Regulation of hypoxia-inducible factor-1α by nitric oxide through mitochondria-dependent and -independent pathways

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Nitric oxide (NO) has been reported both to promote and to inhibit the activity of the transcription factor hypoxia-inducible factor-1 (HIF-1). In order to avoid the pitfalls associated with the use of NO donors, we have developed a human cell line (Tet-iNOS 293) that expresses the inducible NO synthase (iNOS) under the control of a tetracycline-inducible promoter. Using this system to generate finely controlled amounts of NO, we have demonstrated that the stability of the α-subunit of HIF-1 is regulated by NO through two separate mechanisms, only one of which is dependent on a functional respiratory chain. HIF-1α is unstable in cells maintained at 21 % O_2, but is progressively stabilized as the O_2 concentration decreases, resulting in augmented HIF-1 DNA-binding activity. High concentrations of NO (>1 μM) stabilize HIF-1α at all O_2 concentrations tested. This effect does not involve the respiratory chain, since it is preserved in cells lacking functional mitochondria (ρ^0-cells) and is not reproduced by other inhibitors of the cytochrome *c* oxidase. By contrast, lower concentrations of NO (< 400 nM) cause a rapid decrease in HIF-1α stabilized by exposure of the cells to 3 % O_2. This effect of NO is dependent on the inhibition of mitochondrial respiration, since it is mimicked by other inhibitors of mitochondrial respiration, including those not acting at cytochrome *c* oxidase. We suggest that, although stabilization of HIF-1α by high concentrations of NO might have implications in pathophysiological processes, the inhibitory effect of lower NO concentrations is likely to be of physiological relevance.

Key words: cytochrome *c* oxidase, hypoxia, hypoxia-inducible factor-1α (HIF-1α), mitochondria, nitric oxide (NO), oxygen (O_2).

INTRODUCTION

Most cells are able to respond to a decrease in O_2 availability by initiating a series of adaptative responses through the transcriptional activation of hypoxia-inducible genes such as those coding for vascular endothelial growth factor, erythropoietin, iNOS [inducible nitric oxide (NO) synthase] and glycolytic enzymes [1]. The activation of these genes requires the binding of the transcription factor HIF-1 (hypoxia-inducible factor-1) to specific sequences, termed HREs (hypoxia response elements), located in the promoters/enhancers of the hypoxia-inducible genes. This response results in increased O_2 delivery to tissues and maintenance of ATP levels [2].

HIF-1 is a heterodimeric protein consisting of two subunits, HIF-1α and HIF-1β [3]. HIF-1β is constitutively expressed and its levels are not affected by changes in the cellular pO_2 (oxygen partial pressure). By contrast, HIF-1α is tightly regulated by O_2 pressure and accumulates very rapidly in cells exposed to hypoxic conditions [4–6]. Thus, under non-hypoxic conditions, the HIF-1α subunit is promptly and continuously degraded by the ubiquitin–proteasome system after hydroxylation of Pro^{402} and/or Pro^{564} within the O_2-dependent degradation domain of HIF-1α and subsequent binding of the von Hippel–Lindau protein (pVHL). Under hypoxic conditions, prolyl hydroxylation of HIF-1α is impaired, leading to decreased pVHL-ubiquitination and increased HIF-1α stability [7–9].

NO is a highly diffusible gas that mediates a variety of physiological effects, including the maintenance of vascular tone, modulation of synaptic transmission and cellular defence [10]. Although many of the physiological actions of NO are mediated through the activation of soluble guanylate cyclase, at physiological concentrations NO also inhibits the cytochrome *c* oxidase, the terminal enzyme in the mitochondrial electron-transport chain, in competition with O_2 and in a reversible manner [11,12]. This suggests that NO might be a physiological regulator of cell respiration [13,14].

Mitochondria are the major O_2-consuming organelles in the cell. Because of this, it is likely that they play an important role in sensing the cellular O_2 concentration. Indeed, by using pharmacological inhibitors of the respiratory chain, and cells lacking mitochondrial DNA and electron-transport activity (ρ^0-cells), it has been suggested [15–18] that the hypoxic regulation of HIF-1 activity is dependent on mitochondrial function. It is thus possible that NO, by acting on cytochrome *c* oxidase, might modulate cellular responses involving HIF-1. Several studies have shown an effect of NO on HIF-1α accumulation. However, at present, these reports are controversial and both stabilization [19–24] and destabilization [19,25–29] have been reported.

In the present study, using a system in which NO is generated inside the cells in a finely controlled manner, we demonstrate that concentrations of NO below 400 nM prevent the accumulation of HIF-1α in hypoxia in a mitochondria-dependent manner. Additionally, we show that NO at high concentrations (> 1 μM) always results in HIF-1α stabilization, both under hypoxic and non-hypoxic conditions, and that this effect is independent of the mitochondrial respiratory chain. Furthermore, we show that

Abbreviations used: HIF-1, hypoxia-inducible factor-1; NO, nitric oxide; iNOS, inducible NO synthase; S-EITU, *S*-ethylisothiourea; Tet-iNOS 293, tetracycline-inducible iNOS-expressing HEK-293 cells; DETA-NONOate, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; oxyHb, oxyhaemoglobin; pVHL, von Hippel–Lindau protein; FRT, Flp recombination target; PNPP, *p*-nitrophenyl phosphate; HRE, hypoxia response element; BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluorobenzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine; $ONOO^-$, peroxynitrite; ρ^0 cells, cell lacking functional mitochondria.

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stabilization of HIF-1α by hypoxia and by high concentrations of NO is dependent on two different, but synergistic, mechanisms. We suggest that, while the NO-induced decrease in HIF-1α accumulation may have physiological implications, its stabilization by high concentrations of NO may be involved in pathophysiology.

EXPERIMENTAL

Cell culture and reagents

Tetracycline-inducible HEK-293 cells stably expressing human iNOS were cultured in Dulbecco's modified Eagle's medium (Gibco Invitrogen, Barcelona, Spain) containing 4.5 g/l D-glucose, 10 % (v/v) foetal-calf serum, 200 μg/ml hygromycin B and 15 μg/ml blasticidin, at 37 °C in a humidified atmosphere with 5 % CO_2. Cells devoid of mitochondrial DNA (ρ^0-cells) were generated by incubation of the cells in the medium described above containing ethidium bromide (50 ng/ml) and uridine (50 μg/ml) for 2–3 weeks. The ρ^0 status of the cells was confirmed by the lack of mitochondrial-dependent O_2 consumption by comparing the rates of O_2 consumption in the absence or presence of myxothiazol (0.5 μM) as a specific inhibitor of mitochondrial respiration. Rotenone, myxothiazol, antimycin A, cyanide, S-EITU (*S*-ethylisothiourea), L-arginine, ethidium bromide and uridine were from Sigma–Aldrich (St Louis, MO, U.S.A.). Hygromycin B and blasticidin were purchased from Invitrogen. Tetracycline was from Calbiochem (Darmstadt, Germany). The NO donor DETA-NONOate {(*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate} was obtained from Alexis Biochemicals (Lausen, Switzerland).

Plasmid preparation and transfection

The cDNA encoding the complete coding region of iNOS (3465 bp) from human chondrocytes (GenBank® accession no. X73029) was cloned into the inducible expression vector pcDNA5/FRT/TO (Invitrogen) by PCR using primers designed to contain restriction sites for *Hin*dIII and *Xho*I at the 5′ and 3′ ends, respectively. The sense primer was 5′-GAG AAA GCT TGA GAT GGC CTG TCC TTG GAA ATT TCT G-3′ and the antisense primer was 5′-GAG ACT CGA GTC AGA GCG CTG ACA TCT CCA GG-3′. The amplified PCR product was purified (QIAquick PCR Purification Kit; Qiagen, Valencia, CA, U.S.A.), digested with *Hin*dIII and *Xho*I, and ligated into pcDNA5/FRT/TO, previously digested with the same restriction enzymes. The resultant DNA construct was amplified and purified (Qiagen) and designated pcDNA5/FRT/TO-iNOS. To generate tetracycline-inducible HEK 293 cells stably expressing human iNOS, the Flp-In™ T-REx™-293 cell line (Invitrogen), which stably expresses the tetracycline repressor and contains a single integrated Flp recombination target (FRT) site, was cotransfected with pcDNA5/FRT/TO-iNOS and the Flp recombinase expression plasmid pOG44 (Invitrogen); pOG44 mediates insertion of the iNOS gene into the genome at the integrated FRT site through site-specific DNA recombination. The cells, approx. 2×10^6 cells/well in six-well culture plates, were cotransfected by 7.5 μl of LIPOFECTAMINE™ 2000 (Invitrogen) with 0.3 μg of pcDNA5/FRT/TO-iNOS and 3 μg of pOG44, according to the manufacturer's instructions. After 48 h, transfected cells were selected for hygromycin B and blasticidin resistance by supplementing the growth medium with 200 μg/ml hygromycin B and 15 μg/ml blasticidin. Tetracycline-inducible clonal cell lines that stably express the iNOS enzyme were obtained. The cells generated were designated Tet-iNOS 293 cells.

Induction of endogenous NO production and hypoxia

Tet-iNOS 293 cells were plated in 60-mm-diameter culture dishes at a density of 4×10^6 cells/dish. We induced the expression of iNOS by overnight incubation of the cells (14–15 h) in complete growth medium (without selection antibiotics) supplemented with tetracycline (10–1000 ng/ml) in the presence of the potent inhibitor of the synthesis of NO by iNOS, S-EITU (500 μM). Inhibition by S-EITU is readily reversible after wash-out and does not interfere with iNOS dimerization. We confirmed that iNOS activity was fully inhibited by S-EITU by the lack of accumulation of nitrite overnight in the extracellular medium. Following induction with tetracycline, the cells were washed with L-arginine-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1 % dialysed fetal-calf serum to avoid any source of L-arginine and undesired production of NO. The cells were maintained in L-arginine-free medium for 60 min to allow complete wash-out of S-EITU without production of NO, and were then washed with assay buffer [20 mM Hepes (pH 7.4)/125 mM NaCl/5.2 mM KCl/2 mM $CaCl_2$/1.2 mM $MgCl_2$/5.5 mM D-glucose]. Production of NO was initiated by addition to the cells of assay buffer containing L-arginine (1 mM) for the indicated times and O_2 concentrations. Hypoxia was achieved by incubation of the cells in a CO_2/O_2 incubator (model BB6060-O_2; Heraeus, Stuttgart, Germany) with a blend of 5 % CO_2, the desired percentage of O_2 and N_2 to total 100 %.

Preparation of nuclear and cytoplasmic extracts

After treatment, the assay buffer was recovered for nitrite determination and the cells were scraped off in 1 ml of ice-cold PBS freshly supplemented with phosphatase inhibitors [10 mM NaF/10 mM β-glycerophosphate/10 mM PNPP (*p*-nitrophenyl phosphate)/1 mM $NaVO_3$] and a protease-inhibitor cocktail (Roche Diagnostics, Barcelona, Spain). The cells were centrifuged at 300 ***g*** for 5 min at 4 °C and the pellet was resuspended in 100 μl of Buffer A (10 mM Hepes/1.5 mM $MgCl_2$/10 mM KCl/1 mM dithiothreitol/0.2 % Nonidet P40/10 mM NaF/10 mM β-glycerophosphate/10 mM PNPP/1 mM $NaVO_3$/protease-inhibitor cocktail tablet/1 mM pefabloc, pH 7.4), vortex-mixed for 10 s, kept on ice for 10 min, then vortex-mixed again for 10 s. After a short centrifugation (16 000 ***g*** for 30 s at 4 °C), the supernatant was collected as the cytoplasmic extract. To obtain the nuclear fraction, the pellet was resuspended in 25 μl Buffer C [20 mM Hepes/1.5 mM $MgCl_2$/400 mM NaCl/1 mM dithiothreitol/0.2 mM EDTA/25 % (v/v) glycerol/protease-inhibitor cocktail tablet/1 mM pefabloc, pH 7.4], vortex-mixed for 15 s and kept on ice for 15 min. The suspension was vortexed-mixed for 15 s prior to centrifugation (16 000 ***g*** for 60 s at 4 °C) and the supernatant was recovered. The protein concentration was determined by the BCA (bichichoninic acid) protein assay kit (Pierce; from Perbio Science, Tattenhall, Chester, Cheshire, U.K.) using BSA as the standard.

Immunoblot analysis and HIF-1 DNA-binding activity

For HIF-1α and iNOS protein detection, nuclear (50–100 μg) and cytoplasmic (50 μg) extracts respectively were separated by SDS/7.5 %-(w/v)-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Madrid, Spain) using standard procedures [30]. Membranes were blocked with 5 % (w/v) non-fat dry milk in TBS-T [20 mM Tris/HCl (pH 7.2)/150 mM NaCl/0.1 % Tween 20] and incubated overnight with monoclonal antibodies against HIF-1α (1:250; Transduction Laboratories, BD Biosciences, Erembodegem, Belgium) or polyclonal antibodies against iNOS (1:2000, Transduction Laboratories, BD Biosciences) in blocking solution at 4 °C. Protein bands were detected by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2500; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or goat anti-rabbit IgG (1:5000, Vector Laboratories, Burlingame, CA, U.S.A.), followed by enhanced chemiluminescence (ECL®; Amersham, Uppsala, Sweden). HIF-1 activation was quantified in 5–10 μg of nuclear extracts by specific binding of HIF-1 to an oligonucleotide containing the HRE from the Epo gene (5′-TACGTG CT-3′) by means of the TransAM HIF-1 Kit (Active Motive, Rixensart, Belgium) according to the manufacturer's instructions.

Nitrite measurement

The amount of NO formed was estimated by measuring nitrite (NO_2^-) levels in the extracellular medium using the Griess reagent kit (Molecular Probes, Leiden, The Netherlands). Since NO_2^- formation from NO is diminished in hypoxia [31], when comparing NO production at different O_2 concentrations, total $NO_2^- + NO_3^-$ was determined after reduction of NO_3^- to NO_2^- with nitrate reductase by using the Nitric Oxide Fluorometric Assay Kit (Calbiochem).

Measurement of O_2 consumption and NO generation

Oxygen consumption and NO production were determined in parallel in cells suspended in Hanks balanced salt solution at a density of 1×10^7 cells/ml. Measurements were taken in 1 ml of cell suspensions in gas-tight vessels gently agitated and kept at 37 °C. Consumption of O_2 was assessed using an oxygen electrode (Hansatech, King's Lynn, Norfolk, U.K.) after 1 h incubation with L-arginine (1 mM). The oxygen electrode was calibrated with air-saturated incubation medium kept at 37 °C, assuming an O_2 concentration of 200 μM. NO production was monitored for 1 h after the addition of L-arginine (1 mM) using an NO electrode (ISO-NOP; World Precision Instruments, Stevenage, Herts., U.K.). The NO electrode was calibrated by addition of known concentrations of $NaNO_2$ under reducing conditions (KI/H_2SO_4) at 37 °C. The reversibility of NO-induced inhibition of respiration was assessed using the NO scavenger oxyHb (oxyhaemoglobin; 8–30 μM). OxyHb was prepared by reduction of human metahaemoglobin (Sigma) with 10-fold molar excess of sodium dithionite, followed by dialysis against PBS.

RESULTS

Generation of known amounts of endogenous NO

Increasing concentrations of tetracycline administered to Tet-iNOS 293 cells led to a concentration-dependent expression of iNOS protein, generation of NO and increased concentration of NO_2^- in the medium (Figure 1). The quantity of NO released was dependent on the amount of iNOS that had been expressed. Furthermore, the kinetics of release showed that addition to the cells of the substrate for iNOS (L-arginine, 1 mM) resulted in an immediate (< 10 s) release of NO, which increased rapidly, reached a maximum at about 3 min and then declined progressively (Figure 1B). Under these conditions the release of NO was maintained for at least 60 min and could be completely blocked by oxyHb. The highest concentration of NO obtained at peak time with the greatest expression of iNOS was 1.5 μM (Table 1).

Table 1 shows the generation of NO by induction of the cells with different concentrations of tetracycline and the inhibition of respiration by NO at three different concentrations of O_2.

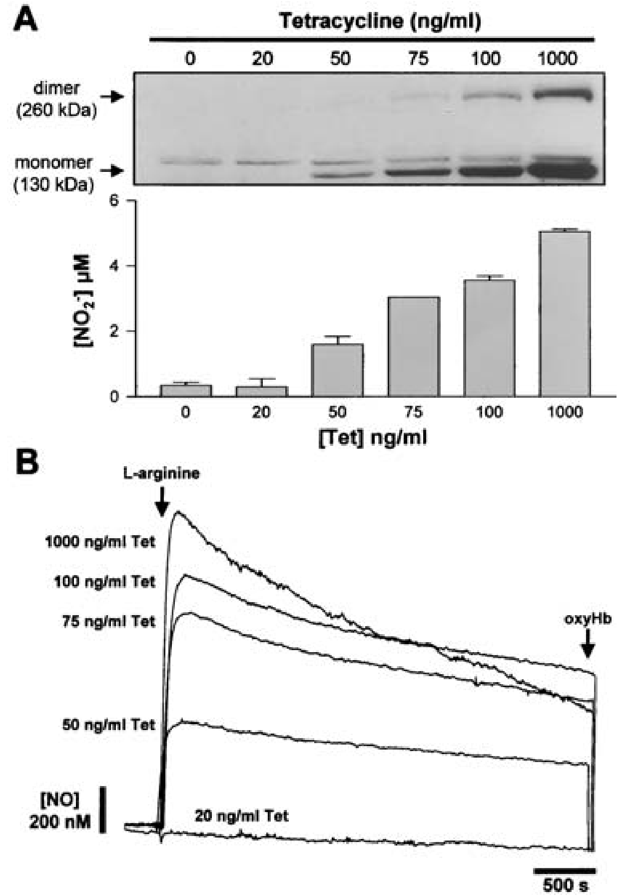

Figure 1 Expression of iNOS and generation of endogenous NO in Tet-iNOS 293 cells

(**A**) Western-blot analysis showing the expression of iNOS protein after overnight induction of the cells with a range of concentrations of tetracycline (Tet) and subsequent accumulation of NO_2^- in the extracellular medium after 1 h of NO production in the presence of L-arginine (1 mM) at 21 % O_2. The blot shows the presence of both the dimer and the monomer of iNOS protein separated in a non-reducing gel (no 2-mercaptoethanol added). (**B**) Online electrochemical detection of authentic NO gas production in cells treated as described above. Production of NO was detected immediately after the addition of L-arginine to a 37 °C chamber containing 1×10^7 cells in suspension, and was monitored for 60 min. Addition of oxyHb decreases levels of NO to basal values. The data presented in each panel are representative of results obtained in three separate experiments.

Table 1 Inhibition of cell respiration produced by different amounts of NO at various O_2 concentrations

Tet-iNOS 293 cells (1×10^7) were treated for 60 min with L-arginine (1 mM) after the overnight induction of iNOS expression with tetracycline at the indicated concentrations, and NO generation and O_2 consumption were measured in parallel. Values are means ± S.D. for at least four identical experiments.

		Inhibition of cell respiration (% of control without NO)		
[Tetracycline] (ng/ml)	[NO] (μM)	13 % O_2 (130 μM)	6 % O_2 (60 μM)	3 % O_2 (30 μM)
1000	1.48 ± 0.07	67 ± 8	85 ± 5	92 ± 4
100	1.15 ± 0.07	57 ± 9	80 ± 6	89 ± 4
75	0.91 ± 0.14	58 ± 7	82 ± 6	91 ± 3
50	0.44 ± 0.10	32 ± 11	54 ± 23	69 ± 20
20	N.D.*	2 ± 3	2 ± 3	6 ± 7

*Not detectable.

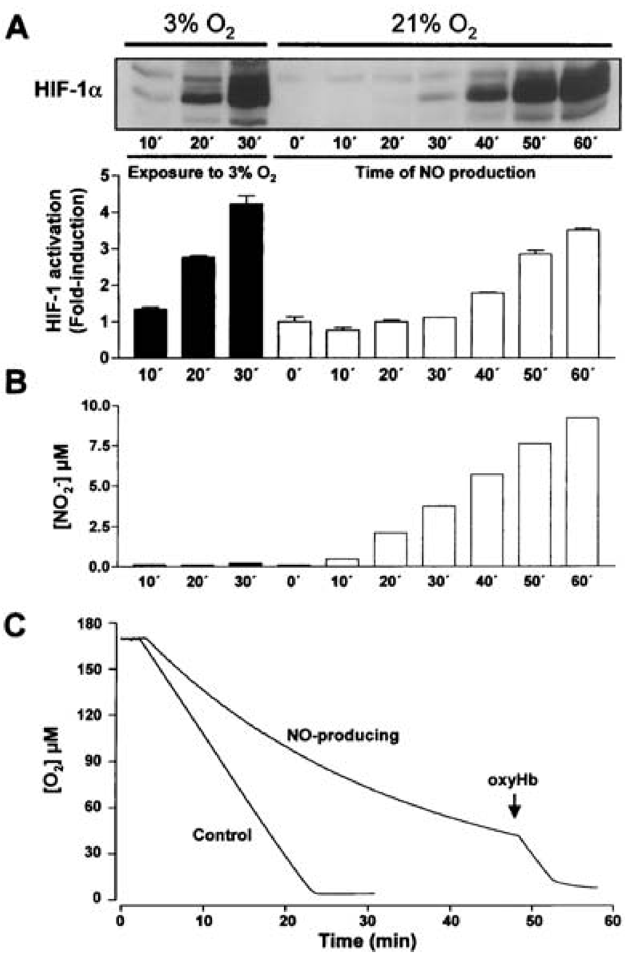

Figure 2 Accumulation of HIF-1α and HIF-1 activation by endogenous NO at 21 % O_2

(**A**) HIF-1α protein accumulation and HIF-1 DNA-binding activity in the nuclear extracts of non-induced Tet-iNOS 293 cells exposed to 3 % O_2 for up to 30 min (black bars) and in cells maximally induced with tetracycline (1000 ng/ml) to produce high levels of NO for increasing times at 21 % O_2 (white bars). (**B**) Time-course of NO_2^- accumulation in the extracellular medium as a consequence of NO production. Black bars show the lack of NO_2^- accumulation in non-induced cells exposed to 3 % O_2. White bars represent NO_2^- concentration after the indicated time in maximally induced cells. (**C**) Online recordings showing the rate of oxygen consumption in cells (1×10^7) treated with tetracycline (1000 ng/ml) overnight and either given L-arginine (1 mM; NO-producing group) or not (control) 60 min prior to the onset of recording. Addition of oxyHb shows the complete reversal of the NO-induced inhibition of O_2 consumption. The data are representative of results obtained in three separate experiments.

Induction of HIF-1 activation by endogenous NO

To determine whether endogenous NO can modulate HIF-1 activity by regulation of HIF-1α accumulation, cells were maximally induced (1000 ng/ml tetracycline) to produce NO for different periods up to 1 h, and nuclear extracts were prepared for immunoblot analysis of HIF-1α protein accumulation and HIF-1 DNA-binding activity. As shown in Figure 2(A), endogenous NO produced in these cells a rapid accumulation of HIF-1α at 21 % O_2. The NO-mediated stabilization of HIF-1α was accompanied by a substantial increase in HIF-1 activation (HIF-1 DNA-binding activity). Both HIF-1α stabilization and HIF-1 binding activity correlated with accumulation of NO_2^- in the extracellular medium (Figure 2B). Under these conditions, HIF-1α accumulation was evident 30 min after initiating NO generation and it continued to accumulate as long as L-arginine was present. Furthermore, the HIF-1α stabilization and the subsequent activation of HIF-1 under these conditions was similar to that obtained by exposure of the cells to 3 % O_2. This amount of NO (> 1 μM) produced a significant inhibition of cell respiration that was dependent on the extracellular O_2 concentration (see Table 1) and was entirely reversible by blocking NO with oxyHb (Figure 2C).

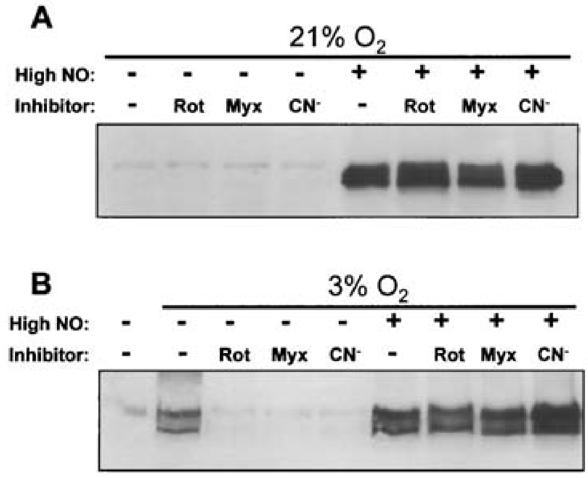

Figure 3 Effect of inhibitors of the respiratory chain on NO- and hypoxic-mediated accumulation of HIF-1α

(**A**) Immunoblot detection of HIF-1α protein in nuclear extracts of Tet-iNOS 293 cells treated with or without rotenone (Rot; 5 μM), myxothiazol (Myx; 1 μM) or cyanide (CN^-; 1 mM) for 1 h, in the presence (+) or absence (−) of high levels of NO (same induction as in Figure 2) at 21 % O_2. (**B**) Similar experiment to that shown in (**A**), but in cells exposed to 3 % O_2. Control cells exposed to 21 % O_2 are shown in lane 1. Blots are representative of results obtained in three separate experiments.

NO-induced accumulation of HIF-1α is a mitochondria-independent process

To investigate further the effects of high concentrations of endogenous NO, cells treated with 1000 ng/ml tetracycline were used to examine the effects of pharmacological inhibitors of the respiratory chain on HIF-1α accumulation. Rotenone (Complex I), myxothiazol (Complex III), and cyanide (Complex IV) were tested at concentrations at which they fully inhibited the respiratory chain in our cells (see below). At 21 % O_2, NO induced a strong stabilization of HIF-1α that was neither mimicked nor altered by any of the other inhibitors tested (Figure 3A). At 3 % O_2, this concentration of NO also stabilized HIF-1α, and this effect was again not reproduced or not obviously affected by the inhibitors (Figure 3B). However, the pharmacological inhibitors themselves decreased the amount of stabilized HIF-1α in cells exposed to 3 % O_2 in the absence of NO (Figure 3B). Since these inhibitors do not imitate the HIF-1α stabilizing action of NO or have any action on it, this suggests that this effect is independent of the respiratory chain.

To confirm that NO-induced HIF-1α stabilization is a mitochondria-independent process, we generated the iNOS-inducible 293 cell line lacking mitochondrial DNA (ρ^0-cells); the rate of O_2 consumption in these cells is greatly reduced (≈80–90 %) and also their ability to generate NO is diminished by 40 % (results not shown). As shown in Figure 4, ρ^0-Tet-iNOS 293 cells did not respond to low O_2 (3 % O_2) with an increase in HIF-1α stabilization. However, they maintained their ability to accumulate HIF-1α when they were treated to generate high concentrations of NO.

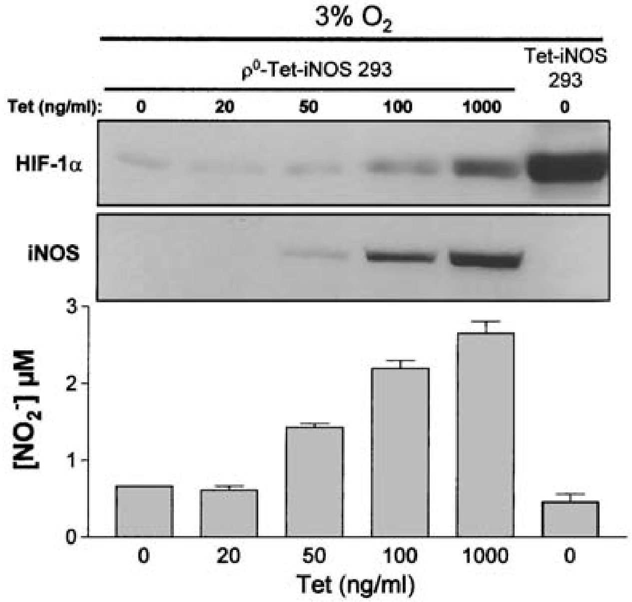

Figure 4 HIF-1α regulation by NO in cells lacking functional mitochondria

Cells lacking a functional electron-transport chain (ρ^0-Tet-iNOS 293) were induced overnight with increasing concentrations of tetracycline (Tet). The cells were then allowed to produce NO for 60 min at 3 % O_2 by addition of L-arginine (1 mM), and HIF-1α protein accumulation and iNOS expression were analysed by Western blotting in nuclear and cytoplasmic extracts respectively. Cells with functional mitochondria (Tet-iNOS 293) were used as a control for the hypoxic response in the absence of NO (no tetracycline). Production of NO was assessed by NO_2^- determination in the extracellular medium. Results shown are representative of those obtained in four separate experiments.

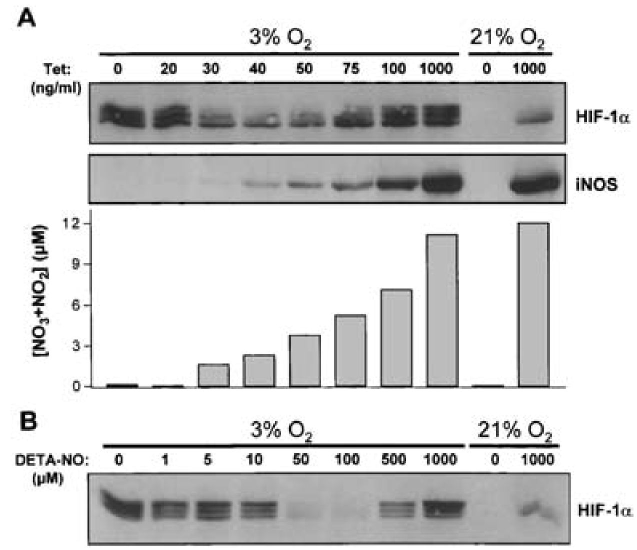

Figure 5 Biphasic effect of NO on HIF-1α accumulation at 3 % O_2

(**A**) Effects of endogenous NO. Cells were induced overnight with increasing concentrations of tetracycline and allowed to produce NO for 60 min by addition of L-arginine (1 mM) at 3 % or were maximally induced with 1000 ng/ml tetracycline (Tet) at 21 % O_2. HIF-1α and iNOS protein levels were detected by Western blot in nuclear and cytoplasmic extracts respectively, and NO production was followed indirectly as the total $NO_2^- + NO_3^-$ concentration in the extracellular medium. Results shown are representative of those obtained in six separate experiments. (**B**) Effects of exogenous NO. Western-blot analysis of HIF-1α in nuclear extracts of cells stimulated for 1 h with increasing concentrations of DETA-NONOate (DETA-NO) at 3 % or at 21 % O_2. The blot shown is representative of those obtained in three separate experiments.

Low concentrations of NO inhibit HIF-1α accumulation at a low O_2 concentration through a mitochondria-dependent mechanism

We then investigated the effects of a range of concentrations of NO on HIF-1α stabilization by a low concentration of O_2 (3 %). Figure 5(A) shows that HIF-1α accumulation was initially reduced by NO in a concentration-dependent manner. However, beyond the initial low concentrations (< 400 nM NO) at which NO produced its maximal inhibitory effect on HIF-1α accumulation, a reversal of this effect was observed and a progressive stabilization occurred which was also concentration-dependent. This stabilizing effect of NO was similar to the stabilization that occurred at 21 % O_2 and was irrespective of the fact that further inhibition of respiration was observed, and was independent of mitochondria (as shown in Figure 4). Stabilization of HIF-1α by high concentrations of NO was also independent of an effect on the soluble guanylate cyclase, since it was not affected by ODQ (1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one) (Sigma), which inhibits the enzyme, nor reproduced by BAY 41-2272 {5-cyclopropyl-2-[1-(2-fluorobenzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine}, which mimics NO in the activation of this enzyme ($n = 3$; results not shown). Importantly, treatment of the cells with the long-lasting NO-donor DETA-NONOate mimicked entirely the biphasic effect on HIF-1α accumulation at 3 % and its accumulation at 21 % O_2 (Figure 5B).

The progressive decrease in accumulation of HIF-1α at 3 % O_2 by the lower concentrations of NO corresponds with the initiation of inhibition of respiration (see Table 1). Furthermore, we had observed that inhibition of the respiratory chain reduced the amount of stabilized HIF-1α (Figure 3B). Because of this we investigated the effects of these inhibitors on HIF-1α accumulation in detail. Interestingly, all inhibitors reduced the hypoxic stabilization of HIF-1α in a concentration-dependent manner (Figure 6) over a range of concentrations at which they inhibit the respiratory chain. The IC_{50} values for the inhibition of cell respiration by the inhibitors were 3 nM for rotenone, 1.6 ng/ml for antimycin A, 24 nM for myxothiazol and 79 μM for cyanide. These data indicate that inhibition of mitochondrial respiration is required to inhibit the accumulation of HIF-1α at 3 % O_2. Interestingly, unlike NO, all the inhibitors completely prevented accumulation of HIF-1α.

Synergism between low O_2 and a high concentration of NO on HIF-1α accumulation

In order to investigate further the nature of the stabilization of HIF-1α by NO and by low O_2 concentrations, we exposed Tet-iNOS 293 cells to increasing concentrations of O_2 in the presence or absence of a high concentration of NO (1000 ng/ml tetracycline). Figure 7 shows that, at 3 and 6 % O_2, there was stabilization of HIF-1α; this effect was greatly enhanced in the presence of NO. At 12 and 21 % O_2, at which HIF-1α was not stabilized, the effect of high NO persisted. Thus low O_2 concentrations and NO stabilize HIF-1α by separate additive mechanisms.

DISCUSSION

NO has been reported to affect the activity of the transcription factor HIF-1 and the stability of its O_2-regulated HIF-1α subunit; however, at present, the literature is controversial and the role of NO in the regulation of the expression of hypoxia-inducible genes remains unclear, as do its implications in cellular O_2 sensing

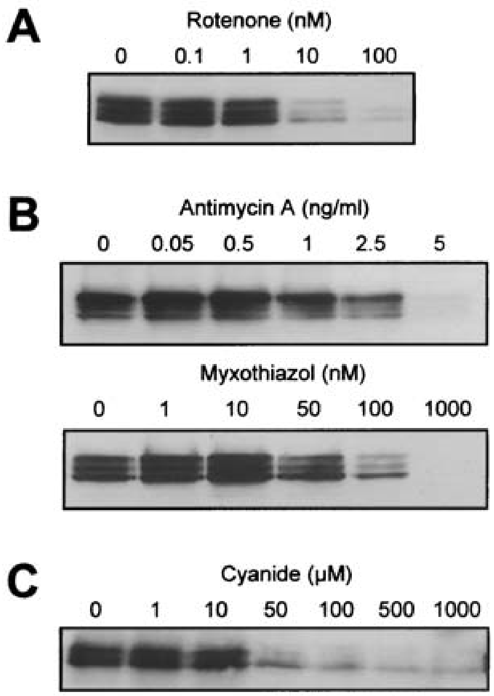

Figure 6 Inhibition of HIF-1α accumulation by various inhibitors of cell respiration at 3 % O_2

Immunoblot analysis of HIF-1α protein in the nuclear extract of Tet-iNOS 293 cells exposed to 3 % O_2 for 60 min in the presence of increasing concentrations of inhibitors of the electron-transport chain: (**A**) rotenone, which inhibits Complex I; (**B**) antimycin A and myxothiazol, which inhibit Complex III; and (**C**) cyanide, which inhibits Complex IV. Cells were not treated with tetracycline. The blots are representative of those obtained in at least three separate experiments.

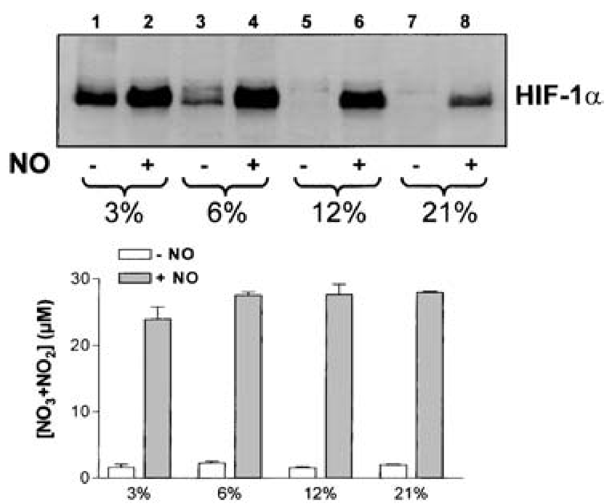

Figure 7 Synergism between hypoxia and a high concentration of NO on HIF-1α accumulation

Immunoblot analysis of HIF-1α in nuclear extracts of Tet-iNOS 293 cells maximally induced with tetracycline (1000 ng/ml) and exposed to increasing concentrations of O_2 for 60 min, in the absence (−) or in the presence (+) of L-arginine (1 mM) to allow generation of a high concentration of NO (≈1 μM). Total $NO_2^- + NO_3^-$ accumulated in the extracellular medium during the 60 min period of incubation in the absence (white bars; no L-arginine added) or presence (grey bars) of NO are shown as an indirect measurement of NO production. The data shown are representative of results obtained in three separate experiments.

[32]. Thus NO, administered exogenously through NO donors or endogenously generated, has been reported either to inhibit [19,25–29] or to enhance [23] HIF-1α accumulation and/or HIF-1 activity in hypoxia (usually ≈1 % O_2). At atmospheric O_2 concentrations (21 % O_2), however, there seems to be agreement that both exogenous and endogenous NO induce HIF-1α stabilization and HIF-1 activity [19–24].

There are a number of possible reasons for such controversial results, the main ones being related to the sources of NO and to the different experimental conditions under which the various studies were carried out. Treatment with the majority of NO donors results in the release of high concentrations of NO with variable kinetics [33]. Furthermore, in some cases, NO donors decompose into molecules with additional biological actions. For instance, sodium nitroprusside (SNP), one of the NO donors most commonly used to study the effect of NO on HIF-1, releases cyanide concomitantly with NO [34] and, as is shown in the present study, cyanide has a strong influence on HIF-1α accumulation in hypoxia. Similarly, GSNO (*S*-nitrosoglutathione) generates oxidized glutathione [35], which can modify the redox state of the cell, and SIN-1 (3-morpholinosydnonimine) generates peroxynitrite ($ONOO^-$) [36], a strong oxidant that results from the interaction between NO and superoxide anion (O_2^-). In addition, the range of concentrations of O_2 over which the studies have been carried out, the different durations of exposure to NO, the diverse methods used to achieve hypoxia and the variety of cell types used, make it difficult to compare the results presented in the large number of studies published.

In order to circumvent some of these problems, we have developed a modification of a cell line that expresses iNOS in a regulated fashion [37], such that we can induce precisely the concentration of NO desired, emulating more accurately the release of endogenous NO under different circumstances. Furthermore, we have studied the regulation of HIF-1α at shorter times of exposure to NO than those normally used by other workers in order to avoid any downstream consequence of long-term inhibition of respiration by NO [38]. Finally, the lowest concentration of O_2 at which we carried out experiments was 3 %, in order to avoid severe hypoxia, which might be confounded with anoxia under certain circumstances (see [18]).

We have found that, at elevated concentrations of O_2 (21 %), nowadays generally, and probably erroneously, termed 'normoxia' (for discussion about this, see [39,40]), activation of iNOS to yield ≈1 μM NO results in significant accumulation of HIF-1α, even when there is enough O_2 for the degradation of HIF-1α by prolyl hydroxylation to take place. This observation confirms previous results [19–24] and strongly suggests that the accumulation of HIF-1α by NO is independent of O_2 concentration. It is also independent of any action on the respiratory chain, since we have shown that it is not affected by compounds that inhibit mitochondrial respiration at different points and occurs in cells lacking a respiratory chain. In addition, it takes place rapidly (≈30 min), suggesting that it is not due to a persistent or irreversible damaging action on the cell. The fact that the accumulation of HIF-1α by NO did not occur with any of the other inhibitors of the respiratory chain, even when used at concentrations above those required to inhibit respiration, further confirms that the stabilizing effect of NO on HIF-1α is not related to an effect on the respiratory chain and is specific to the NO molecule, possibly related to reactions associated with its free-radical nature, such as the formation of $ONOO^-$. If this is the case, then the precise nature of these reactions needs to be clarified, including the possibility that stabilization of HIF-1α is the result of S-nitrosylation of thiol groups in the HIF-1α protein [20,24] or, as has recently been suggested [41], to the direct inactivation of HIF-1α prolyl hydroxylases by NO. A free-radical mechanism has also been proposed for the stabilization of HIF-1α in hypoxia [15,17,18,42,43], but this remains controversial [44–47], and further research will be needed to clarify whether or not reactive oxygen species are involved, either in hypoxia- or NO-induced stabilization of HIF-1α. In this context it is noteworthy that, whereas some workers have demonstrated HIF-1α accumulation in ρ^0-cells in hypoxia [47–49], others, including ourselves, have not [15,17,18]. A simple explanation for this discrepancy in results using ρ^0-cells in hypoxia could be that, without mitochondrial consumption of O_2, there may, in some circumstances, be sufficient O_2 for prolyl hydroxylase activity to persist.

At a lower O_2 concentration (3% O_2), at which HIF-1α accumulates, we found that NO exhibits a potent concentration-dependent inhibition of HIF-1α accumulation at concentrations up to 400 nM. Unlike the stabilizing effect of NO on HIF-1α, the inhibitory effect correlated with inhibition of cell respiration and could be mimicked by all inhibitors of mitochondrial respiration tested, indicating that it is dependent on an action in the respiratory chain. At a certain concentration, however, the effect of NO reverses and HIF-1α starts to accumulate in a way that mimics the stabilizing effect observed with high concentrations of NO at 21% O_2. Interestingly, this explains why, although all other inhibitors destabilize HIF-1α completely, NO does not, since its stabilizing effect, which is not shared by the other inhibitors, already becomes apparent at intermediate concentrations of NO.

In relation to the destabilizing effect of NO on HIF-1α, it has been proposed that this may be due to inhibition of Complex I, dependent on the generation of $ONOO^-$ [29]. However, we have observed decreased accumulation of HIF-1α at low, but not high, concentrations of NO, and our previous results have shown that NO inhibits Complex I at high concentrations of NO [38]. Furthermore, the destabilizing effect of NO was mimicked by other inhibitors of mitochondrial respiration. Finally, the effect occurred rapidly, therefore ruling out any possibility of persistent oxidative damage to any component of the cell. These results indicate that, whatever the mechanism by which NO prevents the accumulation of HIF-1α, it must fulfil some criteria, namely it should take place rapidly, be mitochondria-dependent and occur following inhibition of respiration at any point.

In conclusion, we have demonstrated that, depending on its concentration, NO has two opposite and independent effects. At low concentrations, NO has the ability to destabilize HIF-1α in hypoxia, an effect that is mitochondria-dependent, whereas at high concentrations it stabilizes HIF-1α in a mitochondria-independent manner. It is likely that the paradoxical and overlapping nature of these two actions, as well as the methodological difficulties mentioned above, are responsible for the present controversy in the literature. It is also possible that the effect of NO at low concentrations is part of its physiological regulatory mechanism dependent on inhibition of the cytochrome *c* oxidase (for a review, see [39]). The stabilization of HIF-1α by high concentrations of NO and its synergy with hypoxic stabilization of HIF-1α may play a role in pathological conditions such as inflammation, degeneration and cancer, in which high concentrations of NO, hypoxia and HIF-1α stabilization have been described [50,51].

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REFERENCES

1 Semenza, G. L. (2002) Signal transduction to hypoxia-inducible factor 1. Biochem. Pharmacol. **64**, 993–998

2 Bunn, H. F. and Poyton, R. O. (1996) Oxygen sensing and molecular adaptation to hypoxia. Physiol. Rev. **76**, 839–885

3 Wang, G. L. and Semenza, G. L. (1995) Purification and characterization of hypoxia-inducible factor 1. J. Biol. Chem. **270**, 1230–1237

4 Wang, G. L., Jiang, B. H., Rue, E. A. and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix–loop–helix-PAS heterodimer regulated by cellular O_2 tension. Proc. Natl. Acad. Sci. U.S.A. **92**, 5510–5514

5 Jiang, B. H., Semenza, G. L., Bauer, C. and Marti, H. H. (1996) Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. Am. J. Physiol. **271**, C1172–C1180

6 Jewell, U. R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Gassmann, M. (2001) Induction of HIF-1α in response to hypoxia is instantaneous. FASEB J. **15**, 1312–1314

7 Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V. and Kaelin, W. G. (2000) Ubiquitination of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel-Lindau protein. Nat. Cell Biol. **2**, 423–427

8 Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, Jr, W. G. (2001) HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science **292**, 464–468

9 Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A. V., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al. (2001) Targeting of HIF-α to the von Hippel–Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science **292**, 468–472

10 Moncada, S., Palmer, R. M. J. and Higgs, E. A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. **43**, 109–142

11 Cleeter, M. W., Cooper, J. M., Darley-Usmar, V. M., Moncada, S. and Schapira, A. H. (1994) Reversible inhibition of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. FEBS Lett. **345**, 50–54

12 Brown, G. C. and Cooper, C. E. (1994) Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Lett. **356**, 295–298

13 Brown, G. C. (1999) Nitric oxide and mitochondrial respiration. Biochim. Biophys. Acta **1411**, 351–369

14 Clementi, E., Brown, G. C., Foxwell, N. and Moncada, S. (1999) On the mechanism by which vascular endothelial cells regulate their oxygen consumption. Proc. Natl. Acad. Sci. U.S.A. **96**, 1559–1562

15 Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C. and Schumacker, P. T. (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc. Natl. Acad. Sci. U.S.A. **95**, 11715–11720

16 Duranteau, J., Chandel, N. S., Kulisz, A., Shao, Z. and Schumacker, P. T. (1998) Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. J. Biol. Chem. **273**, 11619–11624

17 Chandel, N. S., McClintock, D. S., Feliciano, C. E., Wood, T. M., Melendez, J. A., Rodriguez, A. M. and Schumacker, P. T. (2000) Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1α during hypoxia: a mechanism of O_2 sensing. J. Biol. Chem. **275**, 25130–25138

18 Schroedl, C., McClintock, D. S., Scott-Budinger, G. R. and Chandel, N. S. (2002) Hypoxic but not anoxic stabilization of HIF-1α requires mitochondrial reactive oxygen species. Am. J. Physiol. Lung Cell. Mol. Physiol. **283**, L922–L931

19 Kimura, H., Weisz, A., Kurashima, Y., Hashimoto, K., Ogura, T., D'Acquisto, F., Addeo, R., Makuuchi, M. and Esumi, H. (2000) Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. Blood **95**, 189–197

20 Palmer, L. A., Gaston, B. and Johns, R. A. (2000) Normoxic stabilization of hypoxia-inducible factor-1 expression and activity: redox-dependent effect of nitrogen oxides. Mol. Pharmacol. **58**, 1197–1203

21 Sandau, K. B., Faus, H. G. and Brüne, B. (2000) Induction of hypoxia-inducible-factor 1 by nitric oxide is mediated via the PI 3K pathway. Biochem. Biophys. Res. Commun. **278**, 263–267

22 Sandau, K. B., Fandrey, J. and Brüne, B. (2001) Accumulation of HIF-1α under the influence of nitric oxide. Blood **97**, 1009–1015

23 Kimura, H., Ogura, T., Kurashima, Y., Weisz, A. and Esumi, H. (2002) Effects of nitric oxide donors on vascular endothelial growth factor gene induction. Biochem. Biophys. Res. Commun. **296**, 976–982
24 Sumbayev, V. V., Budde, A., Zhou, J. and Brüne, B. (2003) HIF-1α protein as a target for S-nitrosation. FEBS Lett. **535**, 106–112
25 Sogawa, K., Numayama-Tsuruta, K., Ema, M., Abe, M., Abe, H. and Fujii-Kuriyama, Y. (1998) Inhibition of hypoxia-inducible factor 1 activity by nitric oxide donors in hypoxia. Proc. Natl. Acad. Sci. U.S.A. **95**, 7368–7373
26 Huang, L. E., Willmore, W. G., Gu, J., Goldberg, M. A. and Bunn, H. F. (1999) Inhibition of hypoxia-inducible factor 1 activation by carbon monoxide and nitric oxide. Implications for oxygen sensing and signaling. J. Biol. Chem. **274**, 9038–9044
27 Yin, J. H., Yang, D. I., Ku, G. and Hsu, C. Y. (2000) iNOS expression inhibits hypoxia-inducible factor-1 activity. Biochem. Biophys. Res. Commun. **279**, 30–34
28 Chun, Y. S., Yeo, E. J., Choi, E., Teng, C. M., Bae, J. M., Kim, M. S. and Park, J. W. (2001) Inhibitory effect of YC-1 on the hypoxic induction of erythropoietin and vascular endothelial growth factor in Hep3B cells. Biochem. Pharmacol. **61**, 947–954
29 Agani, F. H., Puchowicz, M., Chavez, J. C., Pichiule, P. and LaManna, J. (2002) Role of nitric oxide in the regulation of HIF-1α expression during hypoxia. Am. J. Physiol. Cell Physiol. **283**, C178–C186
30 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
31 Archer, S. L., Freude, K. A. and Shultz, P. J. (1995) Effect of graded hypoxia on the induction and function of inducible nitric oxide synthase in rat mesangial cells. Circ. Res. **77**, 21–28
32 Semenza, G. L. (2001) HIF-1 and mechanisms of hypoxia sensing. Curr. Opin. Cell Biol. **13**, 167–171
33 Feelisch, M. (1998) The use of nitric oxide donors in pharmacological studies. Naunyn-Schmiedberg's Arch. Pharmacol. **358**, 113–122
34 Butler, A. R. and Glidewell, C. (1996) Recent chemical studies of sodium nitroprusside relevant to its hypotensive action. Chem. Soc. Rev. **221**, 670–674
35 Williams, D. L. (1996) S-nitrosothiols and role of metal ions in decomposition to nitric oxide. Methods Enzymol. **268**, 299–308
36 Hogg, N., Darley-Usmar, V. M., Wilson, M. T. and Moncada, S. (1992) Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. Biochem. J. **281**, 419–424
37 Xu, W., Liu, L. and Charles, I. G. (2002) Microencapsulated iNOS-expressing cells cause tumor suppression in mice. FASEB J. **16**, 213–215
38 Beltran, B., Orsi, A., Clementi, E. and Moncada, S. (2000) Oxidative stress and S-nitrosylation of proteins in cells. Br. J. Pharmacol. **129**, 953–960
39 Moncada, S. and Erusalimsky, J. D. (2002) Does nitric oxide modulate mitochondrial energy generation and apoptosis? Nat. Rev. Mol. Cell Biol. **3**, 214–220
40 Halliwell, B. (2003) Oxidative stress in cell culture: an under-appreciated problem? FEBS Lett. **540**, 3–6
41 Metzen, E., Zhou, J., Jelkmann, W., Fandrey, J. and Brüne, B. (2003) Nitric oxide impairs normoxic degradation of HIF-1α by inhibition of prolyl hydroxylases. Mol. Biol. Cell **14**, 3470–3481
42 Agani, F. H., Pichiule, P., Chavez, J. C. and LaManna, J. C. (2000) The role of mitochondria in the regulation of hypoxia-inducible factor 1 expression during hypoxia. J. Biol. Chem. **275**, 35863–35867
43 Haddad, J. J. and Land, S. C. (2001) A non-hypoxic, ROS-sensitive pathway mediates TNF-α-dependent regulation of HIF-1α. FEBS Lett. **505**, 269–274
44 Wang, G. L., Jiang, B. H. and Semenza, G. L. (1995) Effect of protein kinase and phosphatase inhibitors on expression of hypoxia-inducible factor 1. Biochem. Biophys. Res. Commun. **212**, 550–556
45 Huang, L. E., Arany, Z., Livingston, D. M. and Bunn, H. F. (1996) Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its α subunit. J. Biol. Chem. **271**, 32253–32259
46 Genius, J. and Fandrey, J. (2000) Nitric oxide affects the production of reactive oxygen species in hepatoma cells: implications for the process of oxygen sensing. Free Radicals Biol. Med. **29**, 515–521
47 Vaux, E. C., Metzen, E., Yeates, K. M. and Ratcliffe, P. J. (2001) Regulation of hypoxia-inducible factor is preserved in the absence of a functioning mitochondrial respiratory chain. Blood **98**, 296–302
48 Srinivas, V., Leshchinsky, I., Sang, N., King, M. P., Minchenko, A. and Caro, J. (2001) Oxygen sensing and HIF-1 activation does not require and active mitochondrial respiratory chain electron-transfer pathway. J. Biol. Chem. **276**, 21995–21998
49 Enomoto, N., Koshikawa, N., Gassmann, M., Hayashi, J.-I. and Takenaga, K. (2002) Hypoxic induction of hypoxia-inducible factor-1α and oxygen-regulated gene expression in mitochondrial DNA-depleted HeLa cells. Biochem. Biophys. Res. Commun. **297**, 346–352
50 Dachs, G. U. and Tozer, G. M. (2000) Hypoxia modulated gene expression: angiogenesis, metastasis and therapeutic exploitation. Eur. J. Cancer **36**, 1649–1660
51 Semenza, G. L. (2001) Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. Trends Mol. Med. **7**, 345–350

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