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Elucidation of the Mechanism by Which Catecholamine Stress Hormones Liberate Iron from the Innate Immune Defense Proteins Transferrin and Lactoferrin[▽]

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The ability of catecholamine stress hormones and inotropes to stimulate the 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 (Tf) 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 electron paramagnetic resonance 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. The 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 so that the normally highly bacteriostatic tissue fluid became significantly more supportive of the growth of bacteria. The relevance of these catecholamine-transferrin/lactoferrin interactions to the infectious disease process is considered.

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 nonspecific host defense mechanism against microbial infection. Tf has one of the highest metal binding affinities recorded, with 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 μM (16). Importantly, serum Tf is not iron replete, with about 70% of it existing in the apo form (16). Work in 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 defense 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-millionfold 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 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 that 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 Tf and Lf. The present study utilized EPR spectrometry, biochemical, and mi-

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crobiological 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, Dop, dobutamine, and isoprenaline were purchased from Sigma Chemical Co. (Poole, Dorset. United Kingdom). $^{55}FeCl_3$ (IES; specific activity, 5 mCi/mg Fe), was obtained from Amersham Life Science, United Kingdom.

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 sera prepared freshly from the blood of healthy donors. Once the Tf/Lf-catecholamine and serum-Tf-catecholamine mixtures were prepared, they 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. The EPR spectrometer analysis parameters were as follows: center 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 77 K. 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-h period using a Varioskan spectrophotometer (Thermo, United Kingdom).

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 μM NE or an equivalent volume of water. Filter-sterilized ^{55}Fe-Tf was prepared as described previously (7) and added at 2×10^5 cpm ml^{-1} (equivalent to a concentration of 10 μg ml^{-1} Tf) either directly into the medium (in contact with the bacteria) or enclosed within a 1-cm-diameter dialysis membrane (4-kDa cutoff). Exponentially 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 phosphate-buffered saline (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 was incubated at 37°C in a humidified, static 5% CO_2 incubator for 18 h; the final cell numbers were determined by mixing, serial dilution, and plating of the cultures on Luria agar.

Chemical structures. The chemical complexes (see Fig. 5) were created using the ChemDraw Ultra 10 Chem Office drawing package (Cambridge Software, United Kingdom).

Statistical analyses. All experiments were performed at least in duplicate and on at least 3 separate occasions, and standard deviations were included. Where appropriate, statistical analysis was performed using an unpaired *t* test in which a two-tailed *P* value was calculated (Instat; GraphPad Software, San Diego, CA). 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 that described the interaction of stress hormones with Tf and Lf focused on it (11). The EPR spectrum of iron-replete Tf in the presence of increasing NE concentrations is shown in Fig. 1A. Tf alone has a dipeak EPR signal, with a *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 (Fig. 1A), with the characteristic dipeak 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 to Tf, the greater the loss of the Tf-iron signal.

The emergence of a new EPR signal when Tf was incubated with NE indicated that modification of the Tf-Fe complex must have occurred; this could be due to either 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 (Fig. 1B); these data show that in the presence of the catecholamine most of the initially iron-replete Tf was converted into the monoferric or apo form of the protein. Since it has been demonstrated that NE can complex with 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 μM, a total iron concentration similar to that used in the Tf-NE experiments shown in Fig. 1A. Figure 1C shows that NE-iron mixtures form an EPR-detectable signal with *g* value characteristics similar to those of the new signal produced when Tf was incubated with NE, suggesting complex formation had 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 reoxidation (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 of 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 gained from Fig. 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 Fig. 1D with Fig. 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 Fig. 1E, which shows that the production of Fe(II) from the NE-treated Tf in Fig. 1D was 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, at 10^{-22} M, has one of the highest binding affinities for iron in the biological world, this affinity is specific for ferric iron, not ferrous iron (16), which may explain the loss of iron observed.

Previous work in our laboratories and that of others has showed that, in addition to NE, a number of other cat-

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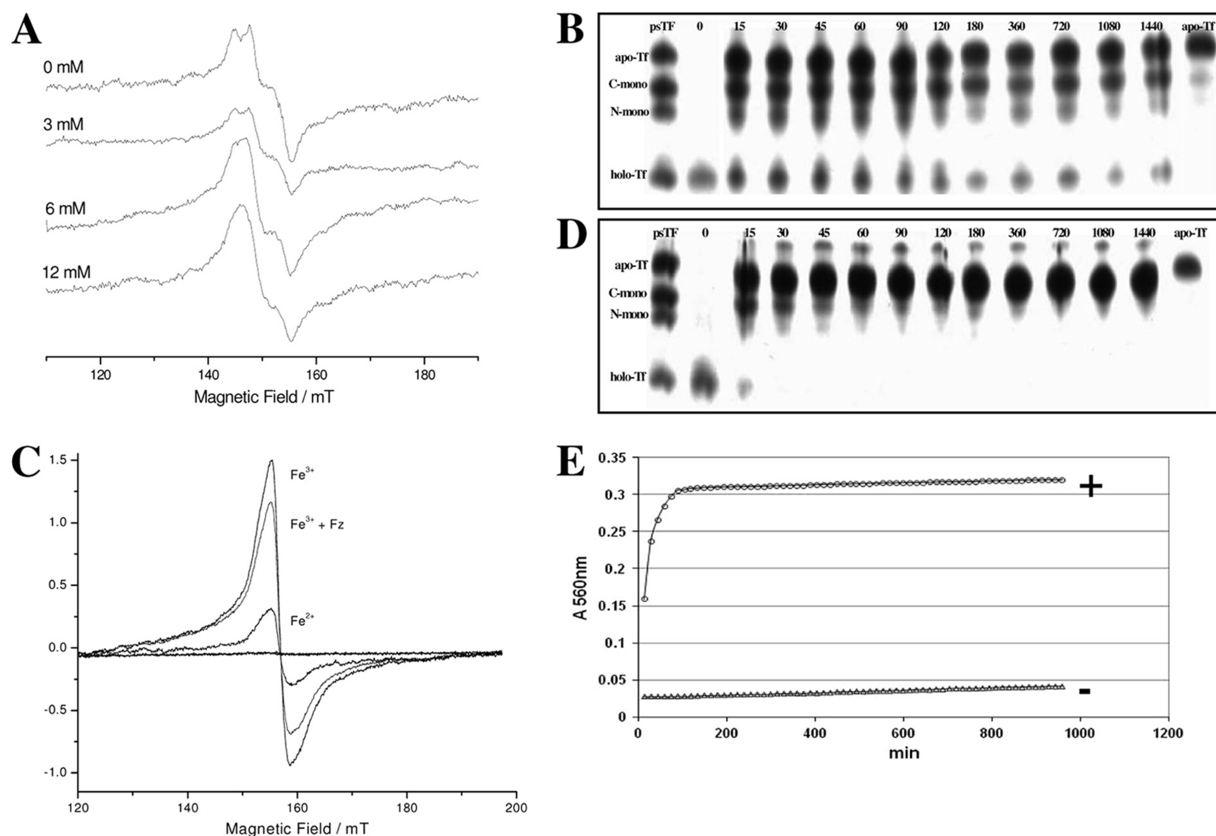

FIG. 1. Transferrin-NE interactions. (A) EPR spectra of 60 μM holo-Tf (equivalent to 120 μM total iron) in the absence and presence of increasing concentrations of NE. (B and D) Urea gels showing the time course of NE-mediated iron removal of 20 μM Tf incubated with 4 mM NE in the absence (B) and presence (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 the length of incubation (minutes). Partially iron-saturated Tf (C-mono and N-mono), iron-saturated holo-Tf, and iron-free apo-Tf were used as protein markers. (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 and NE alone produced no EPR spectrum, as indicated by the linear trace on the EPR profile shown. (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 done using a Varioskan densitometer set at 560 nm.

echolamines 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 Fig. 1 for Epi and Dop (Fig. 2). Epi (Fig. 2A to 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), an analytical approach similar to that used for NE and Epi produced a somewhat different spectrum of data. Figure 2E to 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 h), as well as the presence of a ferrous iron sink (ferrozine), were required to achieve a clear EPR signal change (Fig. 2E). Incubating Dop with Tf and ferrozine did not result in the expected Fe(II)-ferrozine complex formation, and the mixture became dark brown, which also showed absorption at 560 nm (Fig. 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 Fig. 2G and H) was less than that observed with the other catecholamines (Fig. 1B to D and 2B and 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 a hydrochloride salt, and therefore, we were able to carry out the analyses shown in Fig. 2B to 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 the process, probably by competing for the ferric iron pool (by forming $FeCl_3$) and so reducing the iron available for catecholamine complex formation.

Additional analyses of the type shown in Fig. 1 and 2 were also performed using the synthetic catecholamine inotropic agents isoprenaline and dobutamine, which we had previously shown can also provide Tf- and Lf-complexed iron to bacteria

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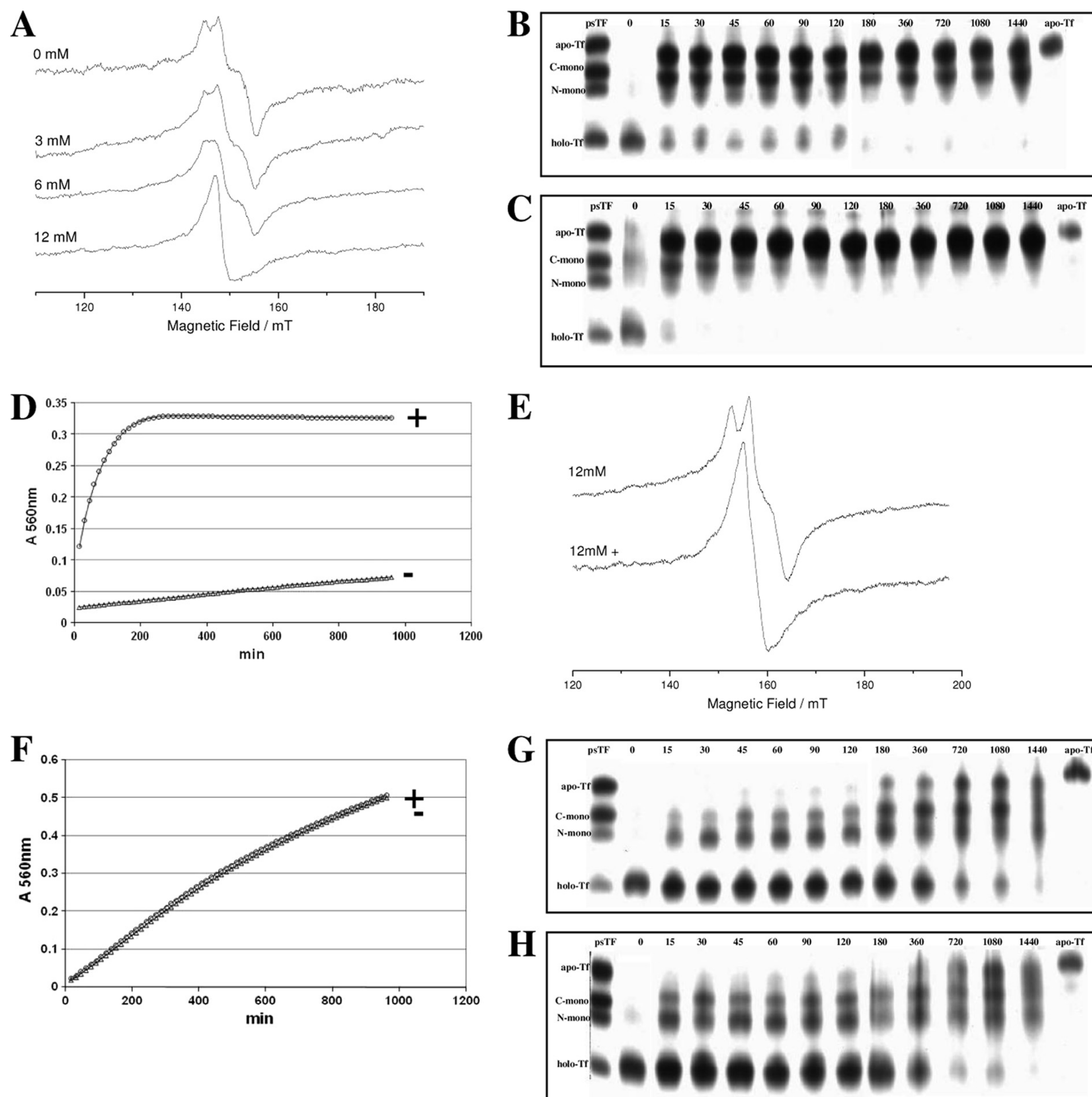

FIG. 2. Tf-Epi and Tf-Dop interactions. (A) EPR spectrum of 60 μM holo-Tf in the absence and presence of increasing concentrations of Epi. (B and C) Urea gels showing the time course of iron removal of 20 μM Tf incubated with 4 mM Epi in the absence (B) and presence (C) of an Fe(II) sink (0.4 mM ferrozine); the numbers at the top of each gel show the length of incubation (minutes). (D) Time course of Fe(II) production from 20 μM Tf incubated with 4 mM Epi in the absence (−, squares) and presence (+, circles) of an Fe(II) sink (0.4 mM ferrozine). Monitoring of ferrozine-Fe(II) complex formation was done using a Varioskan densitometer set at 560 nm. (E) EPR 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) (12 mM +). (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. (G and H) Urea gels showing the time course of iron removal of 20 μM Tf incubated with 4 mM Dop in the absence (G) and presence (H) of an Fe(II) sink (0.4 mM ferrozine).

(8, 21). Both catecholamines produced results that indicated their mechanisms of iron removal from Tf involved ferric iron reduction (data not shown).

Lf-catecholamine interactions. Previous studies (7, 8) had shown that NE and other catecholamines can also facilitate the removal of iron from Lf and can 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 the EPR spectrum of NE-treated Lf, which parallels the profile of the EPR changes observed with Tf (Fig. 1A). Lactoferrin resolves very poorly on urea gels (7), and therefore, we were not able to directly demonstrate Fe loss from Lf using urea gel electrophoresis. However, Fig. 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

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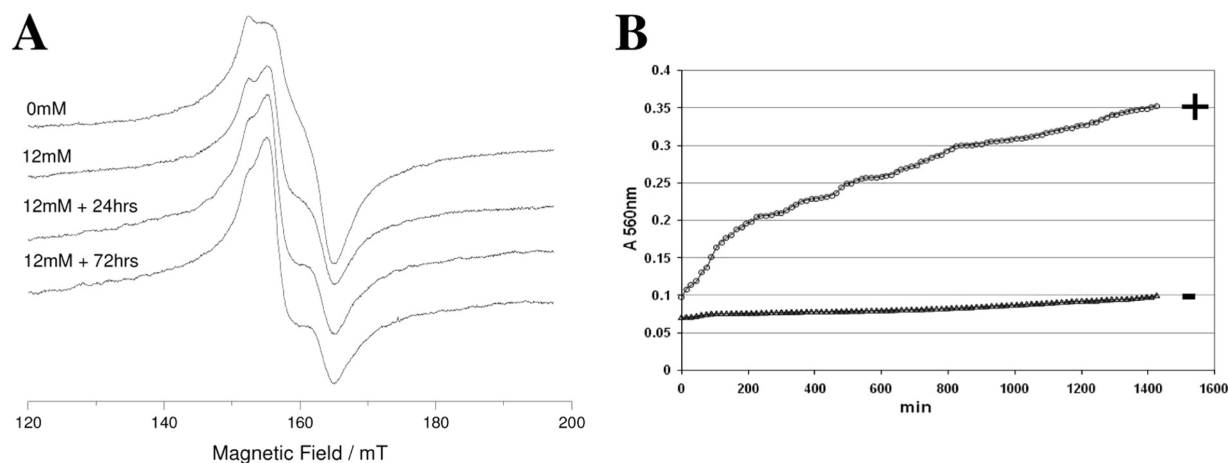

FIG. 3. Lf-NE interactions. (A) EPR spectrum of 60 μM Lf in the presence of increasing concentrations of NE. The symbol + indicates the spectra of Tf incubated with NE and 0.4 mM ferrozine. (B) Time course of Fe(II) production from 20 μM iron-saturated Lf incubated with 4 mM NE in the absence (−, squares) and presence (+, circles) of 0.4 mM ferrozine.

reduction of the Lf-associated Fe(III). What was significantly different between NE-Lf interactions and 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 h, while for Tf this process typically required less than 2 h.

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 the growth induction process (1, 7, 9, 25). Our initial studies suggested that the role of the siderophore is to bind and internalize the catecholamine-released Tf-iron (9).

Table 1 shows the influence of NE on bacterial uptake of ^{55}Fe from ^{55}Fe-Tf on wild-type *E. coli* and ferric iron acquisition mutants (with deletions in *entA* and *tonB*) (9) when in direct contact with the ^{55}Fe-Tf or with the ^{55}Fe-Tf sequestered in a 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 much less ^{55}Fe from the ^{55}Fe-Tf than their wild-type parent, thereby demonstrating for *E. coli* the importance of a complete ferric iron acquisition system in the uptake of Tf-iron. For wild-type bacteria, NE significantly increased uptake of ^{55}Fe under both contact and noncontact 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 the wild type, but the presence of NE could significantly reduce ^{55}Fe incorporation ($P < 0.001$). In contrast, when the siderophore mutants (which are wild type 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 also provide an additional route of entry of Tf-sequestered iron into the bacterial cell.

TABLE 1. Uptake of Fe from Tf by *E. coli* ferric siderophore mutants[a]

Strain	^{55}Fe incorporation (cpm/ml)			
	Noncontact		Contact	
	−NE	+NE	−NE	+NE
Wild type	4,929 ± 112	30,697 ± 887	37,179 ± 213	128,653 ± 1,213
entA	706 ± 56	198 ± 12	10,056 ± 98	17,199 ± 144
tonB	1,246 ± 51	723 ± 15	11,094 ± 319	28,477 ± 210

[a] Exponential cultures of the *E. coli* O157:H7 strains shown were inoculated at 2×10^8 CFU/ml into SAPI ± 100 μM 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 into dialysis tubing (Noncontact). After incubation for 6 h, 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.

The possible clinical significance of the catecholamine-Tf interaction. The experiments described above 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 health care 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 μM) (http//:www.bnf.org), and assessed changes in the EPR spectrum of the serum Tf-Fe and the ability of the same serum to restrict bacterial growth. As can be seen (Fig. 4A), after 72 h of treatment, the clinically attainable concentrations of inotropes consistently caused a

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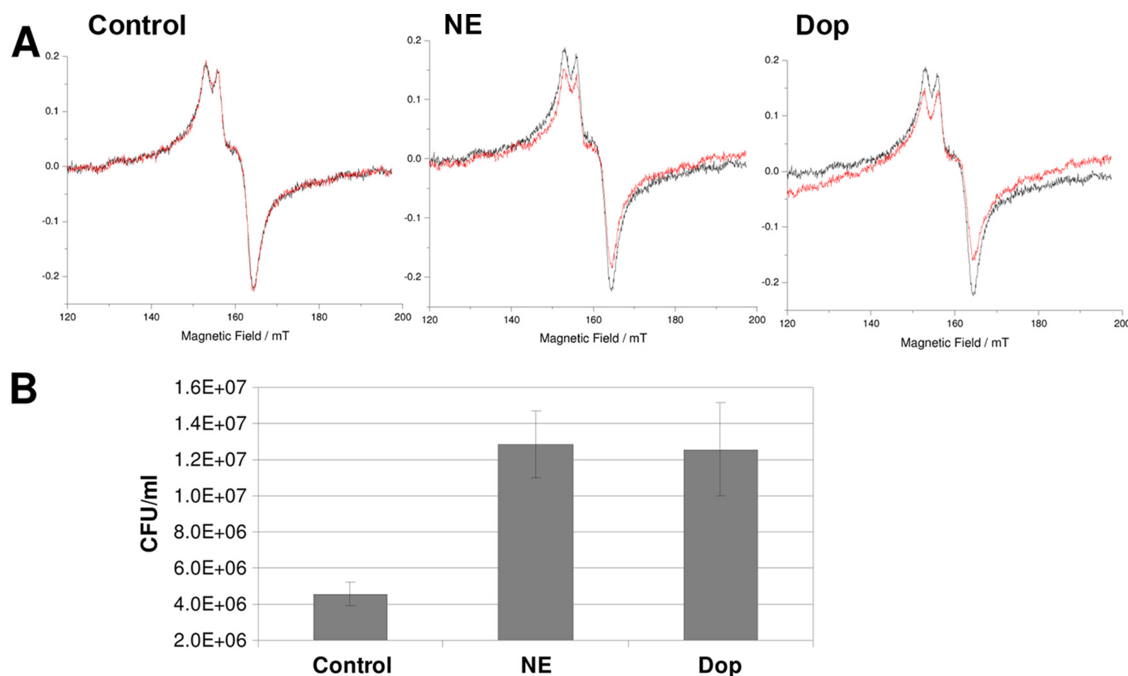

FIG. 4. Human serum-Tf interactions with NE and Dop. (A) Freshly isolated whole sera from healthy volunteers were incubated for 72 h at 37°C with no additions (Control, which comprised the same volume of solvent used for the catecholamines) or with 10 μM additions of NE or Dop. (B) Comparative growth levels of an inoculum of 10^2 CFU/ml *Staphylococcus epidermidis* in the serum samples shown in panel A after 18 h of incubation at 37°C in a humidified static 5% CO_2 incubator; bacteria were enumerated as described in Materials and Methods. The results shown are the combined data from 3 separate growth analyses ± standard deviations.

reduction in the intensity of the serum Tf EPR signal. We also analyzed the ability of the serum to modulate the growth of infectious bacteria (in this case, an 18-h 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 by a factor of between 3- and 7-fold (in the case of Fig. 4B, the reductions were around 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 showed 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 demonstrated 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. In addition to being important for bacterial growth in host tissues (11), reduction of protein-complexed Fe(III) by the catecholamines has also 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 iron form of phenylalanine hy-

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droxylase (20). Our data are also consistent with chemical analyses that showed 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 postulate the following order of events in the iron removal process: catecholamine binds to the Fe(III) within the Tf and reduces it, and the NE-Fe(II) complex then dissociates (we have found that both NE- and Tf-iron are internalized by bacteria) (6, 9). However, this released Fe(II) is likely under aerobic conditions to rapidly reoxidize and therefore to be rebound 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 rebindable iron, and a net loss of iron from the Tf would be observed, which we see in the urea gels of Tf incubated with NE and ferrozine in Fig. 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 (Fig. 1B and C and 2B, C, 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 lobes share 56% similarity, the rate of iron release from the C lobe is considerably lower than that from the N lobe (24). Although Tf and Lf show considerable homology in protein sequence and structure and coordinate iron in similar fashions, they nonetheless display significant differences in their affinities for ferric iron. Wally et al. (24) showed that these differences lie in the variation in the structures of their interlobe linkers; this region, which is helical in Lf, is unstructured in Tf, making the removal of iron from Lf more difficult. This could explain the differences in the potencies 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 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 considered a kind of pseudosiderophore.

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 colocalized, the gut (8, 19). The current work could provide insights into the mechanism(s) 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 more than 24 h is consistent with the findings that the 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 be surprising, then, 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 gastrointesinal (GI) tract (13). Analysis of the gut tissue distribution of the human phenol sulfotransferase family of catechol-inactivating enzymes showed a close correlation with bacterial presence and numbers, with expression lowest in the stomach and greatest in the large intestine and colon (13).

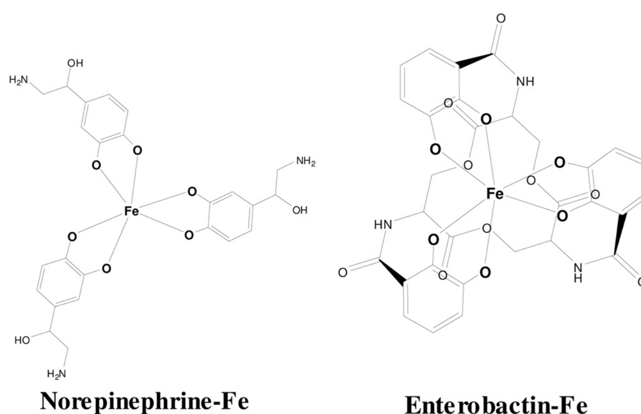

FIG. 5. NE can act as a bacterial pseudosiderophore. The models show the similarity of the structures of norepinephrine-Fe and enterobactin-Fe complexes.

In addition to 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; http//:www.bnf.org); it is also generally recognized that the rates of bacterial infections in intensive care unit patients are significantly higher than those in patients in other hospital wards. Our data (Fig. 4) show 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

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provide a valuable assessment of the infection susceptibility status of severely ill patients. Examining this possibility is a current objective of our laboratories.

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