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Induction of Hypoxia-Inducible-Factor 1 by Nitric Oxide Is Mediated via the PI 3K Pathway

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Adaptation to hypoxic stress provokes activation of the hypoxia-inducible-factor-1 (HIF-1) which mediates gene expression of, e.g., erythropoietin or vascular endothelial growth factor. Detailed information on signaling pathways that stabilize HIF-1 is missing, but reactive oxygen species degrade the HIF-1α subunit, whereas phosphorylation causes its stabilization. It was believed that hypoxia resembles the only HIF-1 inducer but recent evidence characterized other activators of HIF-1 such as nitric oxide (NO). Herein, we concentrated on NO-evoked HIF-1 induction as a heretofore unappreciated inflammatory response in association with massive NO formation. We demonstrated that *S*-nitrosoglutathione induces HIF-1α accumulation and concomitant DNA binding. The response was attenuated by the kinase inhibitor genistein and blockers of phosphatidylinositol 3-kinase such as Ly 294002 or wortmannin. Whereas mitogen-activated protein kinases were not involved, we noticed phosphorylation/activation of Akt in correlation with HIF-1α stabilization. NO appears to regulate HIF-1α via the PI 3K/Akt pathway under normoxic conditions.

Key Words: **hypoxia-inducible-factor-1; nitric oxide; PI 3K; Akt kinase; tubular cells.**

Several proteins such as erythropoietin, vascular endothelial growth factor (VEGF), tyrosine hydroxylase, nitric oxide synthase, glucose transporters, or different glycolytic enzymes are under control of the transcription factor hypoxia-inducible-factor-1 (HIF-1). By regulating these proteins an organism adapts to a situation of reduced oxygen availability (altitude changes or tumor growth) by erythropoiesis, angiogenesis, increased breathing, vasodilatation, and changing to an altered glucose metabolism (1). In this respect, the role of HIF-1 was associated with hypoxic conditions only. More recent evidence suggests other HIF-1 inducers besides hypoxia. For example, insulin, various growth factors (IFG-1/2, EGF, FGF) (2, 3), cytokines (IL-1β, TNF-α) (4), or hormones (angiotensin II, thrombin) (5) have been described to activate HIF-1. Moreover, we and Kimura *et al.* (6) have recently shown that nitric oxide (NO) concentration- and time-dependently induces HIF-1 as well. By using a coculture system of activated macrophages and resting tubular cells to imitate an inflammatory situation, we demonstrated that NO functions as an intercellular inducer of HIF-1, independent of hypoxia.

NO is produced by a family of NO synthases which utilize L-arginine and oxygen to generate NO and L-citrulline (7). Constitutive versus inducible NO synthase isoforms can be destinguished that generate low versus high concentrations of NO. Once NO is produced, it may fulfill either physiological roles as shown in the cardiovascular system or pathophysiological roles as described for different disease states (8). Due to its redox capacities, NO interacts with transition metals, oxygen, or superoxide to function as an effector and modulator of signaling (9).

The newly discovered NO target, HIF-1, is a heterodimer composed of HIF-1α and HIF-1β. In contrast to HIF-1β which is constantly present, the HIF-1α protein is redox-regulated and degraded by the proteasomal system during normoxia (10–12). It is believed that the cellular level of reactive oxygen species affects stabilization or degradation of HIF-1α (13). Therefore, hypoxia and a concomitant decrease of reactive oxygen species promote HIF-1α accumulation, dimerization with HIF-1β, translocation to the nucleus, and binding to the hypoxia responsive element in the promotor region of different genes. Besides reactive oxygen species, phosphorylation events have been postulated and described for HIF-1 regulation (14, 15). Recently, Zhong *et al.* (2) showed the involvement of phosphatidylinositol 3-kinase (PI 3K), Akt, and FKBP-rapamycin-associated protein for hypoxia and growth factor-induced HIF-1 activation. PI 3K catalyzes the conversion of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-biphosphate to phosphatidyl-

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inositol 3,4-biphosphate and phosphatidylinositol 3,4,5-triphosphate. These products are allosteric activators of phosphatidylinositol-dependent kinase 1 which phosphorylates and activates Akt (protein kinase B). Generally, the Akt pathway promotes cell proliferation and inhibits cell death (16).

Herein, we demonstrate that low concentrations of *S*-nitrosoglutathione (GSNO) induced a fast but transient HIF-1α accumulation that correlated with its DNA binding ability. Using genistein as a general kinase inhibitor we blocked HIF-1α up-regulation to emphasize the importance of phosphorylation events for NO actions. Further, we pinpointed PI 3K and concomitant Akt activation as essential components to provoke HIF-1α accumulation/activation in response to NO.

Assuming that HIF-1 accumulates under inflammatory conditions that are characterized by massive NO formation but devoid of hypoxia, it is challenging to investigate signaling pathways being involved.

MATERIALS AND METHODS

Materials. Medium and supplements were purchased from Biochrom, Berlin, Germany. Fetal calf serum was bought from Life Technologies, Berlin, Germany. Nitrocellulose, [γ-^{32}P]ATP, and ECL detection system came from Amersham, Freiburg, Germany. HIF-1α antibody was ordered from Beckton Dickenson, Heidelberg, Germany; phospho-specific (Ser473) Akt and Akt antibodies from New England Biolab, Frankfurt, Germany; and secondary antibodies from Promega, Mannheim, Germany. T4-polynucleotide kinase was purchased from Boehringer Mannheim, Mannheim, Germany, and chromaspin-10 columns were from Clontech, Heidelberg, Germany. Wortmannin and Ly 294002 were ordered by Sigma, Deisenhofen, Germany, and genistein was from Calbiochem, Bad Soden, Germany. All other chemicals were of the highest grade of purity and commercially available.

Cell culture. Proximal tubular LLC-PK$_1$ cells were cultured in DMEM with 1 g/liter glucose, 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 experiments. Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C.

S-Nitrosoglutathione synthesis. S-Nitrosoglutathione (GSNO) was synthesized as described previously (17).

Western blot analysis. HIF-1α or phosphorylated Akt were quantified by Western blot analysis. Briefly, 1×10^6 cells were incubated for the times indicated, scraped off, lysed in 150 μl lysis buffer (50 mM Tris/HCl, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0), and sonicated. After centrifugation (17,000*g*, 15 min) the protein content in the supernatants were analyzed. Finally, 100 μg protein was added to the same volume of 2× sample buffer (125 mM Tris/HCl, 2% SDS, 10% glycerin, 1 mM DTT, 0.002% bromphenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 7.5% or 10% SDS–polyacrylamide gels and blotted onto nitrocellulose. Molecular weights were calibrated in proportion to the running distance of rainbow markers. Transblots were washed twice with TBS (140 mM NaCl, 50 mM Tris/HCl, pH 7.2) containing 0.1% Tween 20 before blocking unspecific binding with TBS/5% skim milk for 1 h. The HIF-1α (1:250 in TBS/0.5% milk) antibody, phospho-specific (Ser473) Akt antibody, or Akt antibody (1:1000 in TBS/0.5% milk) were added and incubated overnight at 4°C. Afterwards, nitrocellulose membranes were washed 5× for 15 min with TBS containing 0.1% Tween 20. For protein detection, blots were incubated with goat anti-mouse secondary antibodies or in case of phospho-Akt and Akt goat anti-rabbit secondary antibodies conjugated with peroxidase (1:10,000 in TBS/0.2% milk) for 45 min, followed by ECL detection.

Nuclear extract preparation. After stimulation, LLC-PK$_1$ cells were washed with ice-cold PBS, scrapped 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 buffer A (10 mM Tris, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM sodium orthovanadate, 0.5 mM DTT, 0.5 mM PMSF) and kept on ice for 25 min and vortexed (2×) in between. For the last 10 s, 25 μl 10% NP-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 buffer C (20 mM Tris, pH 7.8, 1.5 mM $MgCl_2$, 420 mM KCl, 20% glycerol, 1 mM sodium orthovanadate, 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 l buffer D (100 mM KCl, 20 mM Tris, pH 7.8, 2 mM EDTA, 20% glyerol) 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 MWG (Ebersberg, Germany) and contained the sequence of the HIF-1 binding site derived from the human transferrin gene. The sequences were as followed: (sense) 5′-TTCCTGCACGTACACACAAAGCGCACGTATTTC-3′ and (antisense) 5′-GAAATACGTGCGCTTTGTGTGTACGTGCAGGAA-3′. For radioactive labeling, 1.25 pmol annealed oligonucleotides were incubated with 2.5 μl 10× polynucleotide kinase phosphorylation buffer, 1 μl phosphatase-free T4-polynucleotide kinase and 50 μCi [γ-^{32}P]ATP in a final volume of 20 μl at 37°C for 25 min. Labeling reaction was terminated by the addition of 1 μl 0.5 M EDTA and unlabeled oligonucleotides were discarded by using chromaspin-10 columns. The efficiency of radioactive labeling was measured with a counter. Afterwards, HIF-1 binding reactions were set up in a volume of 20 μl and nuclear extracts (5 μg protein) were incubated in a buffer with a final concentration of 50 mM KCl, 10 mM Tris, pH 7.7, 5 mM DTT, 1 mM EDTA, 1 mM $MgCl_2$, 5% glycerol, 0.03% Nonidet P-40, 400 ng salmon testes DNA, and 40,000 cpm ^{32}P-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 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.

RESULTS AND DISCUSSION

To analyze the effect of NO on HIF-1 accumulation/activation we used chemically distinct NO donors. *S*-Nitrosoglutathione (GSNO) induced HIF-1α accumulation in tubular LLC-PK$_1$ cells. This response was reproduced by Dea-NO and spermine-NO but not sodium nitroprusside, most likely because sodium nitroprusside liberates cyanide which might be toxic, prior to the release of NO (data not shown). When using a low and nontoxic concentration of 100 μM GSNO, the HIF-1α protein level increased within 1 h, reached a maximum at 2 h, and decreased afterwards (Fig. 1A). Although HIF-1α is the regulatory component of HIF-1, its accumulation not necessarily correlates with the DNA binding activity of the heterodimer. There-

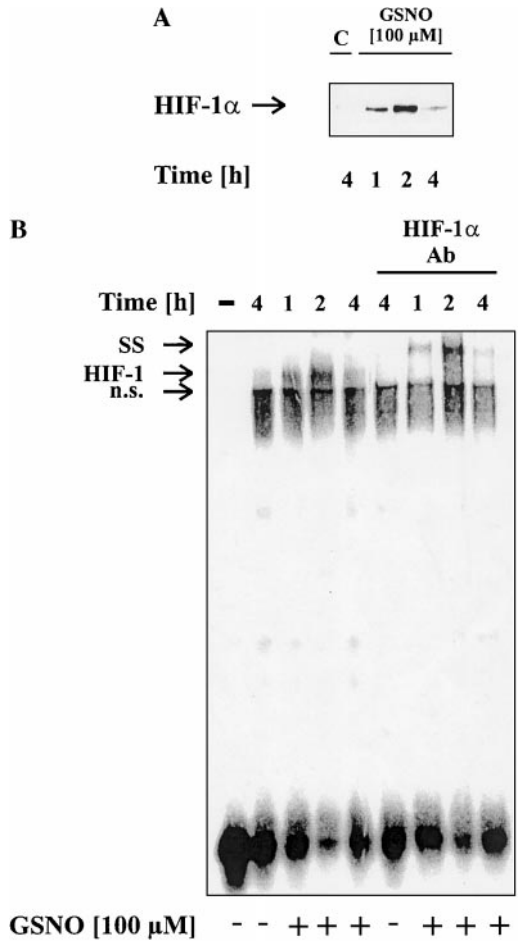

FIG. 1. GSNO-induced HIF-1α accumulation and DNA binding. LLC-PK$_1$ cells were stimulated with vehicle "C" or 100 μM GSNO for 1, 2, or 4 h. (A) Accumulation of HIF-1α was determined by Western blot analysis as outlined under Materials and Methods. (B) For EMSA analysis, nuclei were prepared and incubated overnight with a radioactive-labeled oligonucleotide containing a HIF-1 binding site. Specific (HIF-1) and non-specific (n.s.) bands are indicated. Supershifting (SS) of the HIF-1-hypoxia-responsive-element complex was achieved as described. For details see Materials and Methods. Western blot and gel shift are representative for at least 3 independent experiments.

fore, we performed electrophoretic mobility shift assays (EMSA) with a radioactive-labeled oligonucleotide containing the HIF-1 binding site. Again, LLC-PK$_1$ cells were stimulated with 100 μM GSNO, reactions were terminated after 1, 2, or 4 h, and nuclei extracts were prepared for EMSA analysis. Similar to the HIF-1α protein level, HIF-1 DNA binding activity appeared within 1 h, was strongest after 2 h, and declined afterwards (Fig. 1B). HIF-1 specificity was assured by supershift experiments. Therefore, we supplied 0.25 μg HIF-1α antibody to the protein-oligo binding assay for 1 h. We then noticed supershifting of the HIF-1 band, whereas nonspecific bands (n.s.) remained unaffected.

Having established that NO is a potent inducer of HIF-1, besides hypoxia, growth factors, cytokines, or hormones (2–5, 13), it was our intention to characterize signaling components. Considering that NO scavenges reactive oxygen species, it may appear attractive that this situation imitates hypoxic conditions leading to HIF-1 activation. To test this, we simultaneously generated NO and superoxide but it evoked HIF-1α degradation (unpublished data). Therefore, we excluded this assumption as a likely explanation for NO-induced HIF-1α accumulation.

In further experiments we concentrated on phosphorylation events by using the general kinase inhibitor genistein at a concentration of 100 μM. In corroboration with results obtained for hypoxia-, $CoCl_2$-, and desferroxamine-induced HIF-1 activation (14, 15), genistein completely blocked HIF-1α accumulation in response to 100 μM GSNO incubated for 1, 2, or 4 h (Fig. 2). Since the absence of HIF-1α does not allow the formation of an active HIF-1 complex, we concluded that phosphorylation events resemble a general mechanism for HIF-1 stabilization, including the response towards NO.

Mitogen-activated-protein-kinases (MAPK) were the first phosphorylation cascades analyzed for HIF-1 regulation. Salceda *et al.* (14), Richard *et al.* (18), and Minet *et al.* (19) showed that inhibition of the p42/p44 MAPK cascade abrogated hypoxia-induced HIF-1 activation/phosphorylation independent of whether HIF-1 is composed of HIF-1α or EPAS1 (20). Although c-Jun N-terminal protein kinase (JNK) and members of the p38 protein kinase family can be activated by hypoxia (21), they seem to be less important for HIF-1 regulation (18).

In our experimental setting, the MEK 1 inhibitor PD 98058 and the p38 inhibitor SB 203580 were ineffective in modulating NO-elicited HIF-1α accumulation (unpublished data). In addition, analysis of p42/p44 MAPK, p38, and JNK activity revealed that these pathways were not significantly affected under the conditions of NO-stimulated HIF-1α accumulation in

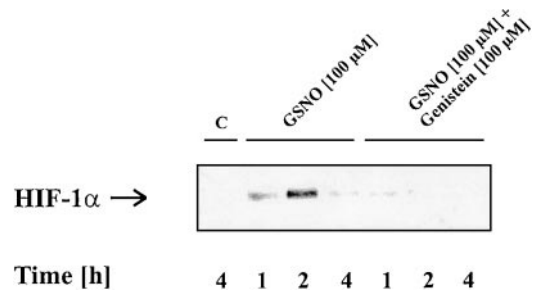

FIG. 2. Genistein blocked NO-evoked HIF-1α stabilization. LLC-PK$_1$ cells were treated with vehicle "C" or 100 μM GSNO in the absence or presence of 100 μM genistein. Genistein was preincubated for 30 min and incubations were terminated after 1, 2, or 4 h. HIF-1α accumulation was analyzed by Western blot as described under Materials and Methods. The blot is representative for at least 3 independent experiments.

LLC-PK$_1$ cells (unpublished data). This is in line with a recent report showing that angiotensin II-induced HIF-1α accumulation was not blocked by PD 98058, although p42/p44 MAPK phosphorylation, which occurred after angiotensin II stimulation, was suppressed (5).

In the next set of experiments we studied signal transmission involving the PI 3K. We stimulated LLC-PK$_1$ cells with 100 μM GSNO in the absence or presence of the PI 3K inhibitors wortmannin or Ly 294002. HIF-1α accumulation in response to GSNO was completely blocked by preincubating cells with either 10 μM wortmannin (Fig. 3A) or 50 μM Ly 294002 (Fig. 3B). This implies an essential role of PI 3K for NO-triggered HIF-1 activation.

A well recognized target of PI 3K is the Akt kinase, also known as protein kinase B. Phosphorylation of its Ser473 residue reflects an active Akt kinase. Therefore, we used a phospho-specific Ser473 Akt antibody to analyze activation of Akt during NO-induced HIF-1α stabilization. After stimulating LLC-PK$_1$ cells with 100 μM GSNO, Akt was phosphorylated within 1 to 2 h and remained in its phospho-form for up to 4 h (Fig. 4A). With the use of the PI 3K inhibitor Ly 294002, we abrogated the HIF-1α response (Fig. 3B) as well as NO-induced Akt phosphorylation, depicted for the 2 h time-point (Fig. 4B). To assure that total amounts of Akt were unaltered, we reprobed Western blots with an antibody that recognizes the phosphorylated and unphosphorylated form. Evidently, no differences in Akt protein levels were detected (Fig. 4A, lower part).

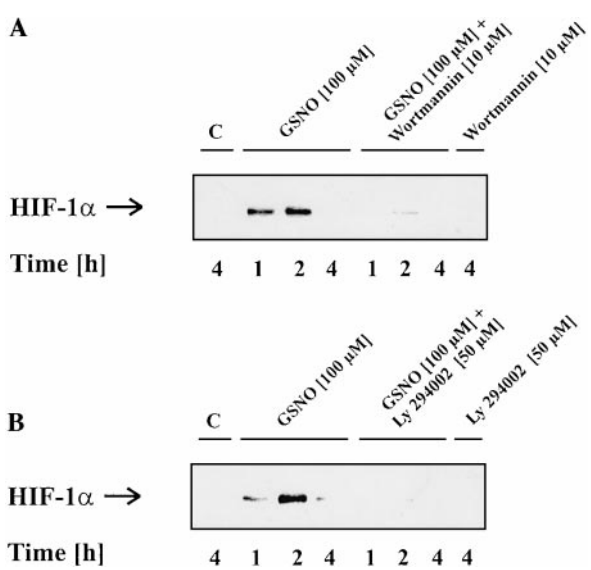

FIG. 3. PI 3K inhibition abrogated NO-elicited HIF-1α accumulation. LLC-PK$_1$ cells were stimulated with vehicle "C" or 100 μM GSNO for 1, 2, or 4 h in the absence or presence of the PI 3K inhibitors wortmannin (A) or Ly 294002 (B) Wortmannin and Ly 294002 were preincubated for 30 min. HIF-1α accumulation was detected by Western blot analysis. For details see Materials and Methods. Blots are representative for at least 3 independent experiments.

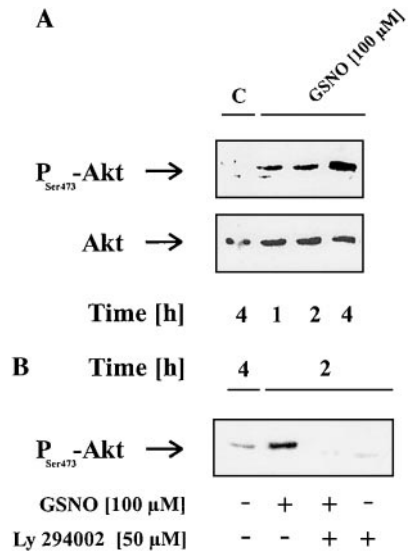

FIG. 4. NO-stimulated Akt phosphorylation is blocked by Ly 294002. (A) LLC-PK$_1$ cells were treated with vehicle "C" or 100 μM GSNO and samples were collected after 1, 2, or 4 h. Phosphorylation of Ser 473 of Akt was detected with a phospho-specific Akt antibody as described under Materials and Methods. Afterwards, blots were stripped and reprobed with an Akt antibody detecting both, the phosphorylated and unphosphorylated form. (B) LLC-PK$_1$ were stimulated with vehicle, 100 μM GSNO, 50 μM Ly 294002, or GSNO in combination with Ly 294002 for 2 h. Ly 294002 was prestimulated for 30 min. Phosphorylated Ser 473-Akt was detected by Western blot analysis. Details are described under Materials and Methods. Blots are representative for at least 3 independent experiments.

Our data on NO signaling and concomitant HIF-1 stabilization are in line with a recent report correlating activation of the PI 3K/Akt pathway to HIF-1 expression under hypoxic conditions and in response to growth factors in prostate cancer cells (2). Along that line, Mazure *et al.* (22) demonstrated that Ha-ras-transformed cells use the PI 3K/Akt pathway for VEGF induction under hypoxic conditions. These results emphasize a role of PI 3K/Akt for the expression of HIF-1 and VEGF in the context of tumor development.

We conclude that NO-evoked HIF-1 induction occurs in a PI 3K-dependent manner that closely correlates with phosphorylation of Akt. Whether Akt directly affects HIF-1α stability/regulation in response to NO will be investigated in future studies. As established for hypoxia, low NO concentrations led to HIF-1α accumulation that demanded the PI 3K pathway. In contrast to hypoxia, MAPK seem to play a minor or no role in signaling pathways that provoke HIF-1α stabilization in response to NO.

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