Borisenko *et al* (3). These workers examined the interaction of Tf with a neurotoxic analogue of dopamine, 6-hydroxydopamine (6-OHDA), as part of a study aimed at understanding both the mechanism of toxicity of 6-OHDA, and its possible use as a chemotherapeutic drug for the treatment of patients with neuroblastoma. Borisenko *et al* showed that the interaction between 6-OHDA and Tf-associated iron resulted in the disappearance of the characteristic double peak EPR signal of iron replete-Tf and the appearance of a single peak EPR signal at 150 mT (3), a value similar to that seen in the current study. As well as being important for bacterial growth in host tissues (11), reduction of protein-complexed Fe(III) by the catecholamines has been shown to regulate host enzyme activity. For instance, control of the rate limiting step in catecholamine biosynthesis in mammalian systems (tyrosine synthesis by phenylalanine hydroxylase) is achieved by complexation and reduction of the enzyme active site Fe(III) by the end products NE, Epi and Dop (20). Interestingly, NE and Epi were not found to bind to the ferrous form of phenylalanine hydroxylase (20). Our data is also consistent with chemical analyses that shown that catecholamines can bind inorganic Fe(III) salts (5, 12).

Transferrin has its greatest affinity for ferric iron (16), and conceptually, the comparatively weaker iron binding affinity of the catecholamine leads us to speculate the following order of events in the iron removal process: catecholamine binds to the Fe(III) within the Tf, reduces it, and the NE-Fe(II) complex then dissociates (we have found that both NE and Tf-iron are internalised by bacteria) (6, 9). However, this released Fe(II) is likely when under aerobic conditions to rapidly re-oxidise, and therefore be re-bound by the now iron-depleted Tf. If a sink for the Fe(II) was present, say in the

form of ferrozine, the Fe(II) would be removed from the pool of re-bindable iron, and a net loss of iron observed from the Tf, which we see in the urea gels of Tf incubated with NE and ferrozine in Figures 1 and 2.

Urea gel electrophoresis of NE, Epi or Dop-treated Tf also showed that all of the catecholamines appeared to preferentially remove Tf-iron from the N-terminal iron-binding domain of Tf (Figures 1B and C, Figure 2B and C and G and H), suggesting that the N-terminal domain could be the initial interaction point for the catecholamines. This result is not entirely surprising since other studies have shown that although the N- and C- lobe share a 56% similarity, the rate of iron release from the C-lobe is considerably slower than that of the N-lobe (24). Although Tf and Lf show considerable homology in protein sequence and structure, and coordinate iron in a similar fashion, they nonetheless display significant differences in their affinity for ferric iron. Wally *et al* (24) showed that these differences lie in the variation in the structure of their inter-lobe linker; this region, which is helical in Lf, and unstructured in Tf, making the removal of iron more difficult from Lf. This could explain the comparative differences in the potency of catecholamine effects on Tf- and Lf-iron removal observed in our current study.

The complexation of Tf-or Lf-iron by the catecholamines provides an important vehicle by which pathogenic bacteria may access normally inaccessible host-sequestered iron stores. We and others have previously shown, using mutants for enterobactin synthesis and ferric uptake systems, that enterobactin is essential for inducing catecholamine-stimulated growth of enteric pathogens such as *Bordetella* (1) *E. coli* (9) and *Salmonella* (25). We also determined that the presence of a dihydroxybenzoyl moiety was an essential element in the ability of stress hormones, inotropes and their metabolites to stimulate bacterial growth through provision of Tf/Lf iron (4, 7, 21). Mechanistically, the dihydroxybenzoyl moiety is also important for Fe(III) binding in catecholate siderophores such as enterobactin (15, 22). Figure 5 shows a molecular comparison of the structure of the norepinephrine-Fe complex with that of the enterobactin-Fe complex. What is most striking is

the obvious similarity in the geometry of iron complexation by the two catechol-containing molecules. Enterobactin, like the catecholamine stress hormones, can also liberate Tf-iron though its specificity is for ferric iron (15, 22). NE possesses both of these properties of enterobactin, but differs in that it reduces the ferric iron it binds. This suggests that NE and the other catecholamines might be best considered as a kind of pseudo- siderophore.

In terms of the further relevance of catecholamine-Tf/Lf interactions to the infectious disease process, we must consider the site where bacteria, catecholamines and Lf are most often co-localized, the gut (8, 19). This current work could provide insights into the mechanism by which elevations in catecholamine levels which occur during acute stress, can cause the often-observed overgrowth and translocation of the gut microflora (2, 17). Our finding that catecholamine-mediated removal of Lf iron requires greater than 24 hrs is consistent with the findings that response of gut microflora to the changes in systemic stress hormone levels can occur a day or more after the stress event (17). It should not then be surprising, given this potentially dangerous scenario of catecholamine-Lf-bacterial interactions, that mammals have evolved mechanisms to tightly regulate levels of gut catecholamines and that catecholamine-degrading enzymes are present throughout the entire length of the GI tract (13). Analysis of the gut tissue distribution of the human phenol sulfotransferase family of catechol-inactivating enzymes show a close correlation with bacterial presence and numbers, with expression lowest in the stomach and greatest in the large intestine and colon (13).

As well as elucidation of the mechanism by which stress hormones can modulate Tf and Lf binding of iron, EPR analysis of catecholamine-Tf interactions could also have potential application in human clinical diagnostics. Surveys of hospital drug use have shown that approximately half of patients in intensive care units may receive several days of catecholamine inotrope support to maintain heart and kidney function (23, 26); it is also generally recognized that the rate of bacterial infections in intensive care unit patients are significantly higher than those patients in other hospital wards. Our data (Figure 4) shows that exposure of serum to pharmacological concentrations of

inotropes can cause changes in the EPR spectrum of serum-Tf that can be correlated directly with a reduction in its ability to inhibit bacterial growth. But what clinical implications would there be if Tf-inotropic drug complex formation was occurring in critically-ill patients? Could such complexation destabilize serum-Tf to such an extent that it would effectively become a bacterial Fe source? The sensitivity and speed of EPR would enable an examination of Tf-Fe binding status to be performed in minutes, and could provide a valuable assessment of the infection susceptibility status of severely ill patients. Examining this possibility is a current objective of our laboratories.

FOOTNOTE

Sara M. Sandrini and Raminder Shergill contributed equally to this paper.

ACKNOWLEDGEMENTS

Raminder Shergill is grateful to the EPSRC for financial support. Sara Sandrini was supported by a University of Leicester PhD studentship

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TABLES AND FIGURES

	Non-contact - NE	Non-contact + NE	Contact - NE	Contact + NE
Wild type	4929 +/-112	30697 +/-887	37179 +/-213	128653 +/-1213
<i>entA</i>	706 +/-56	198 +/-12	10056 +/-98	17199 +/-144
<i>tonB</i>	1246 +/-51	723 +/-15	11094 +/-319	28477 +/-210

Table 1 Uptake of Fe from Tf by *E. coli* ferric siderophore mutants

Exponential cultures of the *E. coli* O157:H7 strains shown were inoculated at 2×10^8 CFU/ml into SAPI +/- 100 μ M NE along with 2.0×10^5 cpm of [^{55}Fe]-TF, either in direct proximity to the bacteria ('Contact') or with the bacteria partitioned away into dialysis tubing ('Non-contact'). After incubation for 6 hours bacteria were harvested and measured for [^{55}Fe]-incorporation (cpm/ml) and cell numbers as described in Materials and Methods. Analysis of viable plate counts revealed no significant differences in cell numbers between control and catecholamine supplemented cultures (data not shown). The data shown are the means of triplicate assays; standard deviations are shown in brackets.

Figure 1 Transferrin-NE interactions

Panel A. EPR spectrum of 60 μM holo-Tf (equivalent to 120 μM total iron) in the absence and presence of increasing concentrations of NE. Panels B and D, Urea gels showing time course of NE-mediated iron removal of 20 μM Tf incubated with 4 mM NE in the absence (B) and presence (D of an Fe(II) sink (0.4 mM ferrozine); 40 μg of Tf was loaded per gel track. The numbers at the top of each gel show length of incubation (minutes). Abbreviations: partially iron saturated-Tf (C-mono, N-mono), iron saturated holo-Tf and iron free apo-Tf were used as protein markers. Panel C., EPR spectrum of 120 μM inorganic Fe(III) in the absence and presence of 12 mM NE +/- 0.4 mM ferrozine compared with a similar concentration of an Fe(II) salt. Note that the Fe salts alone produced no EPR spectrum, indicated by the linear trace on the EPR profile shown. Panel E., Time course of Fe(II) production from 20 μM Tf incubated with 4 mM NE in the absence (squares) and presence (circles) of an Fe(II) sink (0.4 mM ferrozine). Monitoring of ferrozine-Fe(II) complex formation was made using a Varioskan densitometer set at 560 nm.

Figure 2 Tf-Epi and Tf-Dop interactions

Panel A. EPR spectrum of 60 μM holo-Tf in the absence and presence of increasing concentrations of Epi. Panels B and C., Urea gels showing time course of iron removal of 20 μM Tf incubated with 4 mM Epi in the absence (B) and presence (C) of an Fe(II) sink (0.4 mM ferrozine) (see figure 1 for definition of the Tf abbreviations); the numbers at the top of each gel show length of incubation (minutes). Panel D., Time course of Fe(II) production from 20 μM Tf incubated with 4 mM Epi in the absence (closed squares) and presence (closed circles) of an Fe(II) sink (0.4 mM ferrozine). Monitoring of ferrozine-Fe(II) complex formation was made using a Varioskan densitometer set at 560 nm. Panel E. PR spectrum of 60 μM holo-Tf in the presence of 12 mM Dop alone or in the presence of an Fe(II) sink (0.4 mM ferrozine). Panel F., Time course of Fe(II) production from 20

μM Tf incubated with 4 mM Dop in the absence (squares) and presence (circles) of 0.4 mM ferrozine. Panels G and H., Urea gels showing time course of iron removal of 20 μM Tf incubated with 4 mM Dop in the absence (B) and presence of an Fe(II) sink (0.4 mM ferrozine).

Figure 3 Lf-NE interactions

Panel A. EPR spectrum of 60 μM Lf in the presence of increasing concentrations of NE. The symbol '+' shows the spectrum of Tf incubated with NE and 0.4 mM ferrozine. Panel B., Time course of Fe(II) production from 20 μM iron saturated Lf incubated with 4 mM NE in the absence (squares) and presence (circles) of 0.4 mM ferrozine.

Figure 4 Human serum-Tf interactions with NE and Dop

Panel A The Freshly isolated whole serum from healthy volunteers was incubated for 72 hrs at 37°C with no additions (Control, which comprised the same volume of solvent used for the catecholamines), or 10 μM additions of norepinephrine (NE), or dopamine (Dop).

Panel B. The comparative growth levels of an inoculum of 10² CFU/ml *Staphylococcus epidermidis* in the serum samples shown in panel A after 18 hrs incubation at 37°C in a humidified static 5 % CO₂ incubator; bacteria were enumerated as described in Materials and Methods. Results shown are the combined data from 3 separate growth analyses.

Figure 5 NE can act as a bacterial pseudo-siderophore

The models show the similarity of structures of norepinephrine-Fe and enterobactin-Fe complexes.

Figure 1

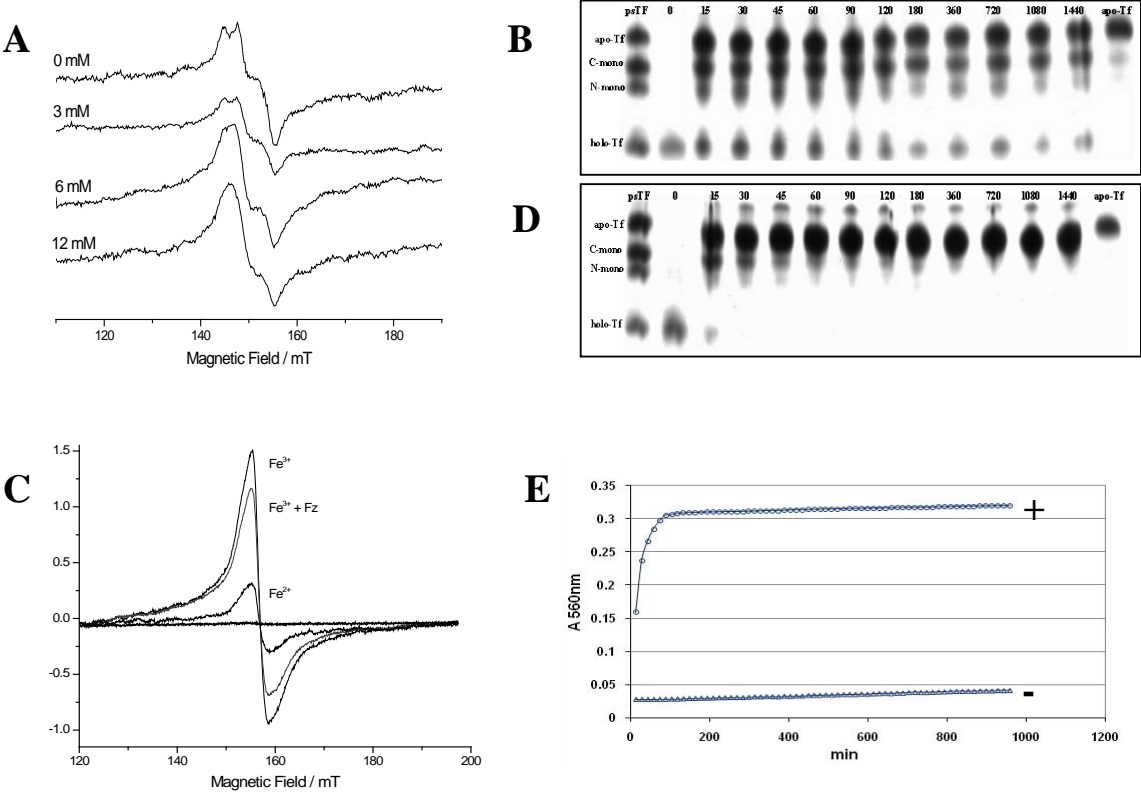


Figure 2

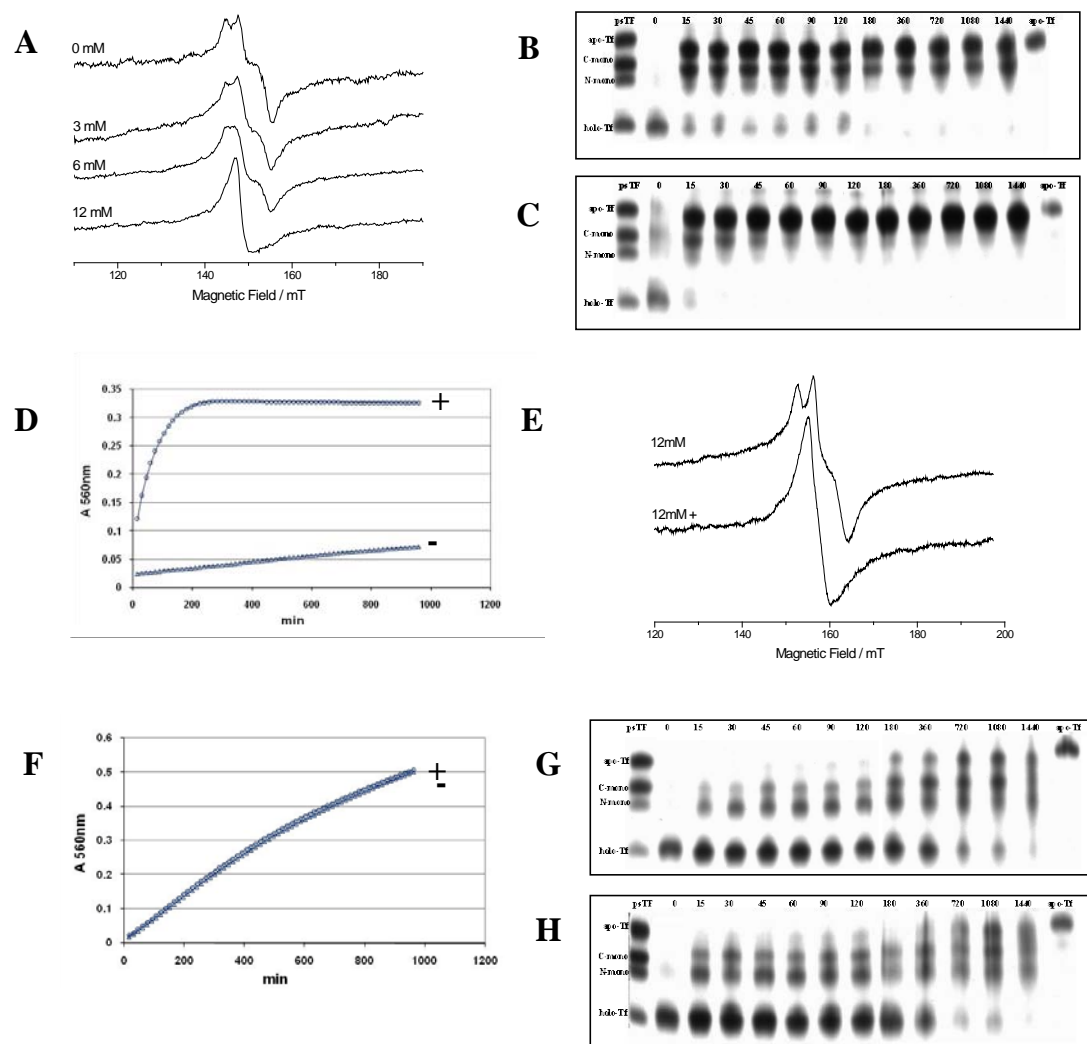


Figure 3

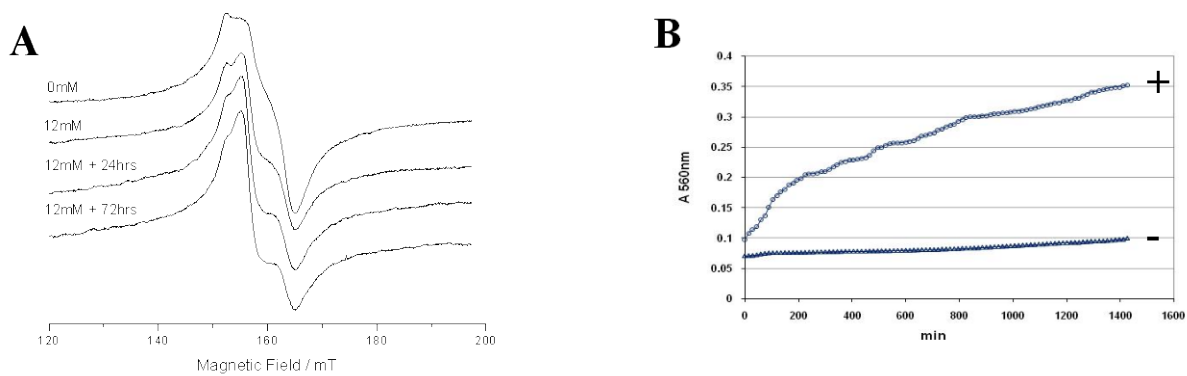


Figure 4

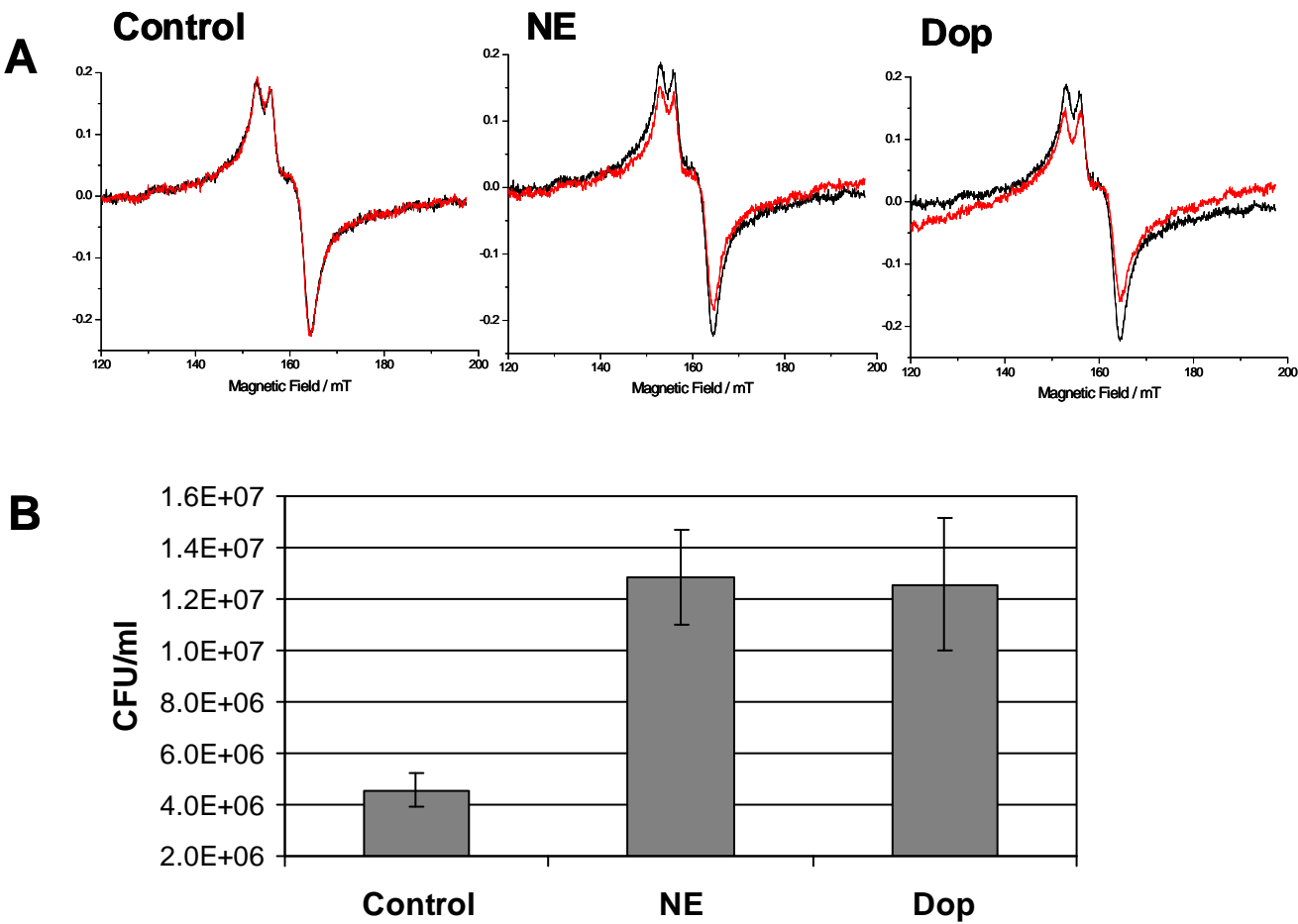
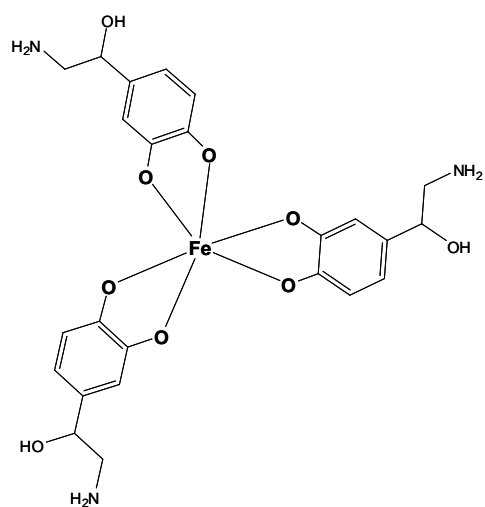
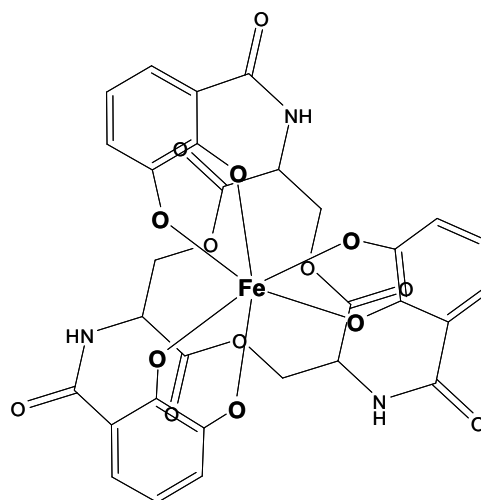


Figure 5



Norepinephrine-Fe



Enterobactin-Fe