Hypoxia-inducible Factor and Target Gene Expression Are Decreased in Patients with Sepsis

Prospective Observational Clinical and Cellular Studies

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ABSTRACT

Background: Hypoxia-inducible factor-1 (HIF-1) is a molecular key player in response to hypoxemic/inflammatory conditions prevailing in sepsis. In a prospective observational study, we tested the hypotheses that sepsis affects HIF-1α messenger ribonucleic acid (mRNA) expression (primary hypothesis) and also (secondary hypotheses) the expression of the HIF-1α target genes adrenomedullin and β₂-integrins. Furthermore, we tested that lipopolysaccharide administration increases HIF-1α mRNA and protein in naive and endotoxin-tolerant monocytes.

Methods: In 99 patients with sepsis and 48 healthy volunteers, leukocyte HIF-1α mRNA expression (real-time polymerase chain reaction), cytokine concentrations (enzyme-linked immunosorbent assay), and intracellular mRNA expression were measured in naive or endotoxin-tolerant (48 h; 60 ng/ml lipopolysaccharide) monocytes, with/without additional lipopolysaccharide (6 h; 1 μg/ml).

Results: In comparison to healthy volunteers, HIF-1α mRNA expression (−67%; \( P = 0.0001 \)) and HIF-1α protein (immunofluorescence stain) were decreased in endotoxin-tolerant cells, which still upregulated HIF-1α mRNA expression, whereas HIF-1α mRNA and protein (−60%; \( P = 0.001 \)) were decreased in endotoxin-tolerant cells, which still upregulated cytokines.

Conclusions: In sepsis, HIF-1α mRNA expression was suppressed and inversely associated with illness severity.

What We Already Know about This Topic
- Sepsis is a leading cause of perioperative morbidity and mortality
- Hypoxia-inducible factor-1α is a marker of cellular hypoxia

What This Article Tells Us That Is New
- In monocytes of patients with sepsis, hypoxia-inducible factor-1α was decreased compared to monocytes of healthy control patients, and the decrease in expression was inversely related to the severity of sepsis
- Disease severity may be linked to an impaired response to hypoxia
While acute lipopolysaccharide administration increased HIF-1α mRNA expression, prolonged stimulation suppressed HIF-1α expression. The clinical implications of decreased HIF-1α may include maladaptation to tissue hypoxia or depressed immune function.

Mammalian immune cells respond to hypoxic conditions by activating the heterodimeric transcription factor complex hypoxia-inducible factor 1 (HIF-1), which is a key regulator of the cellular hypoxia-induced gene expression profile. HIF-1-dependent gene expression enables immune cells to maintain their function when oxygenation is decreased and to withstand concomitant acidosis and hypoxemia. The HIF-1 complex is formed by two subunits, a constitutively expressed nuclear β-subunit (HIF-1β) and the α-subunit (HIF-1α), which is highly regulated by cellular oxygen tension. Under normoxic conditions, HIF-1α is continuously generated but rapidly degraded by the ubiquitin–proteasome system after hydroxylation by oxygen-dependent prolylhydroxylases and recognition by the von Hippel–Lindau protein. During hypoxia, the activity of prolylhydroxylases decreases, and HIF-1α is no longer degraded and accumulates in the nucleus. Consecutively, HIF-1α dimerizes with HIF-1β and binds to hypoxia-responsive elements, thus adapting the cellular gene expression pattern to altered environmental conditions. Besides hypoxia, inflammatory stimuli such as bacterial cell wall components or cytokines released from inflamed tissues impact on HIF-1α regulation.

In vitro, following acute lipopolysaccharide stimulation, HIF-1α protein accumulates and HIF-1 target genes are expressed due to increased HIF-1α mRNA expression. In contrast, following repetitive lipopolysaccharide stimulation, endotoxin tolerance occurs, and HIF-1α decreases in monocytes. In patients with sepsis, circulating monocytes are first exposed to low toxin concentrations (incipient sepsis) and then to increasing toxin concentrations either in the blood stream or when migrating toward foci of pathogens, with higher toxin concentrations likely prevailing in such tissues. Surprisingly, regulation of HIF-1α in human sepsis, a condition often including both local hypoxia and prolonged inflammation, has not been assessed. Accordingly, it remains unclear whether HIF-1α mRNA expression is increased, decreased, or unchanged in patients with sepsis. Furthermore, it also remains unclear whether decreased HIF-1α protein concentration, associated with prolonged or repetitive lipopolysaccharide exposure in vitro, is solely due to decreased mRNA expression, or caused by both increased degradation and decreased mRNA expression. To clarify, this issue was analyzed in this study. Understanding adaptations of mononuclear cells to inflammation is important, as these mechanisms enable immune cells to function at the sites of inflammation.

Therefore, we performed a prospective observational study and tested the primary hypothesis that sepsis affects HIF-1α mRNA expression and also (secondary hypotheses) the expression of the HIF-1α target gene adrenomedullin and of β2-integrins (CD11a, CD11b, and CD18). Furthermore, HIF-1α mRNA expression and protein concentration were analyzed in lipopolysaccharide prestimulated and unstimulated monocytic cells (THP-1) to potentially explain expression changes observed in patients.

Material and Methods

Patient Study

The study was reviewed and approved by the Medical Faculty’s Ethics Committee (No. 06-3078, University of Duisburg-Essen, Essen, Germany). Healthy volunteers were recruited by bulletins posted in our hospital and enrolled following written informed consent, and blood was drawn from a peripheral vein. Furthermore, adult patients with sepsis admitted to an intensive care unit of the University Hospital Essen, Germany, between 2006 and 2011 were evaluated for inclusion. Patients with sepsis were considered eligible when they fulfilled the 2001 American College of Chest Physicians/Society of Critical Care Medicine Consensus conference guidelines. Patients with sepsis were enrolled on the day of first diagnosing sepsis. During the study period, a total of 492 patients were coded for systemic inflammatory response syndrome or sepsis and were attended to in various intensive care units of our hospital (including bone marrow and solid organ transplant recipients undergoing immunosuppression). Of these, 380 had severe sepsis and were, therefore, screened for study inclusion. Of these 380 patients, 281 were not included as they did not meet the inclusion criteria, refused or withdrew participation, suffered from mental dysfunction, or because blood withdrawal and/or immediate preparation and storage of the samples was not available within the 24-h time frame after first diagnosing sepsis, i.e., at weekends and holidays. Eventually, 99 patients were included in this prospective observational trial. Arterial and venous blood samples were taken for blood tests, microbiology cultures, and RNA extraction within the first 24 h after diagnosing sepsis. Furthermore, the Simplified Acute Physiology Score II (SAPS II), results of blood cultures, length of hospitalization, and 30-day mortality were assessed. Characteristics both of patients with sepsis and of healthy volunteers, hemodynamic variables, and results of blood tests are displayed in table 1.

Cell Culture

The human monocytic cell line THP-1 was obtained from the German Collection for Microorganisms and Cells (DSMZ, Braunschweig, Germany). THP-1 cells were cultured in Roswell Park Memorial Institute 1640 medium, supplemented with 10% fetal bovine serum, 1.5 mM L-glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml).
Hypoxia Inducible Factor in Sepsis

and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO₂ in air. For all experiments, 2 × 10⁶ cells were incubated in 35 mm Petri dishes, and lipopolysaccharide was added to the cells as indicated for each experiment. At the end of the experiments, cells were lysed in 4 M guanidine thiocyanate followed by total RNA extraction for the determination of specific mRNA by real-time polymerase chain reaction (RT-PCR). Additionally, total cell lysates were prepared for Western blotting.

**Lipopolysaccharide Prestimulation Experiments and HIF-1α Protein Stabilization**

THP-1 cells were kept in serum-free Roswell Park Memorial Institute 1640 medium only (naïve) or were prestimulated with lipopolysaccharide (0.05 ng/ml) for 48 h. After renewal of the cell culture medium, naïve or lipopolysaccharide prestimulated cells were further incubated with or without lipopolysaccharide (0.05 ng/ml or 1 μg/ml) for 6 h, as described previously. Results of three experiments were combined for analysis and normalized to naïve controls. For Western blotting, the prolylhydroxylase inhibitor dimethylamylglycine (10 μM) was added to samples, as indicated, to analyze whether decreased protein concentrations result from increased prolylhydroxylase activity or decreased HIF-1α mRNA expression. To analyze gene expression of cytokine-related genes and to rule out an overall suppression of gene expression in lipopolysaccharide prestimulated cells, we performed RT-PCR analysis for interleukin 10 and tumor necrosis factor-α.

**RNA Preparation, Qualitative and RT-PCR**

Hypoxic gene regulation was analyzed by qualitative and RT-PCR following RNA extraction, as described. RNA extraction was performed on the patients’ blood samples using the RNeasy Midi kit (Qiagen, Hilden, Germany), and for all cell culture studies by the acidic guanidinium thiocyanate–phenol–chloroform extraction method, as described previously. One microgram of total RNA was reverse-transcribed to complementary deoxyribonucleic acid (cDNA). Primers for qualitative and RT-PCR were designed using National Center for Biotechnology Information primer blast (Rockville Pike, MD) and were obtained from Invitrogen (Invitrogen AG; Karlsruhe, Germany).

### Table 1. Characteristics of Patients with Sepsis and Healthy Volunteers

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients with Sepsis</th>
<th>Healthy Volunteers</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female; No.)</td>
<td>65/34</td>
<td>28/20</td>
<td>0.45*</td>
</tr>
<tr>
<td>Height (cm; mean ± SD)</td>
<td>173 (10)</td>
<td>173 (9)</td>
<td>0.98†</td>
</tr>
<tr>
<td>Body weight (kg; mean ± SD)</td>
<td>81.5 (19)</td>
<td>76.9 (15)</td>
<td>0.18†</td>
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<tr>
<td>Age (yr; median; Q1; Q3)</td>
<td>60; 49; 70</td>
<td>39; 27; 57</td>
<td>0.001‡</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mmHg; median; Q1; Q3)</td>
<td>80; 70; 97</td>
<td>93; 89; 100</td>
<td>0.001‡</td>
</tr>
<tr>
<td>Heart rate (min⁻¹; median; Q1; Q3)</td>
<td>90; 77; 100</td>
<td>70; 64; 75</td>
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</tr>
<tr>
<td>Primary diagnoses, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal cancer</td>
<td>17.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal disease</td>
<td>26.8</td>
<td></td>
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</tr>
<tr>
<td>Cardiovascular disease</td>
<td>22.6</td>
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<tr>
<td>Lung disease</td>
<td>14.5</td>
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<td>Urogenital cancer</td>
<td>4.1</td>
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<td>Intraabdominal pathology, other</td>
<td>5.2</td>
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<tr>
<td>Cancer, other</td>
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<tr>
<td>Hematooncological disease</td>
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<td>Infectious variables</td>
<td></td>
<td></td>
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<tr>
<td>Leukocyte count (×10⁹/l; median; Q1; Q3)</td>
<td>14; 8.4; 20</td>
<td>6.3; 5.2; 7</td>
<td>0.0001‡</td>
</tr>
<tr>
<td>Procalcitonin concentration (µg/l; median; Q1; Q3)</td>
<td>2.3; 0.6; 13</td>
<td>0; 0; 0</td>
<td>0.0001‡</td>
</tr>
<tr>
<td>C-reactive protein concentration (g/l; median; Q1; Q3)</td>
<td>13; 7.3; 24</td>
<td>0; 0; 0</td>
<td>0.0001‡</td>
</tr>
<tr>
<td>Disease severity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAPS III (mean ± SD)</td>
<td>40 (16)</td>
<td></td>
<td></td>
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<tr>
<td>Hospital stay (d; mean ± SD)</td>
<td>34 (24)</td>
<td></td>
<td></td>
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<tr>
<td>30-day mortality (%)</td>
<td>31.3</td>
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</tbody>
</table>

Biometric data, primary diagnoses, infectious variables, and disease severity of 99 patients with sepsis and of 48 healthy volunteers. Data were documented at the time of first diagnosing sepsis and at the time of blood withdrawal in healthy volunteers, respectively.

* Numbers; P value based on Fisher exact tests. † Mean ± SD; P value based on t test for independent samples. ‡ Median with 25 and 75% quartiles (median; Q1; Q3); P value based on Mann–Whitney U test. § No statistical test has been applied here. || Simplified Acute Physiology Score II.

SAPS = Simplified Acute Physiology Score.
First, qualitative PCR was performed for glyceraldehyde-
3-phosphate-dehydrogenase as housekeeping gene to test
the quality of cDNA synthesis.\textsuperscript{13} Second, qualitative PCR
was performed to estimate the amount of specific cDNA for
HIF-1\textalpha, adrenomedullin, a HIF-1 target gene involved in
the regulation of vascular caliber, for the \( \beta_2 \)-integrins CD11a,
CD11b, and CD18 which are involved in cell adhesion, and,
in THP-1 cells for the cytokine-related genes interleukin 10
and tumor necrosis factor-\( \gamma \).\textsuperscript{14,15} Resulting cDNA bands
were visualized using ethidium bromide-stained 1.5% (w/v)
agarose gels.\textsuperscript{3,11}

HIF-1\textalpha, adrenomedullin, CD11a, CD11b, and CD18,
tumor necrosis factor-\( \alpha \), and glyceraldehyde-
3-phosphate-dehydrogenase mRNA expression were
quantified by RT-PCR using SYBR\textsuperscript{\textregistered} green as fluorescent
dye (Eurogentec, Verviers, Belgium) on the iQ5 Multicolor
Real-time PCR Detection System (Bio-Rad, Munich,
Germany) in a two-step RT-PCR. The denaturation steps were
performed at 95°C for 10 min followed by 40 cycles at 95°C
for 15 s, and at 60 or 61°C for 1 min, as described previ-
ously.\textsuperscript{2,3} Amounts of specific cDNA were finally normal-
zied to glyceraldehyde-3-phosphate dehydrogenase using
the \( \Delta \Delta C_t \) method, and results are depicted as 2\( \Delta \Delta C_t \) values, as
described.\textsuperscript{13,16}

**Whole Cell Lysate Preparation**

For whole cell lysate preparation, cells were incubated with
65 \( \mu \)l lysis buffer (150 mm NaCl, 10 mm Tris pH 7.9, 1
mm EDTA, 0.1% Igepal, and 1x protease inhibitor cock-
tail [Roche; Mannheim, Germany]) for 20 min on ice. The
lysates were centrifuged at 1200g at 4°C for 5 min, and
supernatants containing cellular proteins were collected and
stored at −80°C. Protein concentrations of the supernatants
were quantified using the Bio-Rad protein assay reagent.\textsuperscript{5}

**Western Blot Analysis**

Fifty microgram of total cell lysate per lane was subjected
to 7.5% sodium dodecyl sulfate–polyacrylamide gel elec-
trophoresis and transferred onto a nitrocellulose membrane
(0.2 \( \mu \)m pore size; Schleicher and Schuell, Dassel,
Germany). HIF-1\textalpha was detected using a mouse monoclonal
antibody against human HIF-1\textalpha (Transduction Laborato-
ries, San Diego, CA); antibody binding was detected with
an anti-mouse antibody (Novus Biologicals, Denver, CO).
\( \alpha \)-Tubulin (Santa Cruz Biotechnology, Inc., Santa
Clara, CA) served as the loading control.\textsuperscript{3,11}

**Immunofluorescence Staining**

Blood smears from healthy volunteers and patients with sep-
sis were prepared, dried under airflow, and stored at −80°C
within 5 min after blood withdrawal. In addition, blood
samples were incubated with dimethyloxalylglycine (10 \( \mu \)m)
for 2h to achieve HIF-1\textalpha protein stabilization before blood
smears were performed.

For analysis, blood smears were thawed for 10 min at
room temperature and fixed with ice cold methanol/acetone
(1:1) for 10 min at −20°C. Afterward, immunofluorescence
staining was performed using a primary mouse antihuman
HIF-1\textalpha antibody (Transduction Laboratories) followed by
an IgG Alexa-Flour 488 coupled goat anti-mouse antibody
(Molecular Probes, order No. A11001, Eugene, OR), as
described previously.\textsuperscript{17}

An independent investigator, blind for sample origin,
evaluated all immunofluorescence slides in a randomized
order. Fluorescence microscopy was performed on a Nikon
Eclipse E1000 microscope (Nikon GmbH, Düsseldorf,
Germany) using the NIS-Elements F3.0.0 imaging software
(Laboratory Imaging, Prague, Czech Republic). All slides
were analyzed using a standardized procedure. Erythro-
cytes, which account for the major portion of cells in blood
smears, were easily identified due to their homogenous size
(approximately 8 \( \mu \)m), central concavity, and typical non-
fluorescent background staining pattern. All other cells with
a size of 8–12 \( \mu \)m were analyzed. In detail, two blood smear
slides were prepared per sample, and 10 successive images
were captured at 40-fold magnification; leukocytes were
counted and categorized as HIF-1\textalpha positive (entirely fluo-
rescent cells), intermediate (partly fluorescent cells), or nega-
tive (nonfluorescent cells) using the image software Image J
1.43r (National Institute of Health, Bethesda, MD).

**Cytokine Measurements**

Cytokine serum concentrations (interferon \( \alpha \) and \( \gamma \), inter-
leukin 6 and 10, and tumor necrosis factor-\( \gamma \)) were analyzed
using the Procarta Cytokine Assay kit (Affymetrix Inc., Santa
Clara, CA) and measurements were performed according to
the manufacturer’s instructions.

**Statistical Analysis**

Data are presented as means ± SD unless indicated
otherwise. The two-tailed \( t \) test for independent samples or,
in case of violations of the normality assumption (as tested
by Kolmogorov–Smirnov and Shapiro–Wilk tests), the
Mann–Whitney \( U \) test was used to compare patients with
sepsis and healthy volunteers. An \( a \) priori \( \alpha \) error \( P < 0.05
\) was considered statistically significant.

To address multiple testing issues, we focused on HIF-1\textalpha
mRNA expression and tested the following \( a \) priori ordered
hypotheses that sepsis affects HIF-1\textalpha mRNA expression
(primary hypothesis) and also (secondary hypotheses) the
expression of the HIF-1\textalpha target gene adrenomedullin and of
\( \beta_2 \)-integrins (CD11a, CD11b, and CD18). Consequently,
each hypothesis can be tested at a (nominal) significance
level \( \alpha \) of 5% (two-sided) while controlling the family-wise
error in a strong sense.\textsuperscript{18,19} However, if any of the hypotheses
cannot be rejected, all subsequent hypotheses (in the hierar-
chical order) cannot be tested.

Explorative between-group comparisons were performed using
one-way ANOVAs with \( \textit{post hoc} \) two-tailed
independent samples t test to compare differences in HIF-1α protein positive cells in blood smears from healthy volunteers and patients with sepsis with/without dimethyloxalylglycine incubation (\(P = 0.05/n\)). Furthermore, we report the Spearman correlation coefficient for the association between SAPS II and HIF-1α mRNA expression. Finally, logistic regression analysis was performed to analyze 30-day mortality of patients with sepsis dependent on HIF-1α mRNA expression.

For in vitro studies, explorative between-group comparisons were performed using one-way ANOVAs and post hoc two-tailed t tests for independent samples to compare HIF-1α, interleukin 10, and tumor necrosis factor-α mRNA expression in naive and prestimulated cells with or without additional lipopolysaccharide stimulation. To account for multiple testing, the significance level was adjusted (\(P = 0.05/n\)). All statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL) and Graph Pad Prism 5 (Graph Pad Software, La Jolla, CA) software.

Results

**HIF-1α in Leukocytes from Patients with Sepsis**

HIF-1α mRNA expression in leukocytes was decreased in human sepsis. ∆ct values for HIF-1α mRNA expression were −4.6 ± 1.5 in healthy volunteers and −6.5 ± 3.6 in patients with sepsis, corresponding to a 67% decrease in HIF-1α mRNA expression in patients with sepsis compared to that in healthy controls (\(2^{-\Delta\Deltact} = 0.33 \pm 3.79; P = 0.0001\); fig. 1A).

Immunofluorescence staining for HIF-1α protein was successfully established for leukocytes in blood smears from healthy volunteers and patients with sepsis (fig. 1B). Healthy volunteers had significantly more HIF-1α positive leukocytes both under control conditions (46.3% ± 45.8 vs. 15.4 ± 31.9, \(P = 0.01\)) and following incubation with the HIF-1α protein stabilizing agent dimethyloxalylglycine (10 μM) for 2 h (56.5 ± 46.6 vs. 28.1 ± 44; \(P = 0.01\)). Of interest, HIF-1α mRNA expression was not different in patients with septic shock compared to those with severe sepsis (−6.9 ± 3.2 vs. −6.3 ± −3.4; \(P > 0.05\)).

**Adrenomedullin and β2-integrin (CD11a, CD11b, and CD18) mRNA Expression in Leukocytes from Patients with Sepsis**

Expression of the mRNA for adrenomedullin, a known HIF target gene, was decreased by 75% (\(P = 0.0001\)) in patients with sepsis compared to that in healthy volunteers (fig. 2A; ∆ct values −7.6 ± 3.9 in patients with sepsis and −5.3 ± 1.6 in healthy volunteers). Likewise, the expression of the β2-integrin CD11a and CD11b was decreased by 86% and 85%, respectively (fig. 2, B and C; ∆ct values for CD11a: patients with sepsis −6.7 ± 5.4; healthy volunteers −3.9 ± 2.3; \(P = 0.0001\); ∆ct values for CD11b: patients with sepsis −6.7 ± 5.4; healthy volunteers −3.9 ± 2.3; \(P = 0.0001\); ∆ct values for CD11b: patients with sepsis −6.7 ± 5.4; healthy volunteers −3.9 ± 2.3; \(P = 0.0001\); ∆ct values for CD11b: patients with sepsis −6.7 ± 5.4; healthy volunteers −3.9 ± 2.3; \(P = 0.0001\)).
−4.6 ± 3.1; healthy volunteers −2.0 ± 1.1; P = 0.0001). In contrast, mRNA expression of the common β₂-integrin subunit CD18 was 1.6-fold ± 4.3 induced in patients with sepsis compared to that in healthy volunteers (fig. 2D; ∆ct values: patients with sepsis −2.2 ± 4.3; healthy volunteers −2.9 ± 2.5, P = 0.004).

A subgroup analysis revealed that patients with septic shock had decreased expression of CD11a (septic shock, −6.7 ± 3.9; severe sepsis, −4.5 ± −6.9; P = 0.001), CD11b (septic shock, −2.8 ± 4.3; severe sepsis, −1.1 ± −3.6; P = 0.012), and CD18 (septic shock, −7.0 ± 5.4; severe sepsis, −5.4 ± 4.7; P = 0.001) compared to patients with sepsis, respectively.

**Cytokine Serum Concentrations in Patients with Sepsis and Healthy Volunteers**

Interleukin 6 serum concentration was significantly greater (P = 0.0001) in patients with sepsis ([pg/ml; median; Q1; Q3] 69.4; 17.5; 228) than in healthy volunteers (1.0; 1.0; 2.89). Furthermore, interleukin 10 serum concentration was markedly increased in patients with sepsis ([pg/ml; median; Q1; Q3] 3.36; 1.0; 8.54) compared to that in healthy volunteers (1.0; 1.0; 1.0; P = 0.0001). Tumor necrosis factor-α concentration also increased in sepsis ([pg/ml; median; Q1; Q3]: patients with sepsis: 2.09; 1.05; 5.50; healthy volunteers: 1.0; 1.0; 2.09; P = 0.0002). However, interferon serum concentrations were not altered in patients with sepsis compared to healthy volunteers (interferon α [pg/ml; median; Q1; Q3] 1.0; 1.0; 1.22 vs. 1.0; 1.0; 1.22; P > 0.05; interferon γ [pg/ml; median; Q1; Q3] 1.0; 1.0; 5.16 vs. 1.0; 1.0; 1.96; P > 0.05).

**HIF-1α mRNA Expression and Type of Infection**

A total of 28 patients (28.3%) had gram-positive, 26 patients (26.3%) gram-negative, and nine patients (9.1%) fungal sepsis, and in 36 patients (36.3%), no pathogen was detected (negative blood cultures). Of interest, HIF-1α mRNA expression was decreased by 85% in patients with positive blood cultures (known pathogen) compared to patients with negative blood cultures (unknown pathogen) (∆ct values: known pathogen: −7.5 ± 3.3, unknown pathogen: −4.8 ± 3.3; P = 0.0006). SAPS II did not differ between groups (unknown pathogen: 40 ± 16; known pathogen: 43 ± 18; P > 0.05).

**SAPS II, 30-Day Mortality, and HIF-1α mRNA Expression**

The average SAPS II was 40 ± 16 in patients with sepsis (table 1), and there was a significant inverse association between SAPS II and HIF-1α mRNA expression (r = −0.29;
mRNA expression in patients with sepsis (HIF-1α mRNA expression. There was a significant inverse association between SAPS II and HIF-1α mRNA expression in patients with sepsis ($r = -0.29$; $P = 0.0001$). mRNA = messenger ribonucleic acid.

$P = 0.0001$; fig. 3). The 30-day mortality in patients with sepsis was 31.3% (31 patients) and did not correlate with HIF-1α mRNA expression ($P > 0.05$).

**Characteristics of Patients with Sepsis and Healthy Volunteers**

All patients with sepsis were mechanically ventilated at the time of study inclusion. All patients had “severe sepsis” and 52 of 99 patients with sepsis had septic shock. Detailed characteristics of patients with sepsis and healthy volunteers, primary diagnoses, and length of hospitalization are shown in table 1. Leukocyte count, pro-calcitonin, and C-reactive protein concentrations were increased in patients (6.3-fold ± 2.4, $P = 0.0001$; fig. 3). The 30-day mortality in patients with sepsis was 31.3% (31 patients) and did not correlate with HIF-1α mRNA expression ($P > 0.05$).

In **Vitro Studies on HIF-1α mRNA Expression and Protein Following the Induction of Endotoxin Tolerance by Repetitive Lipopolysaccharide Stimulation**

Whereas an acute stimulation with 1 μg/ml lipopolysaccharide of THP-1 cells for 6h increased HIF-1α mRNA expression (4.2-fold ± 0.45; $P = 0.0001$; fig. 4A) compared to control, prolonged prestimulation with low-dose lipopolysaccharide (0.05 ng/ml) for 48 h resulted in decreased HIF-1α mRNA expression (lipopolysaccharide prestimulated cells, 0.19 ± 0.05; $P = 0.01$). Even more important, incubation of such lipopolysaccharide prestimulated cells with a 20,000-fold higher lipopolysaccharide dosage (1 μg/ml) for another 6h no longer increased HIF-1α mRNA expression (0.19 ± 0.05 vs. 0.4 ± 0.13; $P > 0.05$). While a single acute lipopolysaccharide stimulus (0.05 ng/ml or 1 μg/ml) transiently increased HIF-1α mRNA expression, HIF-1α mRNA expression was significantly lower in lipopolysaccharide prestimulated cells following a second high-dose lipopolysaccharide (1 μg/ml) exposure (for 6h even when compared to naïve, lipopolysaccharide unexposed cells; $P = 0.0001$, fig. 4A). However, repetitively stimulated cells did not lose their capacity to upregulate cytokine-related genes. Compared to control (naïve cells not exposed to lipopolysaccharide), interleukin 10 mRNA expression, following a 6-h lipopolysaccharide stimulation (1 μg/ml), was increased both in naïve (6.6-fold ± 4.4; $P = 0.001$) and prestimulated cells (9.9-fold ± 10.2; $P = 0.0001$, data not shown).

Furthermore, compared to control (naïve cells not exposed to lipopolysaccharide), tumor necrosis factor α mRNA expression following a 6-h lipopolysaccharide stimulation (1 μg/ml) was increased both in naïve cells (110-fold ± 54; $P = 0.0001$) and, to a lesser extent, in cells prestimulated by lipopolysaccharide (77-fold ± 41; $P = 0.0001$ vs. control, and $P = 0.01$ vs. prestimulated cells; 0.05 ng/ml lipopolysaccharide for 48 h, data not shown).

In general, HIF-1α protein in *vitro* followed HIF-1α mRNA expression (fig. 4B). Acute lipopolysaccharide stimulation (1 μg/ml) and HIF-1α protein stabilization with the prolylhydroxylase inhibitor dimethyloxalylglycine (10 μM) increased HIF-1α protein only in naïve cells. In contrast, in lipopolysaccharide prestimulated cells, a second lipopolysaccharide stimulation and incubation with dimethyloxalylglycine failed to increase HIF-1α protein concentration.

**Discussion**

This study provides evidence that HIF signaling is altered in humans during sepsis. Specifically, our data show that (1) HIF-1α as well as HIF-1 target gene expression is decreased in leukocytes of human patients with sepsis; (2) HIF-1α expression is inversely associated with sepsis severity. Furthermore, (3) HIF-1α mRNA expression and protein concentration are diminished in lipopolysaccharide prestimulated cells (THP-1) compared to naïve cells, and this was
due to decreased mRNA expression, rather than increased breakdown through prolylhydroxylases.

Almost 150 individuals were included in this prospective observational study, and HIF-1α mRNA expression was decreased by 67% in leukocytes from 99 patients with severe sepsis compared to 48 healthy volunteers, and showed an inverse association with sepsis severity, independent of the type of pathogen detected. We also succeeded in detecting the highly unstable HIF-1α protein in leukocytes from freshly drawn whole blood using blood smears. We found that HIF-1α protein followed decreased mRNA expression and resulted in a significantly lesser number of HIF-1α-positive cells in patients with sepsis. Even the incubation of whole blood samples with the prolylhydroxylase inhibitor dimethylxalylglycine, which normally prevents degradation of the HIF-1α protein, failed to restore HIF-1α protein concentrations in samples of patients with sepsis. This indicates that decreased HIF-1α mRNA expression...
and thus decreased synthesis of HIF-1α protein, but not enhanced degradation, might be responsible for decreased HIF-1α protein in leukocytes from patients with sepsis. The functional consequences of decreased HIF-1α mRNA expression were clear. HIF-1-dependent gene activation in leukocytes from patients with sepsis was significantly decreased for adrenomedullin and also for the β₂-integrins CD11a and CD11b, which are essential for leukocyte migration toward the site of infection and for cellular adhesion.\textsuperscript{14,20–22} Interestingly, expression of the common subunit CD18 was significantly increased excluding a general suppression of gene expression in leukocytes from patients with sepsis or an overall degradation of mRNA during preparation of the samples. Furthermore, a subgroup analysis revealed that integrin expression was further decreased in patients with septic shock compared to that in patients with sepsis without shock. This might contribute to a decreased capability to eliminate pathogens.

Apart from this effect, decreased HIF-1α expression modulates inflammatory pathways by decreasing the transcription of netrin-1, which influences neutrophil migration in an adenosine A₂B receptor-dependent manner.\textsuperscript{6,23,24} In our patients with sepsis, the serum concentration of the anti-inflammatory cytokine interleukin 10 was increased, whereas that of interferon-α and γ was not. This may indicate that our patients with sepsis, despite meeting the clinical criteria of severe sepsis or even septic shock, had passed the initial interferon peak, and that their immune state already may have shifted to an immunosuppressive pattern which in turn might have contributed to decreased HIF-1α mRNA expression.\textsuperscript{15}

The finding that HIF-1α mRNA expression was significantly decreased in circulating leukocytes of patients with sepsis may be influenced by a variety of factors. Apart from specific inflammatory stimuli (i.e., lipopolysaccharide and/or gram-positive toxins, cytokine patterns), duration of exposure, blood concentration of circulating pathogens and their timing, and areas of decreased tissue oxygen partial pressure are all variables that might have influenced HIF-1α mRNA expression, either by inducing or suppressing HIF-1α.

However, despite the heterogeneity of patients with sepsis, HIF-1α mRNA expression was significantly suppressed in sepsis, and there also was an inverse association with the severity of illness as assessed using SAPS II.

As stated above, HIF-1α is differentially regulated and, therefore, the selection of an appropriate control group is important. We believe that normal, non-septic, non-anesthetized, non-ventilated subjects serve as the most appropriate controls as this cohort is the only one that can yield physiological (basal) HIF expression data. All other cohorts are subject to instrumentation, mechanical ventilation, different levels of positive end-expiratory pressure ventilation, inspiratory oxygen fraction, or sedation/anaesthesia, or different surgeries, and thus cannot substitute for healthy subjects as a proper control group.

We can only speculate whether decreased expression of HIF-1α and HIF-1 target genes we examined plays a role in the course of sepsis. However, expression of the HIF-1 target genes adrenomedullin, inducible nitric oxide synthase, or vascular endothelial growth factor has all been implicated to contribute to the sepsis syndrome.\textsuperscript{1,4} Specifically, both adrenomedullin and nitric oxide synthase activity may contribute to hypotension, and it would be worth following whether decreased expression of these two genes results in decreased requirements for vasoconstrictors.

All three genes are also involved in improving oxygen supply to the tissues. However, normal arterial oxygen partial pressures in our cohort with sepsis make us assume that hypoxemia is not a major factor contributing to decreased HIF-1α in circulating mononuclear cells in sepsis.

A further important issue is whether HIF-1α in sepsis is mainly controlled by HIF-1α mRNA expression or by post-translational stabilization. Decreased HIF-1α protein concentrations, as seen in blood smears from patients with sepsis, are unlikely caused by increased degradation, as observed with prolonged exposure to nitric oxide.\textsuperscript{17} This notion is supported by the failure of dimethyloxalylglycine to increase HIF-1α protein expression in blood smears from patients with sepsis, because dimethyloxalylglycine should have prevented degradation if it was caused by elevated prolylhydroxylase activity.

We can only speculate about the course of HIF-1α mRNA expression in humans during incipient sepsis before admission to the intensive care unit, particularly at the moment when infection and/or the systemic inflammatory cascade was initiated. It is well appreciated, however, that when a patient is admitted to an intensive care unit with overt signs of sepsis, the inflammatory processes are going on already for many hours if not days.\textsuperscript{25,26}

In an attempt to better understand the regulation of HIF-1α during sepsis, we also performed in vitro experiments and induced endotoxin tolerance, as described previously by our group.\textsuperscript{3} A single, 6-h stimulation of THP-1 cells with lipopolysaccharide evoked a robust increase in HIF-1α mRNA expression. However, when cells were prestimulated with low-dose lipopolysaccharide, HIF-1α mRNA expression was decreased within 48 h after this stimulus. Furthermore, reexposure to an even 20 000-fold higher lipopolysaccharide concentration failed to increase HIF-1α mRNA expression. Nevertheless, prestimulated cells did not lose their capacity to express cytokine-related genes, as shown for interleukin 10 and tumor necrosis factor-α.

HIF-1α protein expression followed the changes observed for the mRNA. In particular, adding dimethyloxalylglycine to prestimulated THP-1 cells did not increase HIF-1α protein. Thus, we show for the first time that the decrease in HIF-1α protein in endotoxin-tolerant cells is due to decreased mRNA expression rather than increased degradation by prolylhydroxylases.

Our study has limitations. First, although including almost 150 individuals, our study cohort may still be
considered small and the relation between HIF-1α mRNA expression and mortality should be analyzed in an even larger cohort. Second, we obtained blood samples and analyzed HIF-1α mRNA expression only at the time of first diagnosing sepsis, i.e., after intensive care unit admission. This snapshot analysis likely cannot reflect the complete time course of sepsis. This might explain the lack of correlation between HIF-1 expression and mortality.

Third, additional studies should be performed to address whether or not patients benefit from increased or decreased HIF-1α mRNA expression. The only evidence with respect to this issue derives from mice carrying a myeloid HIF-1α knockout which have better wound healing than wild-type animals, and from a T-cell-specific HIF-1α knockout that is associated with better survival following bacterial infections. In our patients, however, positive blood cultures and, therefore, a probably higher pathogen load were associated with a lower HIF-1α mRNA expression. In turn, a lower expression was associated with the severity of illness as assessed using SAPS II. Thus, based on previous in vitro experiments and our cytokine measurements, we speculate that we analyzed blood samples at a time point where leukocytes already contained decreased HIF-1α mRNA due to prolonged or repetitive inflammatory stimuli. 

In conclusion, an acute administration of lipopolysaccharide to monocytes increased HIF-1α mRNA expression in vitro, whereas prolonged lipopolysaccharide stimulation suppressed HIF-1α mRNA expression even below baseline. In vivo, HIF-1α mRNA and protein expression were substantially decreased in patients with sepsis upon their admission to the intensive care unit, and decreased HIF-1α mRNA expression was inversely associated with the severity of illness. Decreased HIF-1α mRNA expression was associated with decreased expression of the HIF target gene adrenomedullin and of β2-integrins.

While the mechanisms of decreased HIF-1α mRNA expression require further study, our data are a first step to characterize HIF-1α gene regulation in patients with sepsis. Attempts to modulate in sepsis HIF-1α expression and related gene expression might enable new therapeutic options.

References


