Regulation of the Hypoxia-inducible Factor 1α by the Inflammatory Mediators Nitric Oxide and Tumor Necrosis Factor-α in Contrast to Desferoxamine and Phenylarsine Oxide*

Karin B. Sandau‡, Jie Zhou‡, Thomas Kietzmann§, and Bernhard Brüne‡‡

From the ‡Faculty of Biology, University of Kaiserslautern, 67663 Kaiserslautern and the §Institute of Biochemistry and Molecular Cell Biology, University of Göttingen, 37073 Göttingen, Germany

Hypoxic/ischemic conditions provoke activation of the hypoxia-inducible factor-1 (HIF-1), which functions as a transcription factor. HIF-1 is composed of the HIF-1α and -β subunits, and stability regulation occurs via accumulation/degradation of HIF-1α with the notion that a prolyl hydroxylase accounts for changes in protein level. In addition, there is evidence that HIF-1 is up-regulated by diverse agonists during normoxia. We investigated the impact of inflammatory mediators nitric oxide (NO) and tumor necrosis factor-α (TNF-α) on HIF-1α regulation. For comparison, LLC-PK1 cells were exposed to hypoxia, stimulated with desferoxamine (DFX, known to mimic hypoxia), and the thiol-cross-linking agent phenylarsine oxide (PAO). Although all stimuli elicited HIF-1α stabilization with differences in the time-dependent accumulation pattern, significant variations appeared with regard to signaling. With the use of a superoxide anion (O2•-) generator, we established an O2•-sensitive pathway that blocked HIF-1α stabilization in response to NO and TNF-α while DFX- and PAO-evoked HIF-1α stabilization appeared O2•-insensitive. NO and TNF-α signaling required phosphorylation events, especially activation of the phosphatidylinositol 3-kinase/Akt, which is in contrast to DFX and PAO. Based on HIF-1-dependent luciferase reporter gene analysis, it was found that, in contrast to NO and TNF-α, PAO resembled a stimulus that induced a dysfunctional HIF-1 complex. These data indicate that diverse agonists activate HIF-1α under normoxic conditions by employing different signaling pathways.

The transcription factor hypoxia-inducible factor-1 (HIF-1)1 is a heterodimer composed of the helix-loop-helix/Per-Arnt-Sim protein HIF-1α and the aryl hydrocarbon nuclear translocator, also known as HIF-1β (1–3). An active HIF-1 complex accumulates in the nucleus; binds to a specific DNA sequence, the HIF-1 binding site within the hypoxia response element (HRE); and enhances transcription of hypoxia-inducible genes, such as erythropoietin or vascular endothelial growth factor. The availability of HIF-1 is mainly determined by the presence/absence of HIF-1α (4, 5). In many cell types, both HIF-1α and HIF-1β appear to be constitutively expressed at the mRNA level, whereas, on protein level, HIF-1α is degraded under normoxic conditions, which contrasts with permanently expressed HIF-1β. Studies in von Hippel-Lindau-defective renal cell carcinomas indicated that the von Hippel-Lindau protein fulfills a critical function in HIF-1α degradation, thus accounting for the extremely short protein half-life (6). However, accumulation of HIF-1α that promotes active HIF-1 complex formation by hypoxia is not fully understood. Oxygen species such as superoxide (O2•-) or hydrogen peroxide (H2O2) have been proposed to limit HIF-1α stability (7). A postulated source for these species is a NAD(P)/H-metabolizing membrane-bound type b cytochrome quite similar to the respiratory burst oxidase in phagocytes. In addition to these intracellular redox changes, phosphorylation cascades such as mitogen-activated protein kinases have been ascribed to stabilize HIF-1α, but precise signaling mechanisms and their cross-talk have not been fully defined (8–10).

Activation of HIF-1 as an adaptive response was first described for conditions of decreased oxygen pressure. Therefore, most mechanistic and functional studies on HIF-1 regulation refer to hypoxic conditions. More recent evidence suggests that HIF-1 can be activated by growth factors, cytokines, hormones, or nitric oxide (NO) as well with very little information on signal transduction pathways being involved (11–15). Zhong et al. (11) verified a role of phosphatidylinositol 3-kinase (PI3K), Akt phosphorylation, and FRAP activation for HIF-1α induction in response to hypoxia and epithelial growth factor treatment. This pathway is negatively regulated by PTEN (phosphatase and tensin homologue deleted in chromosome ten) and loss of function correlated with tumor angiogenesis. For the signaling molecule NO, we noticed that the general kinase inhibitor genistein and, more specifically, the PI3K inhibitors wortmannin or Ly 294002 blocked HIF-1α accumulation, whereas mitogen-activated protein kinases were not involved (16).

Herein, we have compared different stimuli for HIF-1α stability regulation and examined the involvement of phosphorylation events with a focus on the PI3K/Akt pathway. We concentrated on two inflammatory mediators, NO and tumor necrosis factor α (TNF-α). Both agents have been shown to activate HIF-1, but signaling mechanisms largely remained unclear. We compared our results with hypoxia and the iron...
chelator desferroxamine (DFX) often chosen to mimic hypoxia. For further consideration we included a fourth agent, phenylarsine oxide (PAO), known to cross-link vicinal -SH groups since modifications of the -SH moiety of cysteine by S-nitrosation or oxidation are discussed for NO signaling during activation/inhibition of transcription factors such as nuclear factor kB or AP-1 (17, 18).

Although all stimuli elicited HIF-1α accumulation, their signaling pathways differed significantly. The HIF-1α response was blocked by O2 generation or by interrupting the PI3K/Akt pathway in case of hypoxia and the inflammatory mediators NO and TNF-α, whereas the DFX or PAO responses were not affected at all. Interestingly, PAO induced a strong HIF-1α protein accumulation but failed to form an active HIF-1 DNA-binding complex or to provoke reporter gene activation.

Our results suggest that NO-, TNF-α-, DFX-, and PAO-evoked HIF-1 regulation is differently affected by O2 formation, the PI3K pathway, and DNA binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Medium and supplements were purchased from Bioch, (Berlin, Germany). Nutrocellulose, [γ-32P]ATP, and the ECL detection system came from Amersham Pharmacia Biotech (Freiburg, Germany). HIF-1α and p85 antibodies were ordered from Beckton Dickinson (Heidelberg, Germany), the Akt antibody from New England Biolabs (Frankfurt, Germany), and secondary antibodies from Promega (Mannheim, Germany). T4 polynucleotide kinase was purchased from Hoffmann LaRoche (Mannheim, Germany), and chromaspin-10 columns were from CLONTECH (Heidelberg, Germany). Wortmannin, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), sperson NO (SpNO), and phenylarsine oxide were ordered from Sigma (Deisenhofen, Germany), and genistein was from Calbiochem (Bad Soden, Germany). All other chemicals were of the highest grade of purity and commercially available. Plasmids pSRe-Ap65 and pSRe wild-type (WT) p85 were kindly provided by Dr. W. Ogawa (Kobe University, School of Medicine, Kobe, Japan) (19). pCMV5 and pCMV5-m-pPKbK179A were a gift from Dr. B. Hemmings (F. Miescher Institut, Basel, Switzerland). The plasmid pGLEXPOHRE harbors three copies of the erythropoietin HRE in front of the SV40 promoter and was described previously (20).

**Cell Culture**—Proximal tubular LLC-PK1 cells were cultivated in Dulbecco modified Eagle’s medium with 1% glutaric acid, supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Cells were transferred two times a week, and medium was changed prior to harvesting. Cells were kept in a humidified atmosphere of 5% CO2 in air at 37°C.

**Nuclear Extract Preparation**—After stimulation, LLC-PK1 cells were washed with ice-cold PBS, scraped off, and centrifuged for 10 min at 570 × g. Pellets were washed with PBS and centrifuged (4°C, 17,500 × g, 1 min). Pellets were resuspended in 400 μl of buffer A (10 mM Tris, pH 7.8, 1.5 mM MgCl2, 10 mM KCl, 1 mM sodium orthovanadate, 0.5 mM DTT, 0.5 mM PMSF) and kept on ice for 25 min and vortexed (twice) in between. For the last 10 s, 25 μl of 10% Nonidet P-40 was added and suspensions were vortexed before centrifugation (4°C, 17,500 × g, 1 min). To lyse nuclei, pellets were washed with PBS, resuspended in 50 μl of buffer C (20 mM Tris, pH 7.8, 1.5 mM MgCl2, 420 mM KCl, 20% glycerol, 1 mM sodium orthovandate, 0.5 mM DTT, 0.5 mM PMSF) and kept on ice for 25 min. After centrifugation (4°C, 17,500 × g, 10 min), supernatants were dialyzed against 1 liter of buffer D (100 mM KCl, 20 mM Tris, pH 7.8, 2 mM EDTA, 20% glycerol) at 4°C for 1 h. Thereafter, protein concentrations were determined and nuclear extracts were stored at −80°C.

**Gel Shift Assay**—Oligonucleotides for gel shift assays were synthesized by Eurogentec (Heidelberg, Germany) and contained the sequence of the HIF-1 binding site derived from the human transferrin gene. The sequences were as follows: sense, 5′-TTCCCTGACGTACACAAAGCGCAGCTATTTCC-3′; and antisense, 5′-GAAATACGTGCTTTGTGTGACACGTATTTC-3′. For radioactive labeling, 1.25 pmol of annealed oligonucleotides were incubated with 2.5 μl of 10X polynucleotide kinase phosphorylation buffer, 1 μl of phosphatase-free T4 polynucleotide kinase, and 50 μCi of [γ-32P]ATP in a final volume of 20 μl at 37°C for 25 min. Labeling reactions were terminated by the addition of 1 μl of 0.5 M EDTA, and unlabeled oligonucleotides were separated by using Chromaspin-10 columns. The efficiency of radioactive labeling was measured with a counter. Afterward, HIF-1 binding reactions were set up in a volume of 20 μl and nuclear extracts (5 μg protein) were incubated in a buffer of a final concentration of 50 mM KCl, 10 mM Tris, pH 7.7, 5 mM DTT, 1 mM EDTA, 1 mM MgCl2, 5% glycerol, 0.03% Nonidet P-40, 400 ng of salmon testes DNA, and 40,000 cpm 32P-labeled oligonucleotide. Incubations were overnight at 4°C, and samples were resolved by electrophoresis on 5% polyacrylamide gels (polyacrylamide:bisacrylamide, 29:1) at room temperature with 110 V. For supershift experiments, 0.25 μg of HIF-1α antibody was added to the reactions 1 h before running the gel.

**Statistical Analysis**—Each experiment was performed at least three times, and representative pictures are shown. Values presented are mean ± S.E.

**RESULTS**

**HIF-1α Accumulation and the Inhibition by the Reducer Cyclic DMNQ**—A major target cell type affected during renal ischemia/reperfusion injury and several inflammatory processes in the kidney are tubular epithelial cells, whereas in the liver hepatocytes are affected. Therefore, we used tubular LLC-PK1 or HepG2 cells in this study. To examine the impact of the inflammatory mediators NO and TNF-α on HIF-1α accumulation, we applied 100 μM amounts of the chemically distinct NO donors S-nitrosothiolamine or SpNO, or recombinant human TNF-α at a concentration of 500 ng/ml. Furthermore, we exposed cells to 1% hypoxia, and to imitate hypoxia we employed DFX at a concentration of 100 μM. To study the impact of the vimentin- and SH group modification, PAO at a concentration of 1 μM was used. Agonist treatments were neither apoptotic nor necrotic to the cells (data not shown).

**GFP-O2 at a concentration of 100 μM elicited a fast but transient HIF-1α response, as determined by Western blot analysis.** HIF-1α accumulation reached a maximum between 1 and 2 h with a declining signal afterward (Fig. 1A). TNF-α represented a slow inducer for HIF-1α. Protein accumulation in response to TNF-α started around 6 h but remained stabilized up to 18 h (Fig. 1B). Moreover, we noticed HIF-1α stabilization between 2 and 4 h after DFX or PAO stimulation (Fig. 1, C and D) with the further notion that HIF-1α was elevated up to 8 h (data downloaded from http://www.jbc.org/ by guest on July 25, 2018)
penetrates the plasma membrane and releases O₂.

Following incubations, HIF-1α protein levels were detected by Western blot analysis. In the case of GSNO, genistein was tested at 4 or 6 h, only. Similar to GSNO, genistein significantly attenuated the HIF-1α signal triggered by TNF-α (Fig. 3B), whereas the DFX- or PAO-elicited HIF-1α accumulation appeared unaffected by genistein (Fig. 3, C and D).

Zhong et al. (11) depicted the PI3K/Akt pathway to be essential for hypoxia-induced HIF-1α induction, and we described similar results for NO signaling and HIF-1α stabilization. Stimulation of LLC-PK₁ cells with GSNO led to Akt activation/phosphorylation in close correlation to HIF-1α accumulation as inhibition of the PI3K with Ly 294002 suppressed Akt phosphorylation in close correlation to HIF-1α stabilization (16). However, there is little or no information on the role on the PI3K/Akt pathway in response to GSNO or SpNO when assayed after 1, 2, or 4 h (Fig. 3A). When using TNF-α, DFX, and PAO, the effect of genistein was tested at 4 or 6 h, only. Similar to GSNO, genistein significantly attenuated the HIF-1α signal triggered by TNF-α (Fig. 3B), whereas the DFX- or PAO-elicited HIF-1α accumulation appeared unaffected by genistein (Fig. 3, C and D).

HIF-1α Accumulation and the Interference by Phosphorylation Cascades—Besides the regulatory role of intracellular ROS, phosphorylation events have been linked to HIF-1α stabilization. With the use of the unselective kinase inhibitor genistein, we probed for the involvement of phosphorylation cascades. In these experiments, genistein was preincubated for 30 min to assure cell membrane penetration. Genistein at a concentration of 100 μM showed no effect on HIF-1α accumulation by its own (data not shown), but completely attenuated GSNO-induced HIF-1α accumulation detected after 1, 2, and 4 h (Fig. 3A). When using TNF-α, DFX, and PAO, the effect of genistein was tested at 4 or 6 h, only. Similar to GSNO, genistein significantly attenuated the HIF-1α signal triggered by TNF-α (Fig. 3B), whereas the DFX- or PAO-elicited HIF-1α accumulation appeared unaffected by genistein (Fig. 3, C and D).

Wortmannin at a concentration of 100 nM resembles a specific PI3K inhibitor with no effect on HIF-1α by its own (Fig. 4A). Similar to genistein, wortmannin was preincubated for 30 min prior to agonist addition. Wortmannin blocked HIF-1α accumulation in response to GSNO or SpNO when assayed after 1, 2, or 4 h (Fig. 4A). The PI3K inhibitor appeared effective after TNF-α stimulation as well (Fig. 4B) and, as expected, also blocked HIF-1α stabilization in response to hypoxia (Fig. 4C).
The experiments with DFX and PAO in the absence or presence of even an increased concentration of 10 μM wortmannin failed to affect HIF-1α accumulation (Fig. 4, D and E), which neglects the requirement of a wortmannin-sensitive pathway for these agonists. These observations confirmed our results obtained with genistein.

To further establish the role of PI3K and Akt for NO- and TNF-α-induced HIF-1α regulation, LLC-PK1 cells were transfected with the pSRα plasmid encoding the wild type form of the regulatory PI3K subunit p85 (WTp85) or a dominant-negative form (Δp85), which lacks amino acids 479–513 (19). Comparable amounts of p85 due to overexpression of WTp85 or Δp85 were documented in all experiments by Western blot analysis with an antibody against p85 (herein, only shown in combination with GSNO; Fig. 5A). Transfections of WTp85 did not alter HIF-1α accumulation in response to all stimuli tested. This is exemplified for GSNO stimulation when HIF-1α was detected after 1, 2, and 4 h (Fig. 5A). Opposite effects on the HIF-1α signal were achieved by overexpressing the dominant-negative form Δp85. GSNO- and TNF-α-evoked HIF-1α stabilization was significantly attenuated by Δp85 expression (Fig. 5, B and C). In case of TNF-α, the 16-h time point was chosen in these experiments to achieve a stronger HIF-1α signal, whereas GSNO responses are examined after 1, 2, and 4 h. As seen in unstimulated controls, plasmid transfections did not influence basal expression of HIF-1α. Δp85 transfection experiments in combination with the agonist DFX or PAO underlined the results ascertainment with wortmannin (Fig. 5, D and E). Apparently, an inactive PI3K failed to interrupt the signaling pathway of DFX or PAO that leads to HIF-1α accumulation. Whether PI3K signaling is mediated via Akt, also known as protein kinase B (PKB), was tested by overexpression of the dominant-negative Akt protein. Dysfunctional Akt was achieved by mutating the ATP-binding site Lys179 to Ala (23). In addition, a consensus sequence for both myristoylation and palmitylation (m/p) was hooked to the construct as activated protein kinase B (PKB), was tested by overexpression of the dominant-negative Akt protein. Dysfunctional Akt was achieved by mutating the ATP-binding site Lys179 to Ala (23). In addition, a consensus sequence for both myristoylation and palmitylation (m/p) was hooked to the construct as activated PI3K subunit p85 (p85), which lacks amino acids 479–513 (19). Comparable amounts of p85 due to overexpression of WTp85 or Δp85 was documented in all experiments by Western blot analysis with an antibody against p85 (herein, only shown in combination with GSNO; Fig. 5A). Transfections of WTp85 did not alter HIF-1α accumulation in response to all stimuli tested. This is exemplified for GSNO stimulation when HIF-1α was detected after 1, 2, and 4 h (Fig. 5A). Opposite effects on the HIF-1α signal were achieved by overexpressing the dominant-negative form Δp85. GSNO- and TNF-α-evoked HIF-1α stabilization was significantly attenuated by Δp85 expression (Fig. 5, B and C). In case of TNF-α, the 16-h time point was chosen in these experiments to achieve a stronger HIF-1α signal, whereas GSNO responses are examined after 1, 2, and 4 h. As seen in unstimulated controls, plasmid transfections did not influence basal expression of HIF-1α. Δp85 transfection experiments in combination with the agonist DFX or PAO underlined the results ascertainment with wortmannin (Fig. 5, D and E). Apparently, an inactive PI3K failed to interrupt the signaling pathway of DFX or PAO that leads to HIF-1α accumulation. Whether PI3K signaling is mediated via Akt, also known as protein kinase B (PKB), was tested by overexpression of the dominant-negative Akt protein. Dysfunctional Akt was achieved by mutating the ATP-binding site Lys179 to Ala (23). In addition, a consensus sequence for both myristoylation and palmitylation (m/p) was hooked to the construct as activated Akt is recruited to the membrane via its PH domain. m/p has been shown to be sufficient to localize a number of cytosolic proteins to the plasma membrane (24). Therefore, only Akt activation is blocked but not its translocation (23). Control experiments with the empty pCMV5 vector showed no interference with HIF-1α accumulation. Overexpression of PKBeK179A suppressed HIF-1α stabilization initiated by 100 μM GSNO (Fig. 6A). Suppression of HIF-1α accumulation in cells overexpressing PKBeK179A was confirmed toward hypoxia or spermine NO (Fig. 6B). Along that line, TNF-α-evoked HIF-1α stabilization was attenuated in PKBeK179A-transfected cells (Fig. 6C). In contrast, DFX- or PAO-mediated HIF-1α accumulation remained unaffected by PKBeK179A overexpression (Fig. 6, D and E).

![Figure 3](image3.png)

**FIG. 3.** Genistein blocked NO and TNF-α signaling with no influence on DFX and PAO. LLC-PK1 cells were incubated with vehicle (C), GSNO, TNF-α, DFX, or PAO, in the presence or absence of genistein. The kinase inhibitor was prestimulated for 30 min. Reactions were terminated after 1, 2, 4, or 6 h as indicated, and HIF-1α protein levels were analyzed by Western blotting as described under “Experimental Procedures.” Each experiment was performed at least three times, and representative data are shown.

| A | B | C | D |
|---|---|---|---|
| **Time [h]** | **HIF-1α** | **Time [h]** | **HIF-1α** |
| 4 | 2 | 1 | 4 | 2 | 1 | 4 | 2 |
| GSNO | C | 100 μM | DFX | 100 μM | Genistein | [100 μM] | GSNO | C | 100 μM | DFX | 100 μM | Genistein | [100 μM] |
| - + - + | - + - + | - - - + | - + - + | - - - + |

![Figure 4](image4.png)

**FIG. 4.** Wortmannin attenuated HIF-1α accumulation in response to inflammatory mediators but not to DFX and PAO. 100 μM GSNO, 100 μM SpNO, 500 ng/ml TNF-α, 100 μM DFX, 1 μM PAO, or 100 μM 10 μM wortmannin (WT) were added to LLC-PK1 cells. Wortmannin was prestimulated for 30 min. HIF-1α protein levels were analyzed by Western blotting after 1, 2, 4, or 6 h as indicated. For details, see “Experimental Procedures.” Each experiment was performed at least three times, and representative data are shown.
Hypoxia is of major (patho)physiological importance in evoking stabilization and activation of HIF-1. It is also appreciated that HIF-1 is subjected to complex modulation under normoxia as well. Herein, we concentrated on stability regulation of HIF-1α by the inflammatory mediators NO (derived from GSNO or spermine NO) as well as TNF-α and compared the results with classical agonists such as hypoxia or DFX. For mechanistic considerations we included the thiol-modifying agent PAO to obtain information on signaling pathways in regulating HIF-1α accumulation and HIF-1 transcriptional activity.

Initial reports pointed to an inhibitory action of NO on hypoxia-induced HIF-1α stabilization and/or HIF-1 target gene activation (25–27). Subsequently, it turned out that the choice of the NO donor, the concentration, and probably the time of application appeared important in affecting HIF-1α stabilization. Several groups, including our own, have shown in different cell types that, with the exception of sodium nitroprusside, chemically diverse NO donors, transfection of NO synthase, or macrophage-derived NO evoked HIF-1α accumulation and HIF-1 DNA binding followed by target gene expression (14, 15, 28, 29). Signaling pathways that promoted HIF-1α stabilization in response to NO pointed to a genistein-sensitive phosphorylation cascade, which appears in analogy to the impact of genistein on hypoxia-evoked HIF-1α stabilization (10). Along that line, our results show the involvement of the PI3K/Akt pathway as the inhibitor wortmannin or more specifically transfections of inactive kinases attenuated GSNO-induced HIF-1α accumulation (Figs. 4–6). Information about cytokines and HIF-1 appeared similarly diverse. Although interleukin-1β has been shown to initiate HIF-1α accumulation and to potentiate hypoxia-evoked HIF-1 DNA binding (12), we failed to detect HIF-1α stabilization when stimulating tubular LLC-PK1 cells with 25 units/ml interleukin-1β up to 24 h. In addition, interferon-γ and lipopolysaccharide were ineffective as well (data not shown), whereas we found TNF-α to be a slow but strong HIF-1 inducer. This is in contrast to observations of Hellwig-Bürgel et al. (12). They noticed an additive effect of TNF-α during hypoxic stimulation but no impact of TNF-α on HIF-1α mRNA, protein level, or HIF-1 activity by itself when performing experiments in tubular and hepatoma cells for 4 h. Thornton et al. (30) used fibroblasts to show an increase of HIF-1α mRNA at 3 h after TNF-α with no data on protein accumulation or DNA binding of HIF-1. In part these results point to some cell specificity but, in the case of tubular cells, underscore a time-dependent effect. We did not recognize TNF-α-evoked HIF-1α accumulation before 6 h, but a maximal effect was not achieved until 10–18 h (Fig. 1). In general, the stimulatory action of cytokines and GSNO on HIF-1α accumulation may imply a functional role of HIF-1 during inflammatory settings. Herein, we then elucidated signal transducing pathways. Under hypoxic conditions the formation of oxygen species, specifically H2O2, attenuated HIF-1α stabilization (9). We applied the reduct cycle DMNQ to study the influence of O2− and H2O2 (derived from the superoxide dismutase-triggered conversion of O2− to H2O2) on HIF-1α accumulation. It is known that NO reacts with O2− to form peroxynitrite (ONOO−) in a diffusion-controlled reaction, thereby affecting steady-state concentrations of NO, O2−, and ONOO− (31). For ONOO−, this could be discussed as ONOO−-mediated HIF-1α degradation.
transfection, LLC-PK1 cells were stimulated with vehicle, 100 μM GSNO, hypoxia (1%), 250 μM SpNO, 500 ng/ml TNF-α, 100 μM DFX, or 1 μM PAO for times indicated. HIF-1α protein levels were determined by Western blot analysis, followed by reprobing the blot against Akt. Each experiment was performed at least three times, and representative data are shown.

which should be clarified with the use of authentic ONOO⁻ in further experiments. However, the impact of oxygen or nitrogen species on HIF-1α accumulation presently is not fully understood. Obviously, signaling cascades that depend on phosphorylation of the HIF-1α signal, such as NO or TNF-α, appear ROS-sensitive, whereas those of DFX and PAO did not.

The action of DMNQ-derived species seems to contradict studies that show a requirement of mitochondria-derived ROS in stabilizing HIF-1α during hypoxia (3, 32, 33). Chandeli et al. (33) reported a wortmannin-sensitive PI3K pathway to account for HIF-1α stabilization during ROS signaling. One may speculate that a defined cellular redox environment senses redox changes elicited by either NO or O₂⁻ and transmits these changes via phosphorylation cascades into a functional HIF response. Cells equipped with variable amounts of defense systems to fight radical formation may then reveal a variable HIF response.

FIG. 6. Dysfunctional Akt kinase blocked the NO, TNF-α, and hypoxia pathways but appeared ineffective in case of DFX and PAO. 4 × 10⁵ LLC-PK1 cells were seeded 1 day before transfection with 3 μg of either pCMV5 or pCMV5-m/p-PKBαK179. For details, see "Experimental Procedures." 24 h after transfection, LLC-PK1 cells were stimulated with vehicle, 100 μM GSNO, hypoxia (1%), 250 μM SpNO, 500 ng/ml TNF-α, 100 μM DFX, or 1 μM PAO for times indicated. HIF-1α protein levels were determined by Western blot analysis, followed by reprobing the blot against Akt. Each experiment was performed at least three times, and representative data are shown.

In further experiments we provided evidence that PI3K and Akt are essential signaling components for GSNO and TNF-α, whereas DFX and PAO signaled PI3K independently. Previously, the involvement of the PI3K/Akt pathway in affecting HIF-1α stability was identified for hypoxia in addition to activation of the MAPK cascade. To our knowledge, the role of MAPK for NO-, TNF-α-, DFX-, or PAO-triggered HIF-1α accumulation has not been investigated. In case of GSNO, neither p42/p44, p38, nor c-Jun N-terminal kinases were activated and stimulation has not been investigated. In case of GSNO, neither p42/p44, p38, nor c-Jun N-terminal kinases were activated and stimulation has not been investigated. In case of GSNO, neither p42/p44, p38, nor c-Jun N-terminal kinases were activated and stimulation has not been investigated.

FIG. 7. PAO induced a dysfunctional HIF-1 complex in contrast to GSNO, TNF-α, and DFX. LLC-PK1 cells were treated with vehicle, 100 μM GSNO, 500 ng/ml TNF-α, 100 μM DFX, or 1 μM PAO for times indicated. Nuclei were prepared and incubated overnight with a radiolabeled oligonucleotide containing a HIF-1 binding site. Specific (HIF-1) and nonspecific (n.s.) bands are indicated. Supershifting (SS) of the HIF-1-hypoxia-responsive-element complex was achieved as described. For details, see “Experimental Procedures.” Data are representative for at least three independent experiments.

TABLE I

| Luciferase activity | % control |
|---------------------|-----------|
| Control             | 100       |
| Hypoxia (1% oxygen) | 320 ± 53  |
| GSNO (1 mM)         | 308 ± 73  |
| PAO (1 μM)          | 86 ± 27   |
| TNF-α (500 ng/ml)   | 224 ± 48  |

In further experiments we provided evidence that PI3K and Akt are essential signaling components for GSNO and TNF-α, whereas DFX and PAO signaled PI3K independently. Previously, the involvement of the PI3K/Akt pathway in affecting HIF-1α stability was identified for hypoxia in addition to activation of the MAPK cascade. To our knowledge, the role of MAPK for NO-, TNF-α-, DFX-, or PAO-triggered HIF-1α accumulation has not been investigated. In case of GSNO, neither p42/p44, p38, nor c-Jun N-terminal kinases were activated and MAPK inhibitors such as PD 98058 or SB 203580 did not attenuate HIF-1α stabilization. This is in contrast to angiotensin II signaling (13), where HIF-1α activation was attenuated by blocking PI3K or MAPK pathways.

Stimulation with PAO revealed a unique pattern of HIF-1α accumulation and HIF-1α activation. First, HIF-1α protein stabilization appeared with a time pattern remarkably similar to that described for hypoxia, DFX, or cobalt chloride. Interestingly, neither O₂⁻ production nor kinase inhibition disturbed PAO-elicited HIF-1α accumulation. PAO binds with high affinity to vicinal -SH groups. Predicted on the human primary sequence, Cys³³⁴ and Cys³³⁷ may be targeted in HIF-1α, but this protein region is not considered to be essential for HIF-1α stabilization, interaction with other proteins, or DNA binding. However, it cannot be excluded that other potential -SH groups provide targets in the three-dimensional structure. Direct bind-
ing of PAO to HIF-1α would explain a transcriptional dysfunctional HIF-1 complex (Fig. 7, Table I) and the lack of interference by other signaling pathways. Further studies are necessary to explore how binding of PAO stabilizes HIF-1α. Obviously, PAO will be a useful agent to study stabilization/regulation of HIF-1α in more detail.

Our results establish that GSNO and TNF-α, besides hypoxia, stabilize HIF-1α either in tubular LLC-PK1 or HepG2 cells. Evidently, the role of HIF-1 is not restricted to hypoxic conditions and thus may contribute to inflammatory episodes that are characterized by massive NO and/or TNF-α formation. What kind of signal transduction pathways, except of PI3K/Akt, MAPK, or ROS signaling are involved needs further investigation. Signaling cross-talk will influence HIF-1-dependent target gene expression to orchestrate hypoxic and inflammatory settings.

Acknowledgments — We are grateful to Dr. W. Ogawa (Kobe University, School of Medicine, Kobe, Japan) and Dr. B. Hemmings (F. Miescher Institut, Basel, Switzerland) for providing the p85 and PKB plasmids. We highly appreciate the technical assistance of C. Blechner and S. Liechner.

REFERENCES

1. Bunn, H. F., and Poyton, R. O. (1996) Physiol. Rev. 76, 839–885
2. Semenza, G. L. (1999) Cell 99, 281–284
3. Guillemín, K., and Krasnow, M. A. (1997) Cell 89, 9–12
4. Ivan, M., Kondo, K., Yang, H., Kim, W., Yaliando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G. (2001) Science 292, 464–468
5. Jaakkola, P., Mile, D. R., Tian, Y. M., Wisten, M. I., Gielberti, J., Gaskell, S. J., Kriegsheim, A. v., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Science 292, 468–472
6. Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wyckoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. (1999) Nature Lett. 399, 271–275
7. Fandrey, J., and Genius, J. (2000) Adv. Exp. Med. Biol. 475, 153–159
8. Richard, D. E., Berra, E., Gothie, E., Roux, D., and Pouyssegur, J. (1999) J. Biol. Chem. 274, 32631–32637
9. Salceda, S., Beck, I., Srinivas, V., and Caro, J. (1997) Kidney Int. 51, 556–559
10. Wang, G. L., Jiang, B.-H., and Semenza, G. L. (1995) Biochem. Biophys. Res. Commun. 216, 669–675
11. Zhong, H., Chiles, K., Felser, D., Laughner, E., Hanrahan, C., Georgescu, M., J., W. S., and Semenza, G. L. (2000) Cancer Res. 60, 1541–1545
12. Hellwig-Bürgel, T., Rutkowski, K., Metzen, E., Fandrey, J., and Jelkman, W. (1999) Blood 94, 1561–1567
13. Richard, D. E., Berra, E., and Pouyssegur, J. (2000) J. Biol. Chem. 275, 26765–26771
14. Kimura, H., Weiss, A., Kurashima, Y., Hashimoto, K., Ogura, T., D’Acquista, F., Addeo, R., Makucinski, M., and Esumi, H. (2000) Blood 95, 189–197
15. Sandau, K. B., Fandrey, J., and Brune, B. (2001) Blood 97, 1009–1015
16. Sandau, K. B., Gimenetz Faus, H., and Brune, B. (2000) Biochem. Biophys. Res. Commun. 278, 283–287
17. Stamler, J. S. (1994) Cell 78, 931–936
18. Knothen von, A., Callsen, D., and Bruhm (1999) Mol. Biol. Cell 10, 361–372
19. Hara, K., Kondo, K., Sakan, H., Ando, A., Kitani, K., Imita, T., Kitamura, T., Ueda, H., Stephens, L., Jackson, T. R., Waterfield, M. D., and Kasuga, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7415–7419
20. Kiemann, T., Kornes, Y., Brehm, K., Modaresi, S., and Jungermann, K. (2001) Biochem. J. 354, 531–537
21. Hart, T. W. (1997) Tetrahedron Lett. 26, 2013–2016
22. Dypbukt, J. M., Ankarström, M., Burkitt, M., Stijoholm, A., Strom, K., Orrenius, S., and Nicotera, P. (1994) J. Biol. Chem. 269, 30553–30560
23. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. C., Frech, M., Cron, P., Cohen, P., Luccoq, J. M., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 31515–31524
24. Zlatkine, P., Mehl, B., and Magee, A. I. (1997) J. Cell Sci. 110, 673–679
25. Liu, Y., Christou, H., Morita, T., Laughner, E., Semenza, G. L., and Kuremman, S. (1999) J. Biol. Chem. 274, 15257–15262
26. Huang, L. L., Willmore, W. G., Gu, J., Goldberg, M. A., and Bunn, H. F. (1999) J. Biol. Chem. 274, 9038–9044
27. Sogawa, K., Numayama-Tsuruta, K., Ema, M., Abe, M., and Fujii-Kuriyama, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7368–7373
28. Genius, J., and Fandrey, J. (2000) Free Radic. Biol. Med. 29, 515–521
29. Palmer, L. A., Gaston, B., and Johns, R. A. (2000) Mol. Pharmacol. 58, 1195–1203
30. Thornton, R. D., Lane, P., Borghaei, R. C., Pease, E. A., Caro, J., and Meohan, E. (2000) Biochem. J. 350, 307–312
31. Dubé, R. J., Nielsen, M. D., Dittman, A. H., Villacres, E. C., Choi, E. J., and Storm, D. R. (1984) J. Biol. Chem. 269, 7290–7296
32. Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., and Schumacker, P. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11715–11720
33. Chandel, N. S., McIntock, O. S., Feliciano, C. E., Wood, T. M., Melendez, J. A., Rodriguez, A. M., and Schumacker, P. T. (2000) J. Biol. Chem. 275, 25130–25138
Regulation of the Hypoxia-inducible Factor 1α by the Inflammatory Mediators Nitric Oxide and Tumor Necrosis Factor-α in Contrast to Desferroxamine and Phenylarsine Oxide
Katrin B. Sandau, Jie Zhou, Thomas Kietzmann and Bernhard Brüne

J. Biol. Chem. 2001, 276:39805-39811.
doi: 10.1074/jbc.M107689200 originally published online August 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107689200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 21 of which can be accessed free at http://www.jbc.org/content/276/43/39805.full.html#ref-list-1