The Journal of Infectious Diseases

MAJOR ARTICLE

Aspergillus Utilizes Extracellular Heme as an Iron Source During Invasive Pneumonia, Driving Infection Severity

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Background. Depriving microbes of iron is critical to host defense. Hemeproteins, the largest source of iron within vertebrates, are abundant in infected tissues in aspergillosis due to hemorrhage, but *Aspergillus* species have been thought to lack heme import mechanisms. We hypothesized that heme provides iron to *Aspergillus* during invasive pneumonia, thereby worsening the outcomes of the infection.

Methods. We assessed the effect of heme on fungal phenotype in various in vitro conditions and in a neutropenic mouse model of invasive pulmonary aspergillosis.

Results. In mice with neutropenic invasive aspergillosis, we found a progressive and compartmentalized increase in lung heme iron. Fungal cells cultured under low iron conditions took up heme, resulting in increased fungal iron content, resolution of iron starvation, increased conidiation, and enhanced resistance to oxidative stress. Intrapulmonary administration of heme to mice with neutropenic invasive aspergillosis resulted in markedly increased lung fungal burden, lung injury, and mortality, whereas administration of heme analogs or heme with killed *Aspergillus* did not. Finally, infection caused by fungal germlings cultured in the presence of heme resulted in a more severe infection.

Conclusions. Invasive aspergillosis induces local hemolysis in infected tissues, thereby supplying heme iron to the fungus, leading to lethal infection.

Keywords. fungal infection; hemolysis; immunity; innate; immunocompromised host; invasive pulmonary aspergillosis.

The mortality of invasive aspergillosis remains at 30%–50% with the best available therapy [1], underlining the need for better mechanistic understanding of this infection to identify new therapeutic targets. Iron acquisition is integral to microbial pathogenesis and host defense: as part of the innate immune responses, the host suppresses the concentration of extracellular iron ions to exceedingly low levels, thereby withholding this critical element from invading microbes. In contrast, pathogens have evolved sophisticated mechanisms to acquire iron from host tissues. Siderophore-mediated scavenging of extracellular ionic iron is a well defined mechanism of *Aspergillus* iron acquisition that is essential to its growth and virulence [2]. Host mechanisms of iron sequestration, such as neutrophil lactoferrin, inhibit *Aspergillus* growth, and therapeutic iron chelation has an additive benefit to antifungal antibiotics [3, 4]. These mechanisms are clinically important, since iron overload is an independent risk for development of invasive aspergillosis in immunocompromised patients [5, 6].

The host hemeproteins, notably hemoglobin, are the largest iron source in vertebrates. Heme iron is covalently bound to a protoporphyrin ring and is thus unavailable for chelation. Importing extracellular heme is a mechanistically distinct form of iron acquisition in many pathogens [7, 8]. However, a prevailing view in the field is that *Aspergillus* species cannot import heme [9–11]. We found this surprising, because iron acquisition is essential to the growth of *Aspergillus* species, and heme is a major source of iron in decomposing vegetation [12], the natural habitat of saprophytic fungi. We reasoned that *Aspergillus* may have evolved a hitherto undiscovered ability to utilize heme as an iron source. Given the association of invasive aspergillosis with tissue hemorrhage, we tested the hypothesis that heme provides iron to *Aspergillus* during invasive pneumonia, thereby worsening the outcomes of the infection.

MATERIALS AND METHODS

In Vivo Experiments and Tissue Harvest

Animal studies were performed under institutionally approved protocols. Age- and sex-matched 6- to 10-week-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) and hepcidin-deficient mice on C57BL/6 background [13] were used in

Received 18 November 2021; editorial decision 27 February 2022; accepted 1 March 2022; published online 10 March 2022.

Presented in part: American Thoracic Society International Conference, August 2020, Philadelphia, PA.

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The Journal of Infectious Diseases® 2022;225:1811–21

https://doi.org/10.1093/infdis/jiac079

experiments. Aspergillosis was induced as described [14–16], with minor modifications. Neutropenic mice were inoculated with conidia or 6×10^5 germlings. In some experiments, mice were administered 100 μg of hemin (Sigma, St. Louis, MO), equivalent to the heme content of ~1% of mouse blood volume, or a molar equivalent of tin(IV)-protoporphyrin, with conidia and daily thereafter. Intratracheal injection, tissue harvest, bronchoalveolar lavage (BAL), and histology were performed as previously described [17, 18].

In Vitro Studies

Aspergillus fumigatus strain 13073 (American Type Culture Collection, Manassas, VA) or a green fluorescent protein (GFP)-expressing H237 stain (a gift from Dr. David Askew, University of Cincinnati) [19] were cultured at 37°C on Sabouraud's dextrose agar, in Roswell Park Memorial Institute (RPMI) 1640 medium or in minimal media. Conidia were harvested and germlings generated as previously described [20]. Iron-free minimal media were prepared as previously described [21], omitting the addition of ferrous sulfate to Hutner's Trace elements and using a final pH of 6.7. Hemin-supplemented or tin(IV)-protoporphyrin-supplemented media were prepared by dissolving in 1 mL 0.1 N NaOH in phosphate-buffered saline (PBS), then dilution in PBS to a final concentration of 500 μM in minimal media. Cultures were incubated at 37°C in 6- or 24-well or 100-mm culture plates. Iron-free media was used for culture without supplementation or supplemented with 50 μM final concentration of heme, tin(IV)-protoporphyrin, or ferric chloride. Tin(IV)-protoporphyrin was handled under low lighting conditions to avoid phototoxicity.

Conidial susceptibility to hydrogen peroxide was measured as described [22]. Fungal dry mass was determined by removing mycelia from liquid culture media, placing them on preweighed filter paper, air drying overnight, and weighing. Conidiation was determined by removing the mycelium from liquid media, rinsing in 4 mL 2 mM PBS 0.1% Tween and enumeration of conidia under a hemocytometer. For measurements of intracellular iron, germlings were washed in 0.1% Tween in PBS, then homogenized in a high-speed bead basher. For microscopic evaluation of Tin(IV)-protoporphyrin fluorescence in hyphae, sterile pipette tips were used to dissociate hyphal mats. Resulting hyphal strands were placed on glass slides, allowed to dry in the dark for 2 hours, and then imaged under a fluorescent microscope (Axio Imager; Carl Zeiss Microscopy, White Plains, NY) using Zeiss no. 15 filter set (excitation BP 546/12, beamsplitter FT 580, emission LP 590). Tin(IV)-protoporphyrin fluorescence was quantified spectroscopically at excitation and emission wavelengths of 425/590 nm.

To identify candidate *A fumigatus* heme metabolism proteins, we queried the *Aspergillus* (http://www.aspgd.org) and *Candida* (http://www.candidagenome.org) genome databases for the following ontology terms: heme transmembrane transporter activity (GO:0015232), heme transmembrane transport (GO:0035351), heme import into cell (GO:0140420), heme export from vacuole to cytoplasm (GO:0140357), endocytic heme import into cell (GO:0140421), heme import across plasma membrane (GO:1904334), heme transport (GO:0015886), iron acquisition from host (GO:0044847), and hemoglobin binding (GO:0030492). We then searched for annotated genes in *Candida* or non-*fumigatus Aspergillus* species that were orthologous or a best hit to a gene in *A fumigatus* using the *Aspergillus* genome database, and we added orthologous or best hits to PUG1 in *Saccharomyces cerevisiae* due to its role in heme uptake [23]. We prioritized the list using validated polymerase chain reaction primers from the literature, yielding the following final list of possible genes targets: CfmA/Af6g14090, CfmB/Af6g10580, CfmC/Af6g06690, GpdA [24], aspHS/Afu3G00590 [25], MirD/Afu3g03440 [26], FlcA/Afu4g13340, and Afu2g17650, Afu2g06100 [27].

Other Measurements

Fungal colony-forming units (CFUs) were measured by homogenizing freshly excised lungs in 1 mL distilled water at 50 Hz for 10 minutes (TissueLyser LT; QIAGEN, Venlo, Netherlands). Serial 2-fold dilutions of the samples in water were then cultured in duplicate at 37°C on Sabourad's dextrose agar containing 0.05% Triton X-100. After 24 hours, plates were photographed and CFUs were counted. Commercial enzyme-linked immunosorbent assays were used to measure the concentrations of albumin (Abcam, Cambridge, United Kingdom); 1,3 β-glucan (Associates of Cape Cod, East Falmouth, MA); hemoglobin (Cayman Chemical, Ann Arbor, MI); and hepcidin (Intrinsic Life Sciences, La Jolla, CA). Total protein was measured using the Bradford assay (Bio-Rad, Hercules, CA). Fungal messenger ribonucleic acid (RNA) was isolated using a commercial kit (QIAGEN) after grinding in liquid nitrogen, then transcribed into complementary deoxyribonucleic acid (Bio-Rad) and amplified with SYBR Green, using *A fumigatus*-specific primers gathered from the literature (Table 1). Murine tissue RNA was extracted and hepcidin expression quantified as previously described [28]. Plasma iron concentrations were measured with a commercial kit (Sekisui Diagnostics). Tissue and cell culture iron were measured using the ferrozine assay, as previously described [14, 29], with the following modification: 2 aliquots from each sample was run in parallel, 1 treated with an equal volumes 20% trichloroacetic acid 2 N HCl in distilled water, and 4.5% $KMnO_4$ (mixed immediately before use) and 1 with 2 N HCl only. Since $KMnO_4$ is required to liberate iron from heme, the aliquots processed without $KMnO_4$ measured nonheme iron, and the difference between the 2 measurements from each sample represented heme iron.

Table 1. *Aspergillus fumigatus* Primer Sequences Used in the Study

Gene	Forward	Reverse
β-actin	5′ TCA TCA TGC GCG ACA GCT TA	5′ CGT GCT TGG GGT AGC TG
Afu2g06100	5′ TGG TAA CCA GGG ATC TTT CG	5′ TAG GCC TCA ATG CCA GAA AC
Afu2g17650	5′ TGC TGA TGA AAG TGG TCT GC	5′ AAG CCG ATA AAG ACC AGA CG
aspHS	5′ TGG TAC AAG GAC GGT GAC AA	5′ GTC CCA CTG GAC TCT TCC AA
CfmA	5′ ATG TCT CTG CTC CCA AGC CAA CTA	5′ CTA AAG GAT AAT CAA GGC AGC GAG
CfmB	5′ ATG CAC TTC TCT CGT ACT TCC CT	5′ CAA CAA CAC CTG CCA CAT TT
CfmC	5′ CCT GCT CTT CGC GCA TGC CT	5′ GCG CAA TGA GAC CAA TGT CCT TGT
FlcA	5′ GAG GAT GTG GCC AAA CAG AT	5′ GTA ACG GGT ATC GGG GTT TT
GpdA	5′ TCC ACT ATG CTG CCT ACA TGC TCA	5′ TGC CGT TGA GAG AAG GAA TGA CCT
HapX	5′ CCC ATC AGC CAG GCT ACA AA	5′ AGG CGT CGG CAC AAG ATA AA
MirD	5′ ATC GGC ACA AGG CAA AGA AGA G	5′ ATG GAA AAC GGC AGG AGA AAA A

Statistical Analyses

Data were analyzed in Prism software (version 9; GraphPad Software, San Diego, CA). At a single time point, the Mann-Whitney test was used to compare 2 groups, and one-way analysis of variance (ANOVA) with a Tukey multiple comparison posttest was used to analyze more than 2 groups. Change over time was assessed using the Friedman test, and changes between 2 groups over time or over a range of concentrations were compared using 2-way ANOVA with Tukey multiple comparison posttest. Survival data were expressed using Kaplan-Meier curves and compared by log-rank test. In cases where Tukey multiple comparison posttest was used, multiplicity-adjusted *P* values are reported. Two-sided probability values <.05 were considered statistically significant.

RESULTS

Pulmonary Hemorrhage During Invasive Aspergillosis Makes Tissue Iron Available

Pulmonary hemorrhage is a feature of invasive aspergillosis in humans and mouse models. Consistent with this, histologic assessment of lungs of neutropenic mice with invasive aspergillosis showed hyphal penetration of blood vessel walls and extensive hemorrhage into the lung parenchyma (Figure 1A). We quantified the lung hemorrhage and found lung hemoglobin content to progressively increase in the first 3 days of infection (Figure 1B), which correlated with the increase in heme-associated iron in the lungs (including both intra- and extracellular iron pools), and extracellular iron in the bronchoalveolar lavage fluid supernatant (Figure 1C), indicating that infection was associated with hemolysis in the lung. In contrast, lung nonheme-associated iron did not change significantly during the infection (Figure 1D).

Hepcidin is a hormone produced by the liver during infection, which potently reduces plasma iron concentration, limiting the availability of this essential element to invading pathogens [30]. Similar to other infections, hepcidin transcript in the liver was robustly induced during aspergillosis (Figure 2A), associated with progressive increase in plasma and lung hepcidin protein concentration (Figure 2B and C). We were surprised to find, however, that this increase in hepcidin had no detectable effect on plasma iron concentration (Figure 2D). To probe this unexpected result, we examined the effect of hepcidin deficiency on plasma iron levels during the infection. Compared with wild-type animals, hepcidin-deficient mice had elevated plasma iron at baseline, which became further elevated during invasive aspergillosis (Figure 2E), similar to other infections [30]. Despite this, hepcidin deficiency had no effect on the outcome of aspergillosis (Figure 2F). Because iron acquisition is critical to *Aspergillus* pathogenicity [10], we hypothesized that, during infection, *Aspergillus* had access to heme-bound iron within the lung.

Aspergillus fumigatus Can Take Up and Utilize Heme

We began by in vitro examination of *Aspergillus* in iron-deficient media supplemented with various iron sources. We used tin(IV)-protoporphyrin as a heme analog because this molecule is composed of a protoporphyrin-IX ring identical to heme, but it contains a tin, rather than iron, atom that is covalently bound to the center of the ring. Tin(IV)-protoporphyrin is fluorescent, allowing for its identification in cells. We first incubated a transgenic strain of *Aspergillus* expressing GFP in hyphal cytoplasm [19], and we found that fungal hyphae cultured in minimal media with tin(IV)-protoporphyrin acquired the combined fluorescence of tin(IV)-protoporphyrin and GFP (Figure 3A and B). Cell wall and cytoplasmic fluorescence were 8- and 10-fold higher, respectively, in *A fumigatus* incubated with tin(IV)-protoporphyrin compared with minimal media (Figure 3C). Consistent with this, *Aspergillus* hyphae cultured with heme-containing media contained markedly higher levels of heme iron compared with cells cultured in minimal media or in tin protoporphyrin, but only marginally increased nonheme iron compared with tin protoporphyrin (Figure 3D).

To quantify the effect of heme uptake by *Aspergillus*, we began by assessing the expression of the fungal expression of

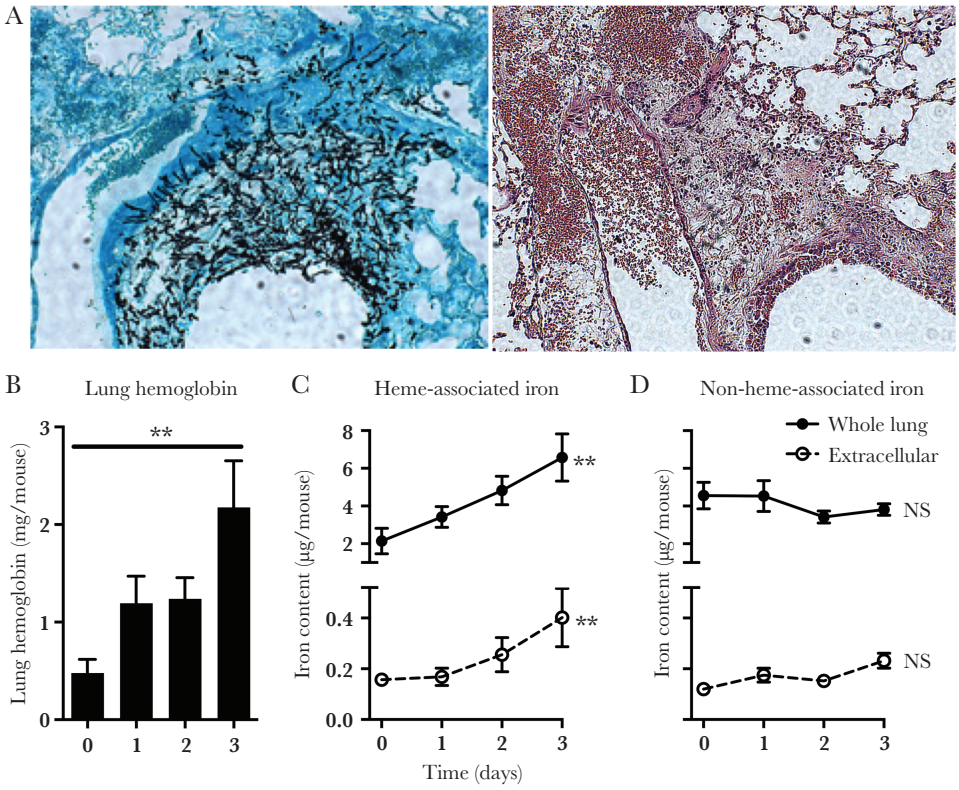

Figure 1. Lung hemorrhage during invasive pulmonary aspergillosis. (A) Lung histology on day 2 of infection. Serial sections of the lung show invasion of a pulmonary venule by fungal hyphae from an adjacent airway and alveoli (left, Gomori's methenamine silver stain), and hemorrhage into the lung parenchyma (right, hematoxylin and eosin stain). Original magnification ×100. (B) Hemoglobin concentration in whole lung homogenates. (C and D) Heme- and nonheme-associated iron in whole lungs (intra- and extracellular) and supernatant of bronchoalveolar lavage fluid (extracellular). Data represent mean ± standard error of the mean of n = 5–10 animals per time point in each panel. **, $P < .01$; time 0 represents neutropenic but uninfected animals. NS, no significant difference.

HapX, an *Aspergillus* transcription factor expressed during iron starvation [31], after exposure of the fungus to equimolar concentrations of heme, tin-protoporphyrin, or ferric chloride. As expected, the addition of ferric chloride to minimal media resulted in down-regulation of *HapX*. Fungal cells cultured with heme displayed a similar suppression of *HapX* expression compared with cells cultured with tin(IV)-protoporphyrin, indicating that exogenous heme provides bioavailable iron to *A fumigatus* and relieves iron starvation (Figure 4A). We further noted that *Aspergillus* grown in heme-containing media produced visibly larger colonies. We quantified this, and we found that fungi cultured in minimal media supplemented with heme or ferric chloride generated markedly more conidia than those cultured in minimal media alone or with tin(IV)-protoporphyrin. In addition, there was no difference between conidia generated from fungal colonies cultured with ferric chloride, heme, and the combination of ferric chloride and tin(IV)-protoporphyrin—the latter indicating that tin(IV)-protoporphyrin does not interfere with conidiation (Figure 4B). The dry mass of the mycelium cultured with heme was greater than both the mycelium cultured in minimal media and with ferric chloride. It is interesting to note that the dry mass of the mycelium cultured with heme was comparable to the dry mass of the mycelium cultured with tin(IV)-protoporphyrin (Figure 4C). We interpret this as evidence that fungal cells under starvation conditions can use the porphyrin ring as an energy source. Conidia from colonies cultured in the presence of heme were also found to display resistance to oxidative killing (Figure 4D).

Next, we sought further evidence that *Aspergillus* heme uptake alters its transcriptional profile. In addition to the iron-sensing transcription factor HapX (Figure 4A), we quantified the *Aspergillus* siderophore transporter MirD and the hemolysin aspHS [26, 32–34]. We also reasoned that any proteins involved in heme uptake and metabolism in *Aspergillus* may share homology with proteins with these functions in other fungi. CfmA, CfmB, and CfmC are *Aspergillus* proteins homologous to *Candida* heme transporters. Likewise, *Aspergillus* FlcA and its paralogs Afu2g17650 and Afu2g06100 were tested because their *Candida albicans* homolog, CaFLC1, mediates heme uptake [27, 35], and glyceraldehyde-3-phosphate dehydrogenase (encoded by *Aspergillus* gene GpdA) acts as a heme chaperone in yeast [36]. We thus quantified the transcription of these 9 genes in fungal cultures in minimal media alone or with minimal media supplemented with heme (Figure 4E). The transcription of 2 genes, CfmB and CfmC, was 20-fold greater in the presence of heme, indicating a transcriptional response of the

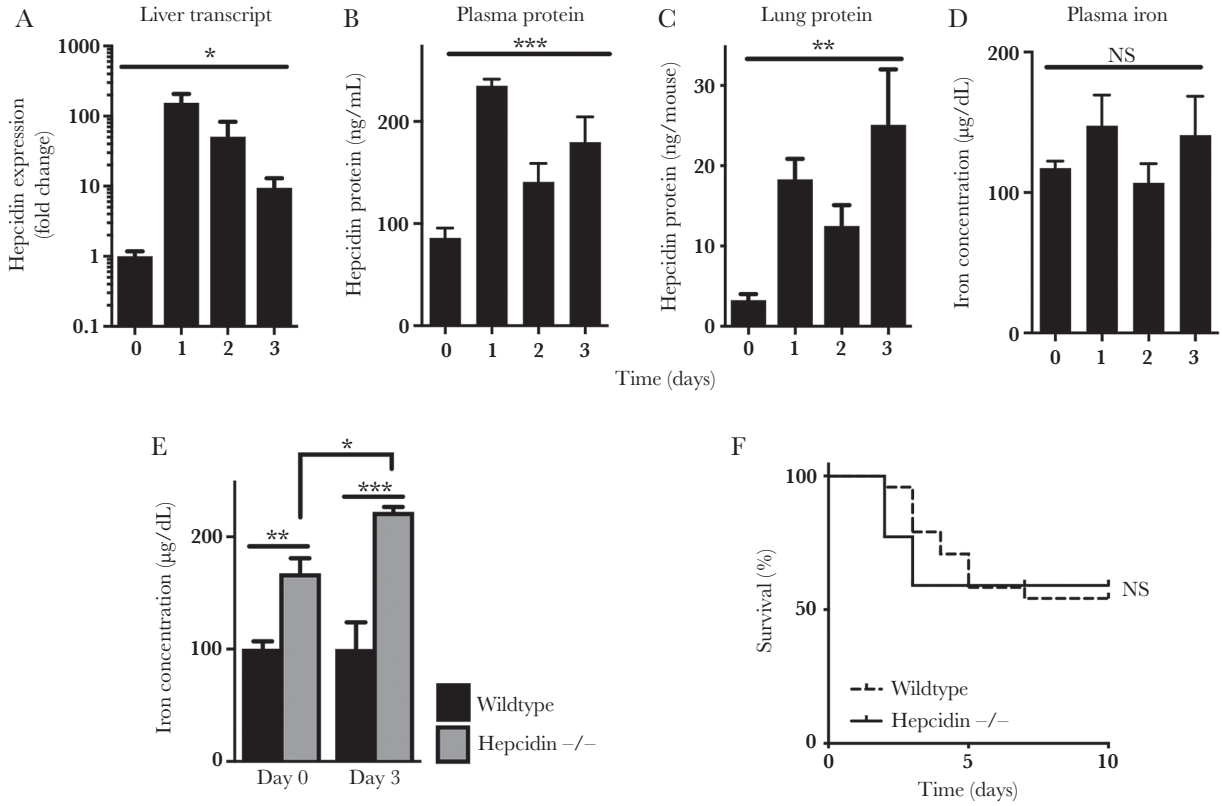

Figure 2. Hepcidin induction and plasma iron during invasive pulmonary aspergillosis. (A) Liver hepcidin messenger ribonucleic acid in the liver normalized to glyceraldehyde 3-phosphate dehydrogenase expression and then to day 0. (B and C) Hepcidin protein concentration in plasma and whole lung homogenate after infection. (D) Plasma iron concentration. (E) Comparison of plasma iron concentration in wild-type and hepcidin-deficient mice before and during invasive aspergillosis. (F) Survival of neutropenic wild-type and hepcidin-deficient mice during invasive aspergillosis. Time 0 represents neutropenic but uninfected animals. Data in panels A–E represent mean ± standard error of the mean of n = 3–10 animals per time point; for panel F, n = 22–24 per group, combined results of 2 experiments. *, **, and *** denote $P < .05$, $P < .01$, and $P < .001$, respectively. NS, no significant difference.

fungus to the presence of heme in its environment. Although some of these changes to fungal phenotype (such as conidiation) do not occur during in vivo infection, taken together, these data indicate that *Aspergillus* heme acquisition is sufficient to alter its biologic behavior.

Heme Worsens the Outcome of Invasive Aspergillosis

To investigate whether heme is relevant to the pathogenesis of invasive pulmonary aspergillosis in vivo, neutropenic mice were intratracheally inoculated with *A fumigatus* alone, heme alone, or conidia together with heme or a molar equivalent of tin(IV)-protoporphyrin. Heme alone had no detectable effect in neutropenic mice, but coadministration of heme with *Aspergillus* resulted in markedly worsened infection outcome compared with vehicle or tin(IV)-protoporphyrin (Figure 5A). To assess whether the detrimental effect of heme was a consequence of exacerbating passive lung injury in response to *Aspergillus* antigens rather than response to an active infection, we also assessed the outcome when animals were inoculated with heme or vehicle together with ethanol-killed *Aspergillus* germlings, and we found that animals inoculated with dead germlings had low mortality, regardless of administration of heme (Figure 5B).

Administration of exogenous heme in these experiments resulted in elevated extracellular heme in the lungs (Figure 5C) and increased lung fungal content, as assessed by CFUs, and BAL (1,3)-β-glucan, a major fungal cell wall component synthesized during exponential hyphal growth [37]. Coadministration of *Aspergillus* with heme resulted in 6-fold higher lung fungal CFUs and 23-fold higher BAL β-glucan (Figure 5D and E). We also quantified the extent of lung injury induced by the infection by comparing the albumin content of bronchoalveolar lavage fluid, a measure of loss of alveolar epithelial integrity. Heme alone did not cause measurable lung injury, and challenge with live *Aspergillus* alone, or ethanol-killed germlings together with heme, resulted in minor lung injury. In contrast, coadministration of heme with live fungi resulted in markedly increased lung injury (Figure 5F). Heme therefore appeared to contribute to both increased fungal growth and increased lung injury during invasive aspergillosis.

We recognize that, during in vivo coadministration of heme with *Aspergillus,* it is not possible to differentiate between the effect of heme on the host and its effect on the fungus. To address this problem, we cultured *Aspergillus* germlings in minimal media supplemented with tin(IV)-protoporphyrin

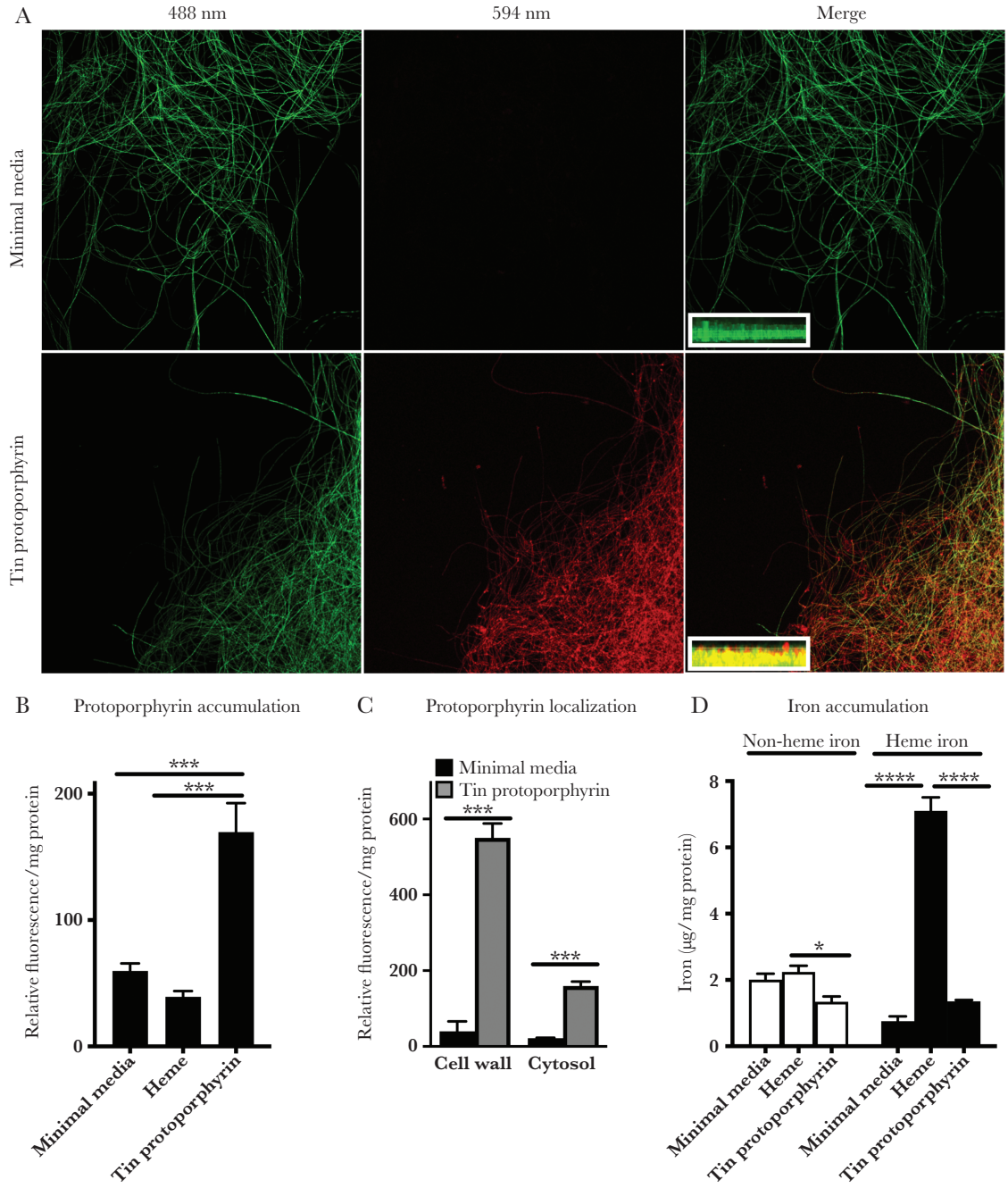

Figure 3. *Aspergillus* uptake of heme and the heme analog, tin-protoporphyrin. (A) Representative confocal images of a green fluorescent protein (GFP)-expressing *Aspergillus fumigatus* hyphae cultured in minimal media or minimal media supplemented with tin(IV) protoporphyrin, obtained with indicated laser emission wavelengths, and 2D Z-stack images of hyphal fragments (insets, 0.5-μm steps) showing colocalization of GFP (green) and tin-protoporphyrin fluorescence (red) in the fungal cytoplasm. Original magnifications ×200 for main panels and ×630 for insets. (B and C) Fluorescence at excitation/emission of 425/590 nm normalized to total protein of *A fumigatus* cultured for 2 days in indicated media. (D) Heme- and nonheme iron recovered from the cytosolic fraction normalized to cytosolic protein content of *A fumigatus* cultured for 2 days in indicated media. Data represent mean ± standard error of the mean of n = 3–6 replicates in each panel. *, ***, and **** denote $P < .05$, $P < .001$, and $P < .0001$, respectively.

or heme, conditions that result in generation of a similar mycelial biomass (Figure 4C). We then washed the resulting fungal cells extensively, and we inoculated them intratracheally into neutropenic mice. Mice challenged with germlings cultured in the presence of heme had a markedly higher lung fungal burden as measured by colony-forming units and β-glucan content, and 2-fold greater lung injury, compared with mice inoculated with germlings cultured with tin(IV)-protoporphyrin (Figure 6). We thus conclude that, independent of the effect of heme on the host, availability of heme to *Aspergillus* is sufficient to result in a more severe infection.

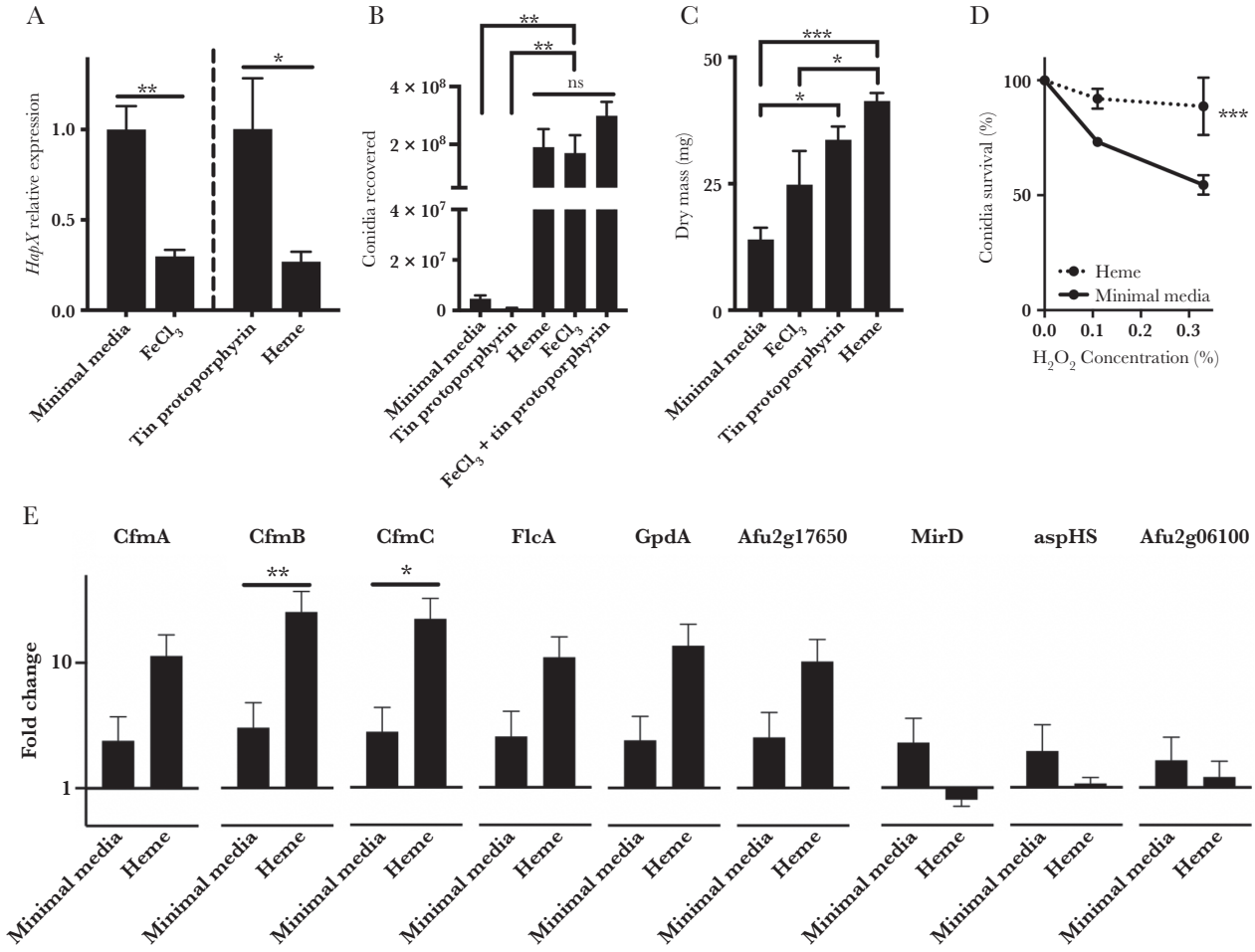

Figure 4. Effect of heme uptake on *Aspergillus*. (A) Expression of fungal *HapX* normalized to β-actin in *Aspergillus fumigatus* cultured for 2 days in indicated media. (B) Conidia recovered after 4 days of culture in indicated media. (C) Dry mass of mycelia after 2 days of culture in indicated media. (D) Survival of conidia after exposure to hydrogen peroxide. (E) Relative expression of candidate fungal heme transport genes normalized to β-actin in *A fumigatus* cultured for 2 days in indicated media. Data represent mean ± standard error of the mean of n = 3–8 replicates in each panel. *, **, and *** denote $P < .05$, $P < .01$, and $P < .001$, respectively. NS, no significant difference.

DISCUSSION

We report a time-dependent release of heme in the lungs during murine invasive aspergillosis, consistent with histologic evidence of alveolar hemorrhage in human infection [38]. In contrast to a prevailing notion in the field, we found (1) that *A fumigatus* can import and utilize exogenous heme and (2) that extracellular heme availability to the fungus enhances its virulence.

Aspergillus heme utilization as an iron source adds to the literature on the importance of iron acquisition in invasive aspergillosis: *Aspergillus* strains deficient in siderophores are avirulent in immunocompromised mice [2]. In a model of *Aspergillus* keratitis, providing iron to the organism in vivo resulted in increased fungal burden, whereas administration of the iron chelator lactoferrin improved outcomes [39]. In an orthotopic tracheal transplant model, which mimics airway-invasive aspergillosis in lung transplant recipients, infection was associated with increased iron availability to the fungus and microhemorrhage in the allograft [40]. In a computational model of invasive aspergillosis, iron availability to the fungus dramatically influenced predicted growth of the fungus [14]. Our data add to this literature by showing heme acquisition to be a nonredundant mode of iron acquisition by the fungus, and that the tissue hemorrhage provides a rich source of iron to the organism, rendering it resistant to systemic hypoferremia orchestrated by the host.

Mechanisms of heme acquisition have been extensively documented as contributing to bacterial virulence [8]. Although less well studied in fungi, heme acquisition has been discovered in *Candida*, *Cryptococcus neoformans*, *Paracoccidioides* species, and *Schizosaccharomyces pombe* [7]. Our findings are consistent with improved protein expression of *Aspergillus oryzae* in heme-containing media [41] and evidence that *Aspergillus niger* is capable of heme utilization, rescuing the lethal phenotype of mutants incapable of endogenous heme synthesis [42, 43]. It is notable that although our observations contradict a

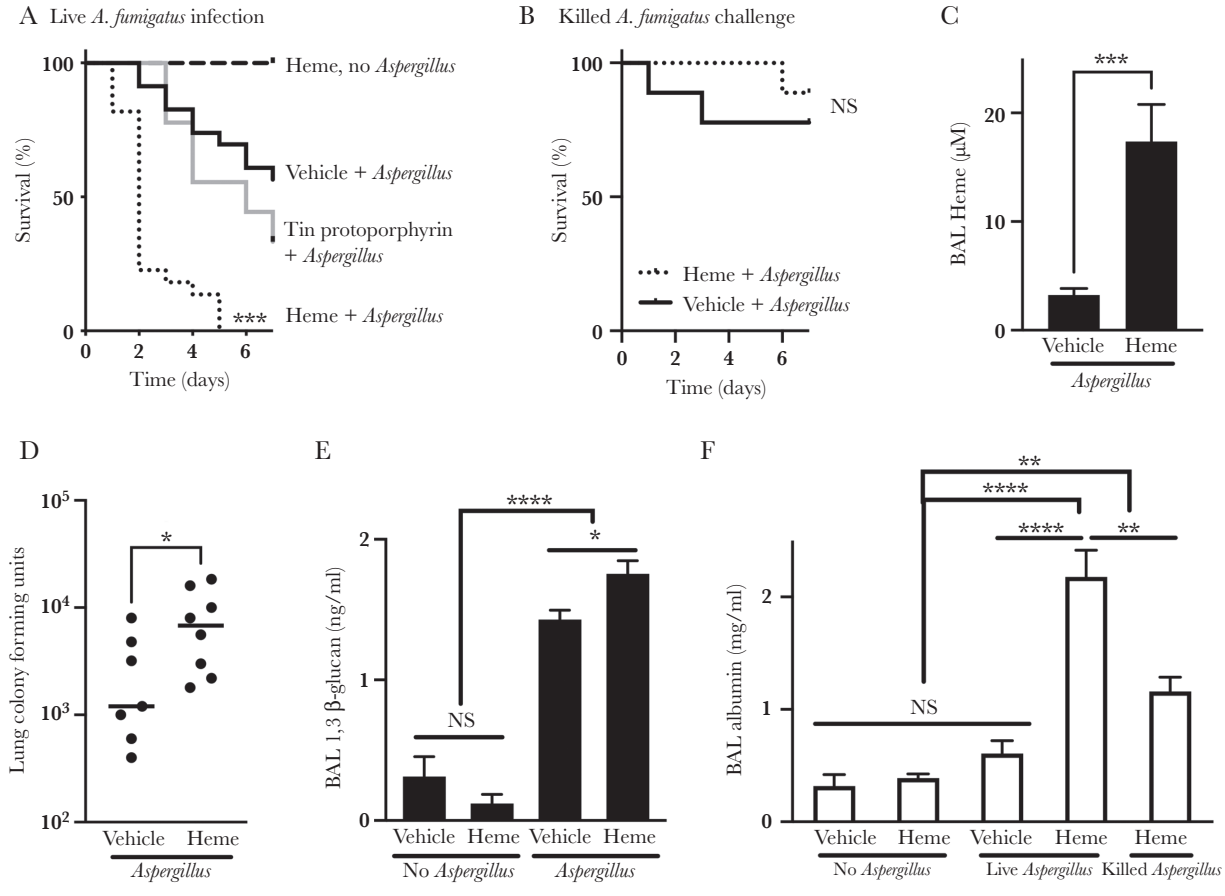

Figure 5. The effect of heme on the outcome of invasive pulmonary aspergillosis. (A and B) Survival of neutropenic mice, intratracheally challenged with indicated inocula. n = 6–23 per group, combined from 2 experiments. (C) Bronchoalveolar lavage (BAL) supernatant heme content in neutropenic mice on day 3 of infection. (D and E) Lung fungal content measured as lung colony-forming units and BAL fluid β-glucan content in neutropenic mice on day 3 of infection. (F) Extent of lung injury, as measured by BAL fluid albumin content, in neutropenic mice on day 3 of infection. Data in C–F represent mean ± standard error of the mean of n = 4–15 animals in each panel, combined from 3 experiments. *, **, and **** denote $P < .05$, $P < .01$, and $P < .0001$, respectively. NS, no significant difference.

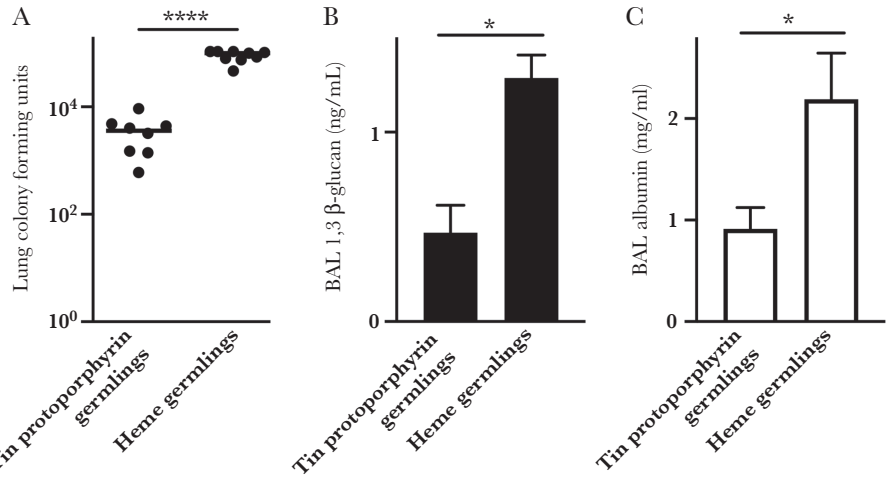

Figure 6. The effect of fungal heme exposure on severity of invasive aspergillosis. Lung fungal content measured as lung colony-forming units (A) and bronchoalveolar lavage (BAL) fluid β-glucan content (B), and extent of lung injury, as measured by BAL fluid albumin content (C) in neutropenic mice on day 2 after intratracheal challenge with *Aspergillus* germlings that were culture in minimal media supplemented with tin(IV)-protoporphyrin or heme. Data represent mean ± standard error of the mean of n = 5–9 animals per group, combined from 2 experiments. * and **** denote $P < .05$ and $P < .0001$, respectively.

prevailing notion in the field, they do not formally conflict with previously published data: the contention that *Aspergillus* species cannot import heme arose from observations that mutant strains with disrupted siderophore synthesis have impaired growth and conidiation that was rescued by culture in iron-supplement media or with exogenous siderophores, but not by culture with blood or heme, and were avirulent in neutropenic mice [21, 44]. This work implicated the fungal siderophore system as indispensable to in vitro growth and in vivo virulence regardless of heme presence, but it did not address heme accessibility to *Aspergillus* strains with intact siderophore systems. In this context, the current report provides evidence that, under conditions of iron starvation, (1) heme is imported and utilized by wild-type *A fumigatus*, providing it with an iron source and enhancing its in vivo pathogenicity and growth, and (2) that siderophore-mediated iron acquisition and heme uptake thus play crucial and nonredundant roles in fungal virulence.

We assessed the transcription of several *Aspergillus* proteins with potential relevance to heme metabolism to document the response of *A fumigatus* to heme, among which HapX, CfmB, and CfmC were found to be differentially regulated (Figure 4). HapX is essential to *Aspergillus* siderophore production, and its downregulation in the presence of heme indicates relief from iron starvation [45]. The Common in Fungal Extracellular Membrane (CFEM) domain identifies a family of heme-import proteins in several fungal species. The *Aspergillus* CfmA, CfmB, and CfmC are CFEM-containing proteins with homology to *Candida* counterparts involved in heme handling [34, 46, 47]. We interpret the upregulation of CfmB and CfmC in the presence of heme as evidence that the fungus can detect and respond to heme, but we recognize this as hypothesis-generating regarding their role in mediating heme import in *Aspergillus*.

Systemic hemolysis has long been recognized to predispose to numerous infections [48]. Our data suggest that invasive aspergillosis represents a special case of local hemolysis, wherein angioinvasion promotes tissue hemorrhage, release of hemoglobin from lysis of red blood cells, and its degradation to heme—resulting in increasing extracellular heme iron as the infection progresses (Figure 1C). *Aspergillus* conidia and germling are phagocytosed, and they are either rapidly killed or kill the phagocytosing cell within hours [19, 49]; *Aspergillus* thus does not persist in an intracellular niche. We therefore speculate that fungal heme uptake occurs only extracellularly during infection, but our data leave open the possibility that phagocytosed fungal elements exposed to heme also kill host cells more rapidly during their brief intracellular phase.

We recognize that, independent of its effects on the fungus, heme affects host cells, resulting in complex pro-oxidant, inflammatory, and immunoregulatory effects [50]. During aspergillosis, extracellular heme thus likely affects both the host and *Aspergillus* concurrently. Our data do not preclude these direct host effects but provide evidence that heme alters the fungal transcriptional profile, increases its growth, and enhances its resistance to oxidative killing. More importantly, fungal exposure to heme before inoculation into neutropenic mice causes a more severe infection, indicating that exposure heme is sufficient to render *Aspergillus* more virulent.

CONCLUSIONS

This work has several implications for future research. First, the mechanisms by which *Aspergillus* takes up heme, such as heme receptors, heme endocytosis, and other extracellular heme binding proteins, are of substantial interest. Second, the direct effects of heme on the host during invasive aspergillosis may be important in dictating the local immune responses during invasive aspergillosis. Finally, strategies aimed at limiting the availability of heme to *Aspergillus* may be relevant therapeutically.

Notes

Financial support. This work was funded by National Institutes of Health Grants EB024501, AI135128, and AI117397.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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