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Hypoxia-Independent Activation of HIF-1 by *Enterobacteriaceae* and Their Siderophores

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Background & Aims: ***Hypoxia inducible factor*-1 (HIF-1) is the key transcriptional regulator during adaptation to hypoxia. Recent studies provide evidence for HIF-1 activation during bacterial infections. However, molecular details of how bacteria activate HIF-1 remain unclear. Here, we pursued the role of bacterial siderophores in HIF-1 activation during infection with *Enterobacteriaceae*. Methods: In vivo, HIF-1 activation and HIF-1-dependent gene induction in Peyer's patches were analyzed after orogastric infection with *Yersinia enterocolitica*. The course of an orogastric *Y enterocolitica* infection was determined using mice with a deletion of HIF-1α in the intestine. In vitro, the mechanism of HIF-1 activation was analyzed in infections with *Y enterocolitica*, *Salmonella enterica* subsp *enterica*, and *Enterobacter aerogenes*. Results: Infection of mice with *Y enterocolitica* led to functional activation of HIF-1 in Peyer's patches. Because mice with deletion of HIF-1α in the intestinal epithelium showed a significantly higher susceptibility to orogastric *Y enterocolitica* infections, bacterial HIF-1 activation appears to represent a host defense mechanism. Additional studies with *Y enterocolitica*, *S enterica* subsp *enterica*, or *E aerogenes*, and, moreover, application of their siderophores (yersiniabactin, salmochelin, aerobactin) caused a robust, dose-dependent HIF-1 response in human epithelia and endothelia, independent of cellular hypoxia. HIF-1 activation occurs most likely because of inhibition of prolylhydroxylase activity and is abolished upon infection with siderophore uptake deficient bacteria. Conclusions: Taken together, this study reveals what we believe to be a previously unrecognized role of bacterial siderophores for hypoxia-independent activation of HIF-1 during infection with human pathogenic bacteria.**

Mammalian cells adapt to oxygen deprivation by the activation of *hypoxia inducible factor* (HIF)-1, the key transcription factor during hypoxia. Subsequently, expression of hypoxia-inducible genes involved in angiogenesis (eg, *vascular endothelial growth factor* [VEGF]), glycolysis (eg, hexokinase [HK]), proliferation and survival (eg, adrenomedullin [ADM]), and erythropoiesis (eg, erythropoietin) is transcriptionally induced[1] or repressed.[2] HIF-1 is a heterodimeric transcription factor composed of the 2 subunits HIF-1α and HIF-1β. Although HIF-1β is constantly present in the nucleus, HIF-1α levels are affected by changes in the cellular oxygen partial pressure (pO_2).[3] 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.[1,3] 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 proteasomal 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.[3]

In addition to HIF-1 activation by hypoxia, previous reports have shown iron deprivation (induced by iron chelating compounds, eg, desferrioxamine [DFO]) as an alternative strategy of HIF-1 activation.[1,3,4] PHDs contain iron as an essential cofactor for their enzymatic activity, and iron chelation inhibits PHD activity resulting in the activation of HIF-1.[5] However, a physiologic role of hypoxia-independent HIF-1 activation by iron chelators has not been described.

Abbreviations used in this paper: act, actinomycin; ADM, adrenomedullin; Aeb, aerobactin; DFO, desferrioxamine; FyuA, ferric yersiniabactin uptake protein A; HIF, hypoxia inducible factor; HK, hexokinase; HMECs, human microvessel endothelial cells; IroN, Iron-regulated outer membrane protein N; IutA, iron uptake transport protein A; MOI, multiplicity of infection; PHD, prolyl hydroxylase domain containing protein; pO_2, cellular oxygen partial pressure; PP, Peyer's patches; Sal, salmochelin; VEGF, vascular endothelial growth factor; Ybt, yersininabactin.

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Many bacteria have developed efficient high-affinity iron uptake systems promoting bacterial growth in iron-restricted environments. The best studied microbial systems are those of low-molecular-weight iron-chelating compounds known as siderophores, which are secreted and taken up again after iron chelation by specific bacterial transport systems. Several bacterial species of *Enterobacteriaceae* (eg, *Yersinia enterocolitica, Salmonella enterica* subsp *enterica, Enterobacter aerogenes*) produce siderophores (yersiniabactin [Ybt], salmochelin [Sal], aerobactin [Aeb]) and the related uptake systems (FyuA, IroN, IutA) to ensure their own iron supply.[6–8] These iron uptake systems have been shown to be essential for bacterial pathogenicity: the deletion of Ybt in *Y enterocolitica* results in a dramatic loss of pathogenicity in a mouse infection model,[9] and similar results were reported for Aeb.[10]

Recently, many parallels between acute inflammation and adaptation to hypoxia have become obvious.[11] As such, we and others have recently shown that HIF-1 activation occurs during bacterial infections. In particular, infections with the angiogenic bacterium *Bartonella henselae* (causing the vasculoproliferative disorder bacillary angiomatosis) result in the activation of HIF-1 in vitro and in vivo (patient samples), presumably via hypoxia-associated metabolic changes.[12] To analyze the phenomenon of HIF-1 activation in bacterial infections in more detail, we now expanded our investigations on diarrhea-causing *Enterobacteriaceae*. Activation of HIF-1 with human pathogenic *Enterobacteriaceae* in vitro and in vivo seems to resemble a general phenomenon in bacterial infections, which involves iron deprivation of host cells by bacterial siderophores, resulting in a hypoxia-independent HIF-1 activation.

Materials and Methods

Bacterial Strains and Culture Conditions

For in vitro experiments, the following bacteria were used: *Y enterocolitica*[13] and *Y enterocolitica* Δ*irp1*[14] (designated as "ΔYbt"), *S enterica* subsp *enterica* (ATCC14028) and *S enterica* subsp *enterica* Δ*iroBC*[8] (designated as "ΔSal"), *E aerogenes* 62-I,[15] and *B henselae* strain Marseille.[16] In vivo experiments with *Y enterocolitica* (serotype O:8) were performed as described.[17]

Y enterocolitica *Infection of Mice*

Female C57BL/6 mice (6–8 weeks) were infected orogastrically with 5×10^8 *Y enterocolitica*, and Peyer's patches (PPs) were removed at days 1 and 3 (transcriptional gene profiling) or at day 2 (HIF-1α immunohistochemistry) upon infection. To study the role of HIF-1, female HIF-$1^{-/-}$ mice with a HIF-1α deletion targeted to the intestinal epithelium and respective littermate controls were used [age: 11–20 weeks; mixed genetic background (129, BALB/c and FVB/N)]. HIF-$1^{-/-}$ mice were based on colonies of colon mutant Fabpl$^{4x\ at-132}$/Cre mice,[18] which had been bred to mice that carried the *Hif1a 2-lox* allele.[19]

HIF-1α Immunohistochemistry

Paraffin-embedded, 4-μm sections of PPs were stained with monoclonal anti-HIF-1α IgG1 antibodies (ESEE122; Novus Biologicals, Littleton, CO) followed by alkaline phosphatase/antialkaline phosphatase (DAKO, Glostrup, Denmark) with hematoxylin counterstaining.

RNA Isolation, Microarray Hybridization, and Cluster Analysis

Five C57BL/6 mice were infected with 5×10^8 *Y enterocolitica*. After 1 and 3 days, total RNA from PPs was prepared, and fragmented complementary RNA (cRNA) was generated and used for hybridization onto GeneChip probe arrays MG-U74Av2 (Affymetrix, High Wycombe, UK). Genechips were read using a GeneChip Scanner 2500 (Affymetrix). Analysis of microarray data was performed using the Affymetrix Microarray Suite 5.0, MicroDB 3.0, and Data Mining Tool 3.0 as described.[12] Expression levels were color coded, with red indicating expression above and green below those levels found in uninfected control mice. Microarray data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/; accession number GSE9558).

Preparation of Bacterial Siderophores: Yersiniabactin, Salmochelin, Aerobactin

All siderophores (purity: ~90%) were prepared by high-pressure liquid chromatography. Ybt was purified from *Y enterocolitica fur* mutant H1852,[9] Sal from *S enterica* H5547, and Aeb from *E aerogenes* 62-I (see Supplemental Materials for details at www.gastrojournal.org).

Cell Culture of HeLa, Caco-2, and HIF-$1^{-/-}$ HMECs and Infection Procedures

HeLa (cervix cancer) cells were grown in RPMI 1640 medium (Biochrom, Berlin, Germany), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma, Deisenhofen, Germany), 1% L-glutamine (Invitrogen, Karlsruhe, Germany), 10 mg/mL streptomycin, and 100 U penicillin (Biochrom). HIF-$1^{+/+}$ and HIF-$1^{-/-}$ human microvessel endothelial cells (HMECs)[20] (see Supplemental Materials for details at www.gastrojournal.org) were maintained in MCDB 131 medium (Invitrogen) supplemented with 10% heat-inactivated FCS, 5% L-glutamine, streptomycin, penicillin, 10 ng/mL epidermal growth factor (Becton Dickinson, Heidelberg, Germany), and 1 μg/mL hydrocortisone (Sigma). Caco-2 cells (human colon adenocarcinoma) were grown in minimum essential medium Eagle (Sigma), supplemented with 20% heat-inactivated FCS, nonessential amino acids (Biochrom), streptomycin, and penicillin.

Cells were seeded the day before infection in cell culture media without antibiotics (to allow bacterial growth). Infection was performed using the following