Activation of Hypoxia Inducible Factor 1 Is a General Phenomenon in Infections with Human Pathogens

Nadine Werth¹, Christiane Beerlage², Christian Rosenberger³, Amir S. Yazdi⁴,⁵, Markus Edelmann¹, Amro Amr¹, Wanja Bernhardt⁶, Christof von Eiff⁷, Karsten Becker⁷, Andrea Schäfer¹, Andreas Peschel¹, Volkhard A. J. Kempf²⁺

¹ Institut für Medizinische Mikrobiologie und Hygiene, Universitätsklinikum Tübingen, Eberhard-Karls-Universität, Tübingen, Germany, ² Institut für Medizinische Mikrobiologie und Krankenhaushygiene, Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany, ³ Medizinische Klinik mit Schwerpunkt Nephrologie und Internistische Intensivmedizin, Charité, Berlin, Germany, ⁴ Département de Biochimie, Université de Lausanne, Epalinges, Switzerland, ⁵ Universitäts-Hautklinik, Eberhard-Karls-Universität, Tübingen, Germany, ⁶ Medizinische Klinik 4 – Nephrologie und Hypertensiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany, ⁷ Institut für Medizinische Mikrobiologie, Universitätsklinikum Münster, Münster, Germany

Abstract

Background: Hypoxia inducible factor (HIF)-1 is the key transcriptional factor involved in the adaptation process of cells and organisms to hypoxia. Recent findings suggest that HIF-1 plays also a crucial role in inflammatory and infectious diseases.

Methodology/Principal Findings: Using patient skin biopsies, cell culture and murine infection models, HIF-1 activation was determined by immunohistochemistry, immunoblotting and reporter gene assays and was linked to cellular oxygen consumption. The course of a S. aureus peritonitis was determined upon pharmacological HIF-1 inhibition. Activation of HIF-1 was detectable (i) in all ex vivo in biopsies of patients suffering from skin infections, (ii) in vitro using cell culture infection models and (iii) in vivo using murine intravenous and peritoneal S. aureus infection models. HIF-1 activation by human pathogens was induced by oxygen-dependent mechanisms. Small colony variants (SCVs) of S. aureus known to cause chronic infections did not result in cellular hypoxia nor in HIF-1 activation. Pharmaceutical inhibition of HIF-1 activation resulted in increased survival rates of mice suffering from a S. aureus peritonitis.

Conclusions/Significance: Activation of HIF-1 is a general phenomenon in infections with human pathogenic bacteria, viruses, fungi and protozoa. HIF-1-regulated pathways might be an attractive target to modulate the course of life-threatening infections.

Introduction

Mammalian cells adapt to oxygen deprivation by the activation of hypoxia inducible factor (HIF)-1, the key transcription factor during hypoxia. Subsequently, the expression of hypoxia-inducible genes involved in angiogenesis [e.g., vascular endothelial growth factor (VEGF)], glycolysis [e.g., hexokinase (HK)], proliferation and survival [e.g., adrenomedullin (ADM)] and erythropoiesis (e.g., erythropoietin) is transcriptionally regulated [1,2]. The heterodimeric transcription factor HIF-1 is composed of the two subunits HIF-1α and HIF-1β. While HIF-1β is constantly present in the nucleus, HIF-1α levels are affected by changes in the cellular oxygen partial pressure (pO₂). The key mechanism involved in HIF-1 activation has been identified to require inhibition of the enzymatic activity of “prolyl hydroxylase domain”-containing proteins (PHDs) during hypoxia. PHDs mediate hydroxylation of the prolyl residues Pro402 and Pro564 of the HIF-1α subunit which results in the binding to the von-Hippel-Lindau protein and subsequent proteosomal degradation under normoxic conditions.

In contrast, hypoxia results in PHD-inhibition and subsequent stabilization of HIF-1α, binding of the HIF-1 heterodimer to promoter regions of hypoxia-inducible genes and corresponding gene induction [2]. Iron deprivation [induced by iron chelating compounds, e.g., desferrioxamine (DFO)] has been found an alternative strategy of HIF-1 activation [3]. The molecular explanation for this phenomenon is that PHDs contain iron as an essential cofactor for their enzymatic activity; therefore, iron chelation inhibits PHD activity resulting in the subsequent activation of HIF-1 [4].

A constantly growing body of evidence suggests that HIF-1 plays a novel and important role in infectious and inflammatory diseases [5]. HIF-1 is essential for the bactericidal capacity of phagocytes and for controlling systemic spread of bacteria in mice [5,6]. Interestingly, HIF-1 activation occurs during bacterial infections with the angiogenic bacterium Bartonella henselae (causing the vasculoproliferative disorder bacillary angiomatosis) [7] and this phenomenon is linked with the expression of the Bartonella adhesin A [8–10]. In infections with humanpathogenic Enterobac-
terraceae, HIF-1 activation is the result of iron-competition between bacteria and host cells caused by secreted bacterial siderophores; here, HIF-1 plays an important role in the defense of *Enterobacteriaceae* infections [11]. Using keratinocytes, it was recently demonstrated that HIF-1 activation results in the expression of cathelicidin, an antimicrobial peptide mediating protection against gram positive group *A. staphylococci* [6]. LPS from gram negative bacteria can trigger HIF-1 activation in macrophages [12,13] and this HIF-1 activation is crucial for the development of a LPS triggered sepsis [14,15]. Interestingly, LPS-dependent HIF-1 activation does not occur using epithelial or endothelial cell-based infection models [9–11].

Although several aspects have been analyzed in the last years, the exact role of HIF-1 in the course of infection with human pathogens remains widely unclear. Therefore, we investigated the activation of HIF-1 in infections with human pathogens more generally using human skin biopsies, cell culture techniques and by employing *Staphylococcus aureus* infection models. Our data reveal that HIF-1 might play an important and previously underestimated role in many infectious diseases.

### Results

**Activation of HIF-1 by human pathogens is a general phenomenon in infections**

First, we analyzed *ex vivo* whether pathogen-triggered HIF-1 activation is detectable in biopsy samples of patients suffering from various bacterial, viral, fungal or parasitic skin infections. For this purpose, paraffin-embedded patient skin biopsies were collected according to the following strict inclusion criteria: (a) a clinically suspected infectious process, (b) the histopathological diagnosis of an infection (no malignancy, no autoimmunological disease) and (c) a microbiologically positive laboratory result confirming the presence of specific pathogens in the lesions. In total, four samples of bacterial skin infections, two samples of viral skin infections, three samples of fungal skin infections and two samples of protozoic skin infections were investigated by HIF-1α-specific immunohistochemistry (Fig. 1). In control samples (healthy patients, biopic sample taken for other reasons, Fig. 1A), a rare and faint nuclear HIF-1α staining occurred in the epidermis whereas the dermis was negative for HIF-1α. In contrast, in all cutaneous infections [here: infections with *S. aureus* (Fig. 1B), coinfection with *A. haemolyticus* and *S. aureus* (Fig. 1C), coinfection with *A. haemolyticus* and *E. coli* (Fig. 1D), infections with *Borellia burgdorferi* (Fig. 1E), *Varicella zoster* virus (Fig. 1F), *Human Herpes Virus-8* (Fig. 1G), *Tinea rubrum* (Fig. 1H, I), *C. albicans* (Fig. 1J) and *Leishmania donovani* (Fig. 1K, L)] a strong nuclear HIF-1α signal was detected in keratinocytes (predominantly of the spinal cell layer), dermal capillaries, neutrophils, dermal lymphocytes and macrophages and sub-corneal neutrophils (for details please see legend of Fig. 1).

Activation of HIF-1 in host cells upon infections with *B. henselae* and several members of the family of *Enterobacteriaceae* is triggered by oxygen-dependent and -independent mechanisms [7,11]. Now, we were interested whether and by which mechanisms various human pathogens lead to the activation of HIF-1 in host cells. For this purpose, we infected HeLa-229 cells and NHEKs (Normal Human Epidermal Keratinocytes) with a collection of bacterial pathogen reference strains (*S. aureus* ATCC 35592, *S. epidermidis* ATCC 12228, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. agalactiae* SK-43 and with the fungal pathogen *C. albicans* ATCC 90028). In all these *in vitro* infection models, a robust activation of HIF-1 was detectable via HIF-1α immunoblotting (Fig. 2, 3) and congruent results were obtained via immunohistochemistry using Hela-229 cells grown on coverslips (Fig. S1, not all data shown). HIF-1 activation was correlated with increased oxygen consumption of the respective infected host cells strongly arguing for oxygen-dependent HIF-1 activation mechanisms. This suggestion is confirmed by the observation that HIF-1 activation by *S. aureus*, *P. aeruginosa* and *E. coli* was overcome by culturing infected cells in gas-permeable dishes (data not shown). Accordingly, induction of the HIF-1-regulated VEGF was detected in bacterial infections with *S. aureus*, *P. aeruginosa* and *E. coli* by VEGF mRNA induction whereas HIF-1α mRNA transcript levels itself again appeared unaffected (Fig. S2). Infections with *S. pyogenes* did not lead to oxygen consumption nor HIF-1 activation (data not shown).

**Infection of host cells with *S. aureus* results in HIF-1-regulated gene programming**

Infection of host cells with *B. henselae* or *Enterobacteriaceae* (*Y. enterolitica*, *Enterobacter aerogenes*, *Salmonella enterica*) results in the activation of HIF-1 and a subsequent HIF-1-dependent angiogenic gene programming in *in vitro* and *in vivo* [7,9–11]. As HIF-1 activation was also detectable in infections with *S. aureus* (see above), a clinically most important pathogen, we focussed on this bacterium to elucidate which mechanisms and which biological consequences underlie this process.

First, we infected HeLa-229 cells with a well characterized *S. aureus* laboratory strain (8325-4). Such infection led to a robust HIF-1 activation and HIF-1 dependent VEGF induction shown by HIF-1α-immunoblotting, HIF-1-dependent luciferase reporter assays and quantitative PCR analysis (Fig. 4A, B, C). HIF-1α mRNA transcript levels itself were unaffected (Fig. 4D) excluding direct effects of *S. aureus* on HIF-1 transcription but suggesting oxygen-dependent posttranslational mechanisms of HIF-1 activation.

Next, we introduced a genetically defined small colony variant of *S. aureus* (*S. aureus* ΔhemB) and the *S. aureus* ΔhemB hemB-complemented strain (*S. aureus* pCX19ΔhemB) in our experiments. *S. aureus* ΔhemB shows a small-colony phenotype and is auxotrophic for the synthesis of hemin [16]. Therefore, as hemin is essential for the biosynthesis of cytochromes, *S. aureus* ΔhemB is deficient in aerobic metabolism [17]. Interestingly, *S. aureus* ΔhemB was neither capable in activating HIF-1 nor in VEGF induction whereas the complemented strain was fully restored in its biological effects (oxygen consumption, HIF-1 activation and VEGF induction) suggesting oxygen-dependent mechanisms in the activation of HIF-1 by *S. aureus*. Furthermore, *S. aureus* strains defective in certain cell wall components known to be important for pathogenicity [e.g., lipoteichoic acid [18], wall teichoic acid [19], extracellular adherence protein [20]] were not impaired in their ability of inducing HIF-1 activation (Fig. 5).

To exclude that the above described phenomenon might be restricted to this particular *S. aureus* laboratory strain, we employed several other *S. aureus* small colony variants (clinical SCV’s) originating from patients suffering from chronic infections and their respective parental wild-type strain (Fig. 6, not all data shown). Data clearly revealed that only wild-type but not clinical SCV bacteria lead to the activation of HIF-1 underlining the general aspect of our observations. Further analysis revealed that the clinical SCV *S. aureus* OM 1b is auxotrophic for menadione [21] and *S. aureus* A22223 II is unable to synthesize hemin [22]. Both auxotrophies are linked to the activity of the electron transport chain and affect thereby the aerobic metabolism.

To prove whether HIF-1 activation by *S. aureus* is an oxygen-dependent process, we determined cellular hypoxia upon a bacterial infection using a hypoxia-sensitive dye and by quantification of the dissolved O₂ concentration in cell culture supernatants. In fact, cellular hypoxia occurred in infections with...
S. aureus 8325-4 and the S. aureus ΔhemB hemB-complemented strain but not when cells were infected with S. aureus ΔhemB. Here, a significantly increased oxygen consumption in cells infected with S. aureus 8325-4 and the S. aureus ΔhemB hemB-complemented strain was detectable (Fig. 7A, B). To analyze whether such HIF-1 activation by S. aureus might be overcome by oxygen, an infection model using conventional versus gas-permeable cell culture dishes was introduced allowing to investigate oxygen-dependent and -independent mechanisms of HIF-1 activation. As expected, activation of HIF-1 by S. aureus 8325-4 and the S. aureus ΔhemB...
Figure 2. Oxygen-dependent HIF-1 activation in HeLa-229 cells and NHEKs (Normal Human Epidermal Keratinocytes) by bacterial and fungal pathogens. Four to six hours upon infection, HIF-1 activation was determined by Western blotting (loading control: actin). pO2 levels were quantified in the medium of control and infected cells (A: S. aureus ATCC 33592, MOI 20, infection time: six hours; B: S. epidermidis ATCC 12228, MOI 20, infection time: six hours; C: E. coli ATCC 25992, MOI 10, infection time: four hours). Negative control: uninfected cells, positive control: hypoxia or DFO (200 μmol/L).
doi:10.1371/journal.pone.0011576.g002
Figure 3. Oxygen-dependent HIF-1 activation in HeLa-229 cells and NHEKs (Normal Human Epidermal Keratinocytes) by bacterial and fungal pathogens. Four to six hours upon infection, HIF-1 activation was determined by Western blotting (loading control: actin). $pO_2$ levels were quantified in the medium of control and infected cells (D: *P. aeruginosa* ATCC 27853, MOI 10, infection time: four hours; E: *S. agalactiae* SK 43, MOI 200, infection time: five hours; F: *C. albicans* ATCC 90028, MOI 20, infection time: six hours). Negative control: uninfected cells, positive control: hypoxia or DFO (200 μmol/L).

doi:10.1371/journal.pone.0011576.g003
A hemB-complemented strain did not occur in gas-permeable culture dishes and this correlated clearly with the oxygen partial pressure in the respective cell culture supernatants. For \textit{S. aureus} \Delta hemB no HIF-1 activation nor oxygen consumption was detectable in infected host cells (Fig. 7G, D). Taken together, these results show that (i) \textit{S. aureus} wild-type but not \textit{S. aureus} SCV induces a HIF-1 regulated gene programming in host cells and that (ii) HIF-1 activation is in fact an oxygen dependent process.

Finally, we employed a murine intravenous \textit{S. aureus} infection model in which the \textit{S. aureus} bacteremia results in kidney abscess formation [23]. After seven days upon infection, macroscopically visible kidney abscesses occurred. Kidneys were removed, fixed and embedded in paraffin. Serial sections were processed by hematoxylin-eosin (H&E)- and HIF-1 staining. Massive abscess formation was also detected in H&E staining (Fig. 8B) and HIF-1 positive nuclei were most abundant in the abscess-surrounding areas (Fig. 8A). HIF-1 activation was also detectable in the peritoneum of mice suffering from a \textit{S. aureus}-peritonitis (see below).

Taken together, we conclude from the above described \emph{in vitro}, \emph{ex vivo} and \emph{in vivo} data that HIF-1 activation is a general phenomenon in infections with human pathogenic bacteria, viruses, fungi and protozoae and that this phenomenon is most likely due to hypoxic signaling.

\textbf{Inhibition of HIF-1 by 17-DMAG increases survival of mice in a \textit{S. aureus} peritonitis model}

Mice are highly susceptible for a \textit{S. aureus} peritonitis [24]. For this purpose, we infected mice intraperitoneally with \textit{S. aureus} wild-
type resulting in a 100% lethality of mice (n = 8). Infection with the related S. aureus SCV (S. aureus ermB\hemB strain (not activating HIF-1, see above) did not cause such a massive infection as all mice (n = 8) survived this infection (Fig. 9A).

As only S. aureus wild-type and not S. aureus SCV infections led to the activation of HIF-1 in vitro (see above), we finally analyzed the biological role of HIF-1 in this infection model. 17-DMAG is known to be a potent HIF-1 inhibitor [25]. Therefore we first established experiments elucidating the inhibition of HIF-1 activation by 17-DMAG in our experimental in vitro setting. In fact, addition of 17-DMAG prevented the activation of HIF-1 by DFO in a dose-dependent manner. A minimum concentration of 10 μmol/L 17-DMAG was effectual for inhibition of DFO-induced HIF-1 activation (Fig. 9B, not all data shown).

In vivo, the administration of 17-DMAG in fact prevented the peritoneal activation of HIF-1 during infection and this phenomenon was accompanied by a significant better survival of mice suffering from a S. aureus peritonitis. In detail, mice treated twice with 17-DMAG survived to a significantly higher percentage (10/38; 26%) compared to mice which did not receive 17-DMAG (1/38; 3%, Fig. 9C).

Discussion

In this work, we demonstrate that HIF-1 activation in infections with human pathogenic microorganisms is a general phenomenon not restricted to certain pathogens. A robust and reliably detectable HIF-1 activation was shown (i) ex vivo in biopsies of patients suffering from skin infections, (ii) in vitro using cell culture infection models and (iii) in vivo using murine S. aureus infection models. Moreover, inhibition of bacterial induced HIF-1 activation resulted in higher survival rates in a S. aureus peritonitis model suggesting that the modulation of HIF-1-regulated pathways might influence the course of infections greatly.

In the past, hypoxia and iron-deprivation have been identified as the major conditions leading to HIF-1 activation [2,3]. PHDs (mainly PHD-2) were shown to represent the molecular regulators of HIF-1 activity regulating ubiquitination and subsequent proteasomal degradation of the HIF-1α subunit [26–28]. According to these observations, we demonstrated earlier that HIF-1 activation by bacteria is either the result of cellular hypoxia following bacterial infections with e.g., B. henselae [7] or of iron-competition between bacteria and host cells in infections with certain Enterobacteriaceae [11]. From the herein described experiments it became obvious that HIF-1 activation by pathogens is a general phenomenon occurring in infections with bacteria, viruses, fungi and protozoa (see Fig. 1–4). This hypothesis is supported by various reports showing that HIF-1 activation occurs also in infections with, e.g., Chlamydia pneumoniae [29], Helicobacter pylori
and respiratory syncytial virus (RSV) [31]. Using bacterial and fungal infection models (HeLa-229 cells, NHEKs), we were able to link this HIF-1 activation with increased oxygen consumption [via (i) pimonidazole staining, (ii) direct quantification of oxygen partial pressure in cell culture supernatants and (iii) by overcoming cellular hypoxia using gas-permeable cell culture dishes, see Fig. 2, 3, 7] and strongly arguing for a role of PHD-2 and against transcriptional HIF-1 induction (excluded by quantification of HIF-1α mRNA, see Fig. 4, Fig. S2). Interestingly, in our experiments no increased HIF-2 activation was detectable when cells were infected with *P. aeruginosa, E. coli* and *S. aureus* (data not shown) and, until now, no reports are published indicating a role of HIF-2 in such settings.

Infections with *S. aureus* strains deficient in their aerobic metabolism [*S. aureus ΔhemB* [16], clinical *S. aureus* SCVs [21,22]] did not influence the oxygen partial pressure in cell cultures and neither led to HIF-1 activation and oxygen consumption. HIF-1 activation was fully restored when infections with the respective parental wild-type or genetically complemented *S. aureus* were performed. 

**Figure 8. HIF-1 activation in kidneys of mice infected with *S. aureus*.** (A) HIF 1α immunohistochemistry and (B) hematoxilin-eosin staining of renal mouse abscesses induced by intravenous infection with *S. aureus* seven days upon infection (following sections). HIF 1α positive nuclei are most abundant within the abscesses borders, some interstitial staining also occurs within the renal parenchyma. In addition, renal tubules in the vicinity of the abscess stain also positively (halfed arrow in B). A: arcuate artery, G: glomerulus, V: arcuate vein; arrowheads: margin of abscesses. Magnifications: (A) 250×, (B) 100×. doi:10.1371/journal.pone.0011576.g008

**Figure 9. Role of HIF-1 in infections with *S. aureus* in a murine peritonitis model.** (A) Survival of NMRI mice after intraperitoneal infection with *S. aureus* (wt; *S. aureus* 8325-4; n = 8) or *S. aureus ΔhemB* (SCV, *S. aureus emrΔhemB* 8325-4; n = 8). Note the higher susceptibility of mice infected with *S. aureus* 8325-4 compared to the mice infected with *S. aureus ΔhemB*. *significant difference: P<0.001. (B) Inhibition of DFO induced HIF-1 activation in HeLa-229 cells by 17-DMAG. Cells were incubated with 17 DMAG (10 μmol/L) for 16 hours following induction of HIF 1 activation by the iron chelator DFO (200 μmol/L) for six hours. HIF 1α protein was analyzed in Western blots (loading control: actin). Negative control: uninfected cells. (C) Survival rate of NMRI mice after intraperitoneal infection with *S. aureus* (*S. aureus* 8325-4). One group of mice was treated 24 h and 16 h before infection with the HIF-1 inhibiting compound 17-DMAG (25 mg/g body weight). Note the higher survival rate of 17-DMAG-treated mice (n = 45) compared with control mice (n = 45). *significant difference: P = 0.005 (Kaplan-Meier analysis). doi:10.1371/journal.pone.0011576.g009
ed S. aureus strains were performed (Fig 4, 6, 7). Obviously, cell wall components of S. aureus (LTA, WTA, EAP) are not involved in HIF-1 activation (Fig 5) arguing again for a crucial role of infection-triggered hypoxia. Interestingly, when using a murine peritonitis model [24], SCV-strains (deficient in their capacity of HIF-1 activation) turned out to be non-pathogenic (see Fig 9A).

Clinical SCVs are usually isolated from patients suffering from chronic infections (e.g., from lung infections due to cystic fibrosis or from osteomyelitis) [32]. It might be speculated that acute infections (e.g., with S. aureus wild-type) are linked to a rapid and strong HIF-1 activation whereas such activation is missing in chronic infections (e.g., with SCV bacteria) suggesting that the activation of HIF-1 in infections correlates directly with pathogenicity and the course of infection.

Although there is solid evidence for the impact of HIF-1 on the host response, the exact role of HIF-1 in infectious diseases is still largely unknown. The activation of HIF-1 by bacteria was demonstrated in vitro and ex vivo for the angiogenic bacterium B. henselae causing the vasculoproliferative disorders bacillary angiomatosi and peliosis hepatis [7,10]. Here, B. henselae induces an angiogenic reprogramming of host cells via HIF-1. Infected cells secrete angiogenic compounds (e.g., VEGF) leading to the proliferation of endothelial cells [9], the assumed habitate of B. henselae [33,34]. In contrast, for some members of the family of Enterobacteriaceae (Y. enterocolitica, E. aerogenes and S. enterica spp. enterica) an oxygen-independent HIF-1 activation was demonstrated. Here, bacterial siderophores (secreted by these Enterobacteriaceae) compete with host cells for iron affecting PHD2-activity and resulting in the activation of HIF-1 [11]. As mice with deletion of HIF-1α in the intestinal epithelium showed a significant higher susceptibility to orogastric Y. enterocolitica infections, bacterial HIF-1 activation appears to represent a host defense mechanism [11]. In fact, HIF-1 plays an important role in cellular host defense and innate immunity as HIF-1 is crucial for activation and stimulation of T cells by dendritic cells [35], regulates the bactericidal capacity of phagocytes [6] and the production of antimicrobial peptides (e.g., cathelicidin) [14]. Such defenses of the intestinal mucosa have been demonstrated to provide an antibacterial barrier to prevent infections with, e.g., Enterobacteriaceae [36]. Therefore, a lacking intestinal HIF-1 activity should finally result in a higher susceptibility for infections with, e.g., Y. enterocolitica. Similar mechanisms might also be operating in inflammatory bowel diseases (IBDs) where HIF-1 regulates the barrier function of the intestinal mucosa [37].

In contrast to the protective effect in intestinal infections, HIF-1 activation or induction of HIF-1 regulated genes seems to be detrimental in severe systemic infections. Strongly elevated VEGF serum levels were detected in septicaemic patients [e.g., suffering from a methicillin-resistant S. aureus (MRSA) infection] and in patients with severe meningitis [38–40]. HIF-1 activation was also observed in a lipopolysaccharide (LPS)-induced murine sepsis model [14]. Interestingly, functional blocking of serum VEGF by soluble VEGF-receptors decreased the mortality in a LPS-septicaemia model dramatically [15]. Concluding these observations and our results gained from the murine S. aureus peritonitis model (Fig 9A, G) we suggest that an overwhelming HIF-1 activation (and subsequent VEGF secretion) is detrimental in severe infections. A mechanistic explanation for this hypothesis might be hidden in the vasculopermeability-increasing effects of VEGF which was originally described as vasculopermeability factor (VPF) [41]. In fact, VEGF-dependent vascular leakage was already demonstrated in patients suffering from septic shock [42].

Taken together, HIF-1 activation is obviously a general phenomenon in severe infectious diseases caused largely by hypoxia-dependent mechanisms. Our ex vivo, in vitro and in vivo data from infections with humanpathogenic bacteria, fungi, viruses and protozoan point towards a most important role of HIF-1 in the host defense against various pathogens. Extensions of these findings will determine the exact mode of HIF-1 activation by pathogens and the related biological effects in critically ill patients. The application of HIF-1 inhibiting compounds for the treatment of severe infections (such as peritonitis) remains to be elucidated in further studies.

Materials and Methods

Bacterial Strains

For in vitro experiments, the following microorganisms were used: the laboratory strains S. aureus ATCC 33592, S. aureus 8325-4, S. aureus ermB, S. aureus pCX19B, S. aureus SA113, S. aureus Newman, S. aureus ATCC 25923 and the clinical isolates S. aureus A22223 I (wt; patient isolate 1a), S. aureus A22223 II (clinical SCV; patient isolate 1b), S. aureus OM 1a (wt; patient isolate 2a) and S. aureus OM 1b (clinical SCV; patient isolate 2b). Additionally, the following strains defective in cell wall components were used: S. aureus SA113 Agyf::ermB (about 87% reduced lipoteichoic acid content), S. aureus SA113 AgtG (defective in producing wall teichoic acid) and S. aureus Newman maH12 (defective in producing the extracellular adherence protein EAP). Other strains used for in vitro infection experiments were Staphylococcus epidermidis ATCC 12298, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Strepococcus agalactiae SK 43 and Candida albicans ATCC 90028.

Cell Culture and Infection Procedures of HeLa-229 cells and NHEKs

HeLa-229 cervix carcinoma cells were grown in VLE RPMI 1640 medium supplemented with 2 g/L NaHCO3 (Biochrom, Berlin, Germany), 10% heat-inactivated fetal calf serum (FCS; Sigma Aldrich, Taufkirchen, Germany), 1% L-glutamine (Gibco, Karlsruhe, Germany) and 10 mg/mL streptomycine and 100 U penicillin (Biochrom, Berlin, Germany). Normal Human Epidermal Keratinocytes (NHEKs) were grown in Keratinocyte Growth Medium 2 (PromoCell, Heidelberg, Germany) supplemented with the appropriate SupplementMix (supplied with Keratinocyte Growth Medium 2; PromoCell), 10 mg/mL streptomycine and 100 U penicillin (Biochrom).

For performing infection experiments, cells were detached with 0.05% Trypsin-EDTA (Gibco). After trypsinization of NHEKs, Trypsin-EDTA was neutralized by adding Trypsin Neutralizing Solution (PromoCell). Cells were seeded the day before infection in cell culture media without antibiotics (to allow bacterial growth). Infection experiments were performed in cell culture media without antibiotics and without FCS to avoid unspecific HIF-1 activation. The following pathogens were used: S. aureus, S. epidermidis, S. pyogenes, C. albicans [multiplicity of infection (MOI): 20, infection time: six hours], S. agalactiae (MOI: 200, infection time: six hours), E. coli, P. aeruginosa (MOI: 10, infection time: four hours). Uninfected cells were used as negative controls, desferrioxamine (DFO, 200 μmol/L, Sigma Aldrich) -treated cells or cells infected with the heat shock protein (Hsp) 90-inhibitor 17-(dimethylamino)-17-demethoxygeldanamycin (17-DMAG; LC labo-
ratories, Woburn, MA, USA)[25]. Cells were incubated with...
Detection of HIF-1 activation

For the detection of HIF-1 activation by immunoblotting, proteins from cell cultures were extracted as described [43], separated by 8% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Schwalbach, Germany). Mouse anti-HIF-1α antibodies (Becton Dickinson, Heidelberg, Germany) were used as primary antibodies and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibodies (Dako, Hamburg, Germany) as secondary antibodies. Signals were visualized with the enhanced chemiluminescent (ECL)-reagent (PJK, Kleinbittersdorf, Germany). For loading control, mouse actin-specific antibodies (Sigma Aldrich) were used. In some experiments, mouse anti-human HIF-2α specific antibodies were used (NB 100–132; Novus Biologicals, Littleton, CO, USA).

To detect HIF-1α protein in infected cells, high-amplification immunohistochemistry was used as described previously [44]. HeLa-229 cells or NHEKs were seeded on coated glass slides (Superfrost Plus, Menzel, Germany) the day before infection. Four hours upon infection, cells were fixed with freshly prepared 3.75% paraformaldehyde (PFA; pH 7.4; dissolved in PBS) for 30 minutes and rinsed in PBS. Antigen retrieval was performed for 2 minutes in preheated target retrieval solution (Dako) using a pressure cooker. For detection of HIF-1α, monoclonal mouse anti-human HIF-1α (m167; Novus) and biotinylated secondary anti-mouse antibodies (Dako) were used. For signal amplification and visualisation, a catalyzed signal amplification system (CSA-Kit, Dako) based on a streptavidin-biotin-peroxidase reaction was used according to the manufacturers instructions. Between incubations, specimens were washed two to three times (50 mmol/L 17-DMAG before stimulating HIF-1 activation by adding 200 μmol/L DFO.

Detection of Cellular Hypoxia and Oxygen Consumption

NHEKs and HeLa-229 cells were cultivated using conventional polystyrene dishes or special gas-permeable dishes (Lumox; Greiner Bio-One, Frickenhausen, Germany) with a hydrophilic tissue culture treated bottom membrane as described [46]. The pO2 (mmHg) was quantified in the medium of infected or uninfected control cells using the blood gas analyzer ABL-77, sensor cassettes and calibration packs (Radiometer, Willich, Germany). For internal control, cells were harvested in parallel four to six hours after infection, and whole cell extracts were prepared for the detection of HIF-1 activation by immunoblotting as described above.

Detection of hypoxia was also displayed by visualization of the hypoxic cell state using the hypoxia-sensitive marker pimonidazole hydrochloride (Natural Pharmacia International, Burlington, USA). Pimonidazole hydrochloride (which is a 2-nitroimidazole) forms adducts with thiol groups in proteins, peptides and amino acids in hypoxic cells (pO2 <10 mmHg) [47]. Here, 200 μmol/L pimonidazole hydrochloride (Hypoxyprobe-1) was added to HeLa-229 cells immediately before infection as described earlier [7]. After six hours of infection, cellular hypoxia was visualized using a primary monoclonal antibody IgG1 that detects protein adducts of pimonidazole in hypoxic cells (Hypoxyprobe-1Mab1; NPI, Burlington, USA) and secondary Cy3-conjugated goat anti-mouse IgG antibodies (Dianova, Hamburg, Germany). Visualization was performed using a Leica DM IRE2 confocal laser scanning microscope (CLSM; Leica Microsystems, Wetzlar, Germany).

Intraperitoneal infection of mice with S. aureus

Female NMRI mice (33–35 g) were infected intraperitoneally with S. aureus 8325-4 or S. aureus emrΩhemB 8325-4 (SCV) by injecting 0,3 × 10^7 bacteria/g body weight in conditioned medium, as described before [24]. To study the role of HIF-1α in vivo, mice were inoculated additionally with the HIF-1 inhibitor 17-DMAG (25 mg/g body weight; application 24 and 16 hours before infection) as described previously [25]. Survival rates were measured half-hourly. Peritoneal HIF-1α activation was analyzed by immunoblotting of the shock-frozen peritoneal specimens using the Nuclear Extract Kit (Active Motif; Rixensart, Belgium), a mouse monoclonal anti-HIF-1α (H106/7) antibody (Novus Biologicals, Littleton, USA) and horseradish peroxidase (HRP-conjugated rabbit anti-mouse IgG antibodies (Dako). Signals were visualized with the enhanced chemiluminescent (ECL)-reagent (PJK). Normalization of the samples used for immunoblotting was performed using a Pierce protein quantification assay (BCA Protein Assay; Thermo Fisher Scientific, Bonn, Germany).

Intravenous infection of mice with S. aureus

Mouse infection experiments were done in accordance with the animal experiment proposal (H2/06, Tuebingen, Germany, approved by the Regierungspäsidium Tübning, Germany). The Institutional Animal Care and Use Committee approved this protocol. Female NMRI mice (33–35 g) were infected intravenously with S. aureus 8325-4 (0,3×10^7 bacteria/g body weight) resuspended in 200 μl conditioned medium as described previously [23,24]. Seven days after infection, mice were euthanized and kidneys were taken out when a macroscopically visible abscess formation had occurred. Organ fixation was performed using 3.75% PBS-buffered (pH 7.4) paraformaldehyde (PFA).
Selection of patient samples

Formalin-fixed, paraffin-embedded diagnostic tissue biopsies were collected from the files of the Department of Dermatology, University of Tuebingen. Only samples for which a microbiological diagnosis of the underlying infection was made in parallel were included in the study. Microbiological pathogen identification was performed using standard microbiological techniques [46].

Histology and HIF-1 immunohistochemistry of patient and murine samples

For murine kidney samples, serial sections (thickness of the sections: 2 μm) were done and processed with hematoxylin-cosin and HIF-1α staining. Human samples were processed directly from paraffin-embedded blocks.

Paraffin sections (2 μm) were dewaxed in xylene, rehydrated in a series of ethanol washes, and placed in distilled water before staining procedures. Slides were coated with 3-aminopropyl-triethoxysilane. For detection of HIF-1α isoforms, a monoclonal mouse anti-human HIF-1α antibody was used for patient samples (267; Novus Biologicals) and polyclonal rabbit anti-mouse HIF-1α antibodies were used for murine samples (PM16, obtained from a rabbit immunized with a peptide containing amino acids 553 to 609 of mouse HIF-1α; kindly gift from Patrick Maxwell, Imperial Hospital, London, UK). Detection of bound antibodies was performed by using biotinylated secondary anti-mouse (patient samples) or anti-rabbit (murine samples) antibodies and a catalyzed signal amplification system (Dako) based on the streptavidin-biotin-peroxidase reaction, according to the instructions provided by the manufacturer. Antigen retrieval was performed for 90 s in preheated target retrieval solution (Dako) using a pressure cooker. All incubations were performed in a humidified chamber. Between incubations, specimens were washed two to four times in buffer (50 mmol/L Tris-HCl, 300 mmol/L NaCl, 0.1% Tween-20, pH 7.6). For counterstaining, hematoxylin-cosin staining of the corresponding section was done using standard laboratory procedures.

Statistical analysis

For statistical analysis, the unpaired, two-tailed Student t test was used. Analysis of the survival rates of mice was performed by Kaplan-Meier analysis using the GraphPad Prism Software (GraphPad Software Inc., La Jolla, USA). For all assays, a value of P<0.05 was considered statistically significant.

Supporting Information

Figure S1 Induction of HIF-1 activation in HeLa-229 cells by bacterial pathogens. Hela-229 cells were seeded on glass slides infected with P. aeruginosa ATCC 27853 or C. albicans ATCC 90028. HIF-1α activation was detected by nuclear accumulation of HIF-1α via immunohistochemistry six hours upon infection. Negative control: uninfected cells; positive control: DFO (200 μmol/L). Scale bar: 20 μm. Found at: doi:10.1371/journal.pone.0011576.s001 (1.47 MB TIF)

Figure S2 Induction of HIF-1-dependent gene programming by bacterial pathogens in HeLa-229 cells. Transcriptional analysis of (A) VEGF or (B) HIF 1α upon infection. Total mRNA was prepared four to six hours upon infection, transcribed into cDNA, and mRNA was quantified by real-time Light-Cycler-PCR (ratio: VEGF/actin or HIF 1α/actin transcripts; triplicate means given). * significant difference to control cells (P<0.05). Found at: doi:10.1371/journal.pone.0011576.s002 (0.82 MB TIF)

Acknowledgments

The authors thank Charlotte Reitmeier (Universitäts-Hautklinik, Tübingen) for excellent technical assistance.

Author Contributions

Conceived and designed the experiments: NW AP VK. Performed the experiments: NW CB CR ASY ME AA WB AS VK. Analyzed the data: NW CR ASY ME AA AS AP VK. Contributed reagents/materials/analysis tools: CvE KB AP VK. Wrote the paper: NW VK.

References

1. Shimoda LA, Manalo DJ, Sham JS, Semenza GL, Sylvestre JT (2001) Partial HIF-1α deficiency impairs pulmonary arterial myocyte electrophysiological responses to hypoxia. Am J Physiol Lung Cell Mol Physiol 281: 202–208.
2. Pugh CW, Ratcliffe PJ (2003) Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 9: 677–684.
3. Ivan M, Kondo K, Yang H, Kim W, Valiando J, et al. (2001) HIFα alpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292: 464–468.
4. Mazure NM, Brahami-Horn MC, Berta MA, Benzi E, Bilston RL, et al. (2004) HIF-1: master and commander of the hypoxic world. A pharmacological approach to its regulation by siRNAs. Biochem Pharmacol 68: 971–980.
5. Cramer T, Yamamichi Y, Chaeus BF, Forster F, Pawiński R, et al. (2003) HIF-1α is essential for myeloid cell-mediated inflammation. Cell 112: 645–657.
6. Peysonnaux C, Datta V, Cramer T, Doedens A, Theodorakis EA, et al. (2005) HIF-1α expression regulates the bacterial capacity of phagocytes. J Clin Invest 115: 1096–1105.
7. Kempf VA, Lebiedzidzevski M, Altitoz K, Waldein JH, Ehlach U, et al. (2005) Activation of hypoxia-inducible factor-1 in basillary angiomas: evidence for a role of hypoxia-inducible factor-1 in bacterial infections. Circulation 111: 1054–1062.
8. Kaiser PO, Riess T, Wagner CL, Linke D, Lupas AN, et al. (2008) The head of Bartolomea adhesin A is crucial for host cell interaction of Bartolomea hirne. Cell Microbiol 10: 2223–2234.
9. Kempf VA, Volkman B, Schaller M, Sander CA, Altitoz K, et al. (2003) Evidence of a leading role for VEGF in Bartolomea hirne-induced endothelial cell proliferations. Cell Microbiol 3: 623–632.
10. Riess T, Anderson SG, Lupas A, Schaller M, Schäfer A, et al. (2004) Bartolomea adhesin A mediates a proangiogenic host cell response. J Exp Med 200: 1267–1278.
11. Hartmann H, Eltzschig HK, Würz H, Hanke K, Rakin A, et al. (2008) Hypoxia-independent activation of HIF-1 by enterobacteriaceae and their siderophores. Gastroenterology 134: 756–767.
12. Blouin CC, Page EL, Soucy GM, Richard DE (2004) Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor-1α. Exp Cell Res 296: 112–119.
13. Frede S, Stockmann C, Freitag P, Fandrey J (2006) Bacterial lipopolysaccharides induce HIF-1α activation in human monocytes via p44/42 MAPK and NF-κappaB. Biochem J 396: 517–527.
14. Peysonnaux C, Cejudo-Martin P, Doedens A, Zinkernagel AS, Johnson RS, et al. (2007) Essential role of hypoxia inducible factor-1α in development of lipopolysaccharide-induced sepsis. J Immunol 178: 7516–7519.
15. Yano K, Liaw PC, Mollington JM, Shi JH, Okada H, et al. (2006) Vascular endothelial growth factor is an important determinant of sepsis morbidity and mortality. J Exp Med 203: 1447–1458.
16. von Eiff C, Heilmann C, Proctor RA, Woltz C, Peters G, et al. (1997) A site-directed mutant of B. henselae hemB is a small-colony variant which persists intracellularly. J Bacteriol 179: 4706–4712.
17. von Eiff C (2000) Staphylococcus aureus small colony variants: a challenge to microbiologists and clinicians. Int J Antimicrob Agents 31: 507–510.
18. Fedtke I, Mader D, Kohler T, Moll H, Nicholson G, et al. (2007) A site-directed inactivation of Eap in Staphylococcus aureus hemB mutant is a small-colony variant which persists intracellularly. J Bacteriol 189: 1067–1070.
19. Weidnermaier C, Koki-Kun JF, Kristian SA, Chanturiya T, Kelhacher H, et al. (2004) Role of teichoic acids in Staphylococcus aureus nasal colonization, a major risk factor in nosocomial infections. Nat Med 10: 243–245.
20. Hussain M, Haggar A, Heilmann C, Peters G, Flock JL, et al. (2002) Insertional inactivation of Eap in Staphylococcus aureus strain Newman confers reduced staphylococcal binding to fibroblasts. Infect Immun 70: 2933–2940.
21. Lammergard J, von Eiff C, Sander G, Cordes T, Seggewiss J, et al. (2008) Identification of the genetic basis for clinical menadioneauxothropic small-colony variant isolates of Staphylococcus aureus. Antimicrob Agents Chemother 52: 4017–4022.
22. Sifri CD, Baresch-Bernal A, Calderwood SB, von Eiff C (2006) Virulence of Staphylococcus aureus extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. Cell Microbiol 9: 2181–2191.

23. Essin K, Salanova B, Kettritz R, Saubier M, Luft FC, et al. (2007) Large-conductance calcium-activated potassium channel activity is absent in human and mouse neutrophils and is not required for innate immunity. Ann J Physiol Cell Physiol 293: 45–54.

24. Chavakis T, Hussian M, Kame SM, Peters G, Bretzel RG, et al. (2002) Staphylococcus aureus-induced sepsis: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 4: 295–305.

25. Lang SA, Moser C, Gaumann A, Klein D, Glockzin G, et al. (2007) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J 22: 4082–4090.

26. Rupp J, Gieffers J, Klinger M, van Zandbergen G, Wrase R, et al. (2007) Methicillin-resistant Staphylococcus aureus extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. Cell Microbiol 9: 1091–1096.

27. Jaakkola P, Molet DR, Tian YM, Wilson MI, Gielbert J, et al. (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylase. Science 292: 468–472.

28. Berra E, Benizri E, Ginouves A, Volmat V, Roux D, et al. (2003) HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J 22: 4082–4090.

29. Ropp J, Gieffers J, Klinger M, van Zandbergen G, Wrase R, et al. (2007) Methicillin-resistant Staphylococcus aureus extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. Cell Microbiol 9: 1091–1096.

30. Yeo M, Kim DK, Han SU, Lee JE, Kim YB, et al. (2006) Novel action of gastric acid variants in the Staphylococcus aureus infection model. Infect Immun 74: 1091–1096.

31. Kempf VA, Hitziger N, Riess T, Autenrieth IB (2002) Do plant and human pathogens have a common pathogenicity strategy? Trends Microbiol 10: 269–273.

32. Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, et al. (2006) Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 4: 295–305.

33. Lang SA, Moser C, Gaumann A, Klein D, Glockzin G, et al. (2007) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J 22: 4082–4090.

34. Jantsch J, Chakravortty D, Tarza N, Prechtel AT, Buchholz B, et al. (2008) Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function. J Immunol 180: 4697–4705.

35. Meyer-Hoffert U, Hornef MW, Henzi-Liu-Norman B, Axelsson LG, Midvold T, et al. (2008) Secreted enteric antimicrobial activity localizes to the mucus surface layer. Gut 57: 764–771.

36. Karhausen J, Furuta GT, Tomaszewski JE, Johnson RS, Golgan SP, et al. (2004) Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. J Clin Invest 114: 1098–1106.

37. Enkhbaatar P (2008) Methylcellulose-resistant Staphylococcus aureus-induced sepsis: role of nitric oxide. In: Yearbook of intensive care and emergency medicine. Traber L, Traber D, eds. Springer-Verlag, Berlin, Heidelberg, New York. pp 404–412.

38. van der Flier M, van Leeuwen HJ, van Kessel KP, Kimpen JL, Hoepelman AI, et al. (2005) Plasma vascular endothelial growth factor in severe sepsis. Shock 23: 35–39.

39. van der Flier M, Stockhammer G, Vonk GJ, Nikkels PG, van Diemen-Stevenoorde RA, et al. (2001) Vascular endothelial growth factor in bacterial meningitis: detection in cerebrospinal fluid and localization in postmortem brain. J Infect Dis 183: 149–153.

40. Seigner DR, Galli SJ, Dvorak CA, Dvorak HV, et al. (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219: 983–985.

41. Pickkers P, Sprong T, Eijk J, Hooeven H, Smits P, et al. (2005) Vascular endothelial growth factor is increased during the first 48 hours of human septic shock and correlates with vascular permeability. Shock 24: 508–512.

42. Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, et al. (1998) Oxygen-independent stabilization of hypoxia inducible factor (HIF)-1alpha in normoxia. EMBO J 22: 4082–4090.

43. Karhausen J, Furuta GT, Tomaszewski JE, Johnson RS, Golgan SP, et al. (2004) Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. J Clin Invest 114: 1098–1106.

44. Doege K, Heine S, Jensen I, Jelkmann W, Metzen E (2005) Inhibition of HIF-1 alpha in normoxia. EMBO J 22: 4082–4090.

45. Sheta EA, Trout H, Gildea JJ, Harding MA, Theodorescu D (2001) Cell density mediated pericellular hypoxia leads to induction of HIF-1alpha via nitric oxide and Ras/MAP kinase mediated signaling pathways. Oncogene 20: 7624–7634.

46. Meyer-Hoffert U, Hornef MW, Henzi-Liu-Norman B, Axelsson LG, Midvold T, et al. (2008) Secreted enteric antimicrobial activity localizes to the mucus surface layer. Gut 57: 764–771.

47. Enkhbaatar P (2008) Methylcellulose-resistant Staphylococcus aureus-induced sepsis: role of nitric oxide. In: Yearbook of intensive care and emergency medicine. Traber L, Traber D, eds. Springer-Verlag, Berlin, Heidelberg, New York. pp 404–412.

48. Kerl H, Garbe C, Cerroni L, Wolff H (2003) Histopathologie der Haut. Springer Verlag, Berlin, Heidelberg, New York. pp 3–7.