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# Nitric oxide Produced by Cytochrome c oxidase Helps Stabilize HIF-1α in Hypoxic Mammalian Cells

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## Abstract

The mitochondrial respiratory chain has been reported to play a role in the stabilization of HIF-1α when mammalian cells experience hypoxia, most likely through the generation of free radicals. Although previous studies have suggested the involvement of superoxide catalyzed by complex III more recent studies raise the possibility that nitric oxide (NO) catalyzed by cytochrome c oxidase (Cco/NO), which functions in hypoxic signaling in yeast, may also be involved. Herein, we have found that HEK293 cells, which do not express a NOS isoform, possess Cco/NO activity and that this activity is responsible for an increase in intracellular NO levels when these cells are exposed to hypoxia. By using PTIO, a NO scavenger, we have also found that the increased NO levels in hypoxic HEK293 cells help stabilize HIF-1α. These findings suggest a new mechanism for mitochondrial involvement in hypoxic signaling in mammalian cells.

### Keywords

Cytochrome c oxidase; mitochondria; hypoxia; Hypoxic Inducible Factor; superoxide; nitric oxide

## Introduction

Several studies have implicated the mitochondrial respiratory chain in the induction of hypoxic genes (hypoxic signaling) when eucaryotic cells experience hypoxia and have proposed that increased mitochondrial free radicals produced by hypoxic mitochondria are involved [5;9;20]. As yet, the mitochondrial free radical(s) involved in hypoxic signaling has (have) not been conclusively identified. Early evidence suggested the involvement of superoxide produced by complex III [8;11]. However, it has been demonstrated more recently that hypoxic mitochondria also produce nitric oxide (NO), catalyzed by cytochrome c oxidase (Cco/NO) [6;7], and there is growing evidence that cell and tissue NO levels increase under hypoxic conditions [34]. Moreover, several lines of evidence indicate a role for Cco and NO in hypoxic signaling in yeast cells [6;7;32].

Hypoxic signaling in mammalian cells has been addressed largely by examining HIF-1, a dimeric hypoxic transcription factor, whose alpha subunit, HIF-1α, is susceptible to degradation after prolyl hydroxylation in normoxic cells and which becomes stabilized



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under hypoxia [14]. It is now well established that mitochondria play a role in the hypoxic stabilization of HIF-1α and it has been demonstrated that cytochrome c, which is required for Cco/NO activity, helps stabilize HIF-1α in murine cell lines exposed to hypoxia [26]. Current understanding of the role of NO in HIF-1α stability is still evolving. While several early studies reported that NO destabilizes HIF-1α in hypoxic cells [15;25;33] recent studies have revealed a more complex picture in which NO levels are important. Indeed, it now appears that short term exposure to NO leads to the stabilization of HIF-1α either in normoxic or hypoxic cells while longer term exposure destabilizes HIF-1α [3;4]. Similarly, high doses of NO have been reported to stabilize HIF-1α independently of $O_2$ concentration whereas low levels of NO destabilize HIF-1α under hypoxic conditions [27]. These latter studies have used human osteocarcinoma cells treated with exogenous NO donors (e.g., GSNO) [4] or HEK293 cells with an overexpressed transfected exogenous iNOS isoform or in the presence of exogenous NO donors [27]. Consequently, it is not clear if the intracellular NO levels produced in these studies were in the physiological range or if either cell type experienced an increase in NO from endogenous sources when exposed to hypoxia. Also unclear is whether NO produced from Cco/NO plays a role in the stabilization of HIF-1α in hypoxic cells These questions are addressed in this study with untransfected HEK293 cells.

## Materials and Methods

### Cell Culture

Human embryonic kidney cells, HEK293 (Invitrogen 11625-019: 293F), were grown to >80% confluence in Dulbecco's modified eagle medium (DMEM; Gibco 10569) supplemented with 10% FBS and 1% Penicillin-Streptomycin at 37°C in a humidified atmosphere of air supplemented with 5% $CO_2$. To harvest, confluent HEK293 adherent cells were washed once with PBS pH 7.4 and removed from the culture plate using 0.05% trypsin-EDTA solution. A 10% FBS solution was added and the harvested cells were washed twice with PBS and then suspended in the appropriate buffer.

### Hypoxic Shifts

Confluent HEK293 cells were placed in a Biospherix Pro-ox C- Chamber inside a 37°C incubator with fresh media without FBS supplement. $O_2$ concentration in the Biospherix C-Chamber was controlled using the Pro-ox Model C21 Biospherix regulator that balances air and $N_2$ to achieve the desired $O_2$ concentration while $CO_2$ is regulated independently and held constant at 5% for all experiments. Cells were shifted from atmospheric $O_2$ concentrations to 2%, 1%, or 0% $O_2$ and maintained at the desired $O_2$ concentration for varying lengths of time (typically 3 hours). The media used during the hypoxic shifts were DMEM or Phenol-free DMEM (Gibco 31053) with 100 mg/L Sodium Pyruvate added to match Gibco 10569 DMEM composition.

### Measurement of NO production

**NO Electrode Assay—**HEK293 adherent cells were harvested and then suspended in Electrode Assay Buffer (75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM $KH_2PO_4$, 10 mM Tris, 50 μM EDTA, adjusted to a final pH of 7.0) with 2% n-dodecyl-R-D maltoside to solubilize the cells. The cell lysate was clarified by centrifuged at 3,000 x g, treated with 1X phosphatase inhibitor cocktail (Sigma P5726) to stabilize activity [13], aliquoted for single use, and stored in liquid nitrogen. Samples were assayed immediately after thawing. Cell lysate NO production was measured simultaneously with $O_2$ consumption, with the Innovative Instruments inNOII system using the TMPD/ascorbate method as previously described [2]. Briefly, measurements were performed at 30°C in a closed WPI NOCHM-4 multi-port thermostatic measurement chamber using amiNO-700 and OXY-2 Clark-type

electrodes. Cell lysate (3 mg/mL), superoxide dismutase (5000 units), 10 mM ADP, 10 μM mammalian cytochrome c, 0.5 mM ascorbate, and 0.5 mM TMPD were added to the assay chamber; the $O_2$ concentration was drawn down to 0% by the respiring lysate, and then sodium nitrite ($NaNO_2$) (1 mM) was added to the assay chamber to initiate NO production. The NO scavenger, PTIO (2-Phenyl-4,4,5-tetramethylimidazoline-3-oxide-1-oxyl) was used to validate the NO specificity of the electrode signal. The NO electrode was calibrated with a series of known concentrations of NO (10, 20, 40, 80, 100 nM) generated by mixing 1 μM $NaNO_2$ with 0.1 M $KI_2$ and 0.1M $H_2SO_4$.

**Measurement of NO Levels using DAF-2DA Fluorescent dye—**Confluent HEK293 cells were harvested and resuspended in Reaction Buffer (PBS pH 7.4, 110 mg/L Sodium Pyruvate, 4.5 g/L glucose). The fluorescent probe, DAF-2DA (4,5-Diaminofluorescein diacetate; Cayman Chemical 85165), was added to a concentration of 11 μM [18] and 150 μL (~20 x $10^4$ cells per well) of each fraction was added in replicate to a black 96-well plate. NO related fluorescence signals (Ex/Em 485/538) were measured following 1 hour of normoxia (atmospheric $O_2$, 5% $CO_2$, 37°C) [30] and following up to 3 hours of hypoxia (1% $O_2$, 5% $CO_2$, balanced $N_2$, 37°C). Changes in NO levels were determined by calculating the difference in fluorescence in cells prior to entering the hypoxic chamber and immediately after removal.

## Measurement of HIF

**Nuclear Protein isolation—**Nuclear and cytoplasmic extracts were obtained using a Nuclear Extract Kit (Active Motif 40010) per kit instructions. Briefly, media was aspirated from adherent HEK293 cells and the cells were washed and scraped with ice-cold PBS containing Phosphatase Inhibitor (Active Motif 102146). They were then pelleted, resuspended, and incubated in Hypotonic Buffer (Active Motif 100505). Next, detergent (Active Motif 100512) was added, the sample was vortexed 10 seconds at the highest setting, and the sample was centrifuged for 30 seconds at 14,000 x g. The supernatant (cytoplasmic fraction) was removed and saved. The nuclear pellet was lysed (Lysis Buffer, Active Motif 100534) and the nuclear protein fraction was collected. All protein fractions were stored at −80°C and used within one week of collection.

**Active HIF-1 Analysis—**Active HIF results were obtained using a HIF-1 Activation Assay Kit (Active Motif 47096) per kit instructions. Briefly, the kit contains a 96-well plate with immobilized HRE oligonucleotide (5'-TACGTGCT-3') from the EPO gene. Nuclear extract (20 μg/well) was incubated with Binding Buffer and bound HIF was detected by absorbance at 450 nm with HIF-1α antibody followed by an HRP conjugate antibody.

**Total HIF-1α Analysis—**Nuclear and/or cytoplasmic protein fractions were run on a 4–20% polyacrylamide gel and transferred to a PVDF membrane. The membrane was blocked with 5% Blotto-PBS Solution and washed in PBS with 0.1% Tween-20. The following antibodies were used: anti-HIF-1α (BD-Bioscience 610958 at 1:1000); anti-tubulin (Santa Cruz sc-58666 at 1:2,000 or NeoMarkers at 1:8000); anti-Histone H2B (Cell Signaling 2934S at 1:30,000); and anti-mouse HRP (ThermoFisher PI31430 at 1:20,000). Western Lightning (PerkinElmer NEL105001) was used to visualize bands on Kodak X-OMAT Blue XB Imaging Film. Bands were quantified using a previously described Photoshop Method [29].

## Results and Discussion

### HEK293 cells possess Cco/NO activity

Although Cco/NO activity is enhanced under hypoxic conditions it is operable over a wide range of $O_2$ levels [2;7] and functions at physiological $NO_2^-$ concentrations (~10 μM) [2]. The $NO_2^-$-reductase activity of Cco and other proteins (e.g., xanthine oxidase, and hemoglobin) [6;16;23] are generally assayed at $NO_2^-$ concentrations that exceed 1 mM in order to achieve high signal to noise ratios. So far, Cco/NO activity has been demonstrated in yeast, rat, mouse brain, plant, and human endothelial cell mitochondria as well as with purified Cco [2;6;17;21;32]. In order to determine if HEK293 cells also possess Cco/NO activity we used the previously described TMPD/ascorbate assay [2]. To improve signal level assays are generally done at $O_2$ concentrations below 15 μM $O_2$. As shown in Figure 1A, solubilized HEK293 cell lysates begin producing NO in this assay immediately upon the addition of $NO_2^-$. This signal returns to baseline with the addition of the NO scavenger, PTIO. It is notable that no activity is observed when the phosphatase inhibitor cocktail is omitted from the lysate during cell lysate preparation. As expected, NO synthesis is inhibited by azide but not L-NAME (Figure 1B).

To our knowledge this is the first report which demonstrates that HEK293 cells possess Cco/NO activity. It is likely that the failure of an earlier study to find this activity in HEK293 cells transfected with iNOS [31] was the result the dephosphorylation of Cco during detergent lysis of the cells. Indeed, as shown in Figure 1B, no Cco/NO activity could be measured in the absence of a phosphatase inhibitor. This finding suggests that Cco/NO activity, like Cco/$H_2O$ activity, is regulated by phosphorylation [1;13].

### Endogenous NO levels increase in hypoxic HEK293 cells

HEK293 cells have been used extensively to examine mitochondrial involvement in hypoxic signaling and mammalian gene induction and it has been reported that HEK293 cells transfected with i-NOS release NO into the surrounding medium when exposed to hypoxia [31]. While interesting, it is unlikely that these findings with HEK293 cells transfected with an exogenous i-NOS are relevant to normal HEK293 cells because these cells by themselves do not express any NOS isoforms [10]. Similarly, these previous findings do not address whether intracellular NO levels change in hypoxic untransformed HEK293 cells or whether NO generated from an endogenous source affects HIF-1 stability in these cells when they are exposed to hypoxia. To examine these questions we first determined whether exposure to hypoxia increases intracellular NO levels in HEK293 cells by using an NO sensitive dye, DAF-2DA. Previously, we and others have reported that DAF-2DA is specific for NO and NO oxidation products and does not react to any measureable extent with other reactive oxygen species (e.g., superoxide and hydrogen peroxide) *in vivo* under our assay conditions [22;35]. As shown in Figure 2A, HEK293 cells experience an increase in NO-related signals upon exposure to hypoxia. This increase begins after about 30 minutes of exposure to hypoxia, plateaus after about 2 hours, and is unaffected by added L-NAME, a general NOS inhibitor. Moreover, the level of NO produced is elevated in a dose-dependent manner by added exogenous $NO_2^-$ (Figure 2B) and is reduced by cyanide (Figure 2C), which inhibits Cco/NO activity [2]. Oxypurinol, a xanthine oxidase inhibitor, has no effect on the increase in hypoxic NO production (Figure 2D). Together, these findings indicate that hypoxia increases endogenous NO levels in HEK293 cells, which do not express a NOS isoform, and suggest that new NO synthesis, catalyzed by Cco/NO, is responsible for the increase. These results also do not support the view that the increase in intracellular NO observed merely reflects the release on NO from Cco, rather than new NO synthesis by Cco/NO, because cyanide inhibits the increase in intracellular NO levels. Indeed, if Cco bound NO is merely

released from Cco under hypoxia then NO levels should increase in the presence of cyanide, as the enzyme becomes reduced [31].

### HIF-1 expression levels in hypoxic cells are affected by Cco/NO inhibitors and endogenously produced NO

Under hypoxia NO has been reported previously to both increase and decrease HIF-1α stability. Early studies suggested that this dual effect of NO was concentration dependent where high concentrations of NO stabilize HIF-1α irrespective of $O_2$ concentration and low levels destabilize HIF-1α in hypoxic cells [27]. More recent studies have reported that acute exposure to NO stabilizes HIF-1α while longer term exposure to NO destabilizes HIF-1α [3;4]. Because these latter studies made use of an exogenous NO donor it appears that the two phases are a reflection of the amount of NO released to the cells and that the latter phase is a reflection of the reduced release kinetics from the NO donor. Consequently, it is not clear how these results translate to the changes in endogenous NO synthesis during hypoxia that are reported in this study.

In order to ask if the increase in endogenously produced NO in hypoxic cells affects HIF-1 levels we first looked at the expression of HIF in the HEK293 cell line used above. Cells grown in an air- 5% $CO_2$ mixture were exposed to reduced $O_2$ levels of 2% $O_2$, 1% $O_2$, or 0% $O_2$ with 5% $CO_2$ and balanced $N_2$ for 3 hours. Then, both active HIF-1 levels and HIF-1α subunit levels were determined. From Figure 3A it is clear that this cell line expressed HIF activity at nominal levels under aerobic conditions and that this activity increased under hypoxic (1 or 2% $O_2$) or anoxic conditions. As expected, immunoblot analysis of both cytosolic and nuclear fractions revealed the complete absence of HIF-1α in the cytosol at all $O_2$ concentrations, indicating that the increase in HIF-1 activity coincides with an increase in nuclear but not cytosolic HIF-1α levels (Figure 3B,C).

Having demonstrated that HEK293 cells have Cco/NO activity, which is responsible for the increase in endogenous NO levels in hypoxic cells, we next asked if this Cco/NO affects HIF-1 and HIF-1α levels under hypoxia. This was done first by examining whether azide, a Cco/NO inhibitor, affects HIF-1 levels in hypoxic cells. From Figure 4A it is clear that active HIF-1 levels do not increase in cells treated with azide, either in the presence or absence of L-NAME. The finding that azide affects the hypoxic stabilization of HIF-1 supports and extends a previous observation that azide inhibits the accumulation of HIF-1α in hypoxic HEK293 cells [12]. We next asked if the increased NO levels generated by Cco/NO in hypoxic cells affect HIF-1 or HIF-1α levels. This was done by using the cell permeable NO scavenger, PTIO, to deplete intracellular NO levels in HEK293 cells exposed to hypoxia (1% $O_2$). From Figure 4B it is clear that added PTIO diminishes active HIF levels in a concentration dependent manner. This decrease in active HIF levels parallels a decrease in HIF-1α levels in these cells (Figure 4C).

Together, these findings support the conclusion that NO produced by Cco/NO in hypoxic HEK293 cells plays a role in the stabilization of HIF-1α. Previous efforts to understand the effects of NO on HIF-1α stability under hypoxia have focused largely on the effects of NO on the prolyl hydroxylases (PHDs) that hydroxylate HIF-1α. They have revealed that exposing cells to exogenous NO-donors reduces HIF-1α hydroxylation [28] and that NO has an inhibitory effects on PHDs 1–3 *in vitro*. It is not yet clear how NO inhibits PHDs but it has been proposed to compete with $O_2$ for binding to the ferrous iron at the active site on these enzymes [28]. It is not yet known if NO also functions in the nitrosylation or tyrosine nitration of HIF-1 protein side chains in hypoxic cells or if NO exerts its effect on HIF-1α indirectly by affecting proteins with which it interacts or which function in other HIF-1α posttranslational modifications. In this latter regard it is interesting that the hypoxic

deacetylation of HIF-1α is catalyzed by SIRT1 [24] and that NO modulates the deacetylase activity of SIRT1 [19].

### Mitochondrial involvement in hypoxic signaling

It is clear that the identification of mitochondria as the intracellular source of NO generated in hypoxic cells is crucial to understanding the role of NO in HIF-1α stabilization and hypoxic signaling. Indeed, the findings reported here support a new model for mitochondrial involvement in hypoxic signaling [32]. This model takes into account the finding that hypoxic mitochondria produce both superoxide and NO and proposes that the predominant mitochondrially-generated signals in hypoxic cells are either peroxynitrite (ONOO-) or NO. Because these free radicals are highly reactive and unstable it is unlikely that they diffuse over the great distances that separate the mitochondrial reticulum and the nucleus. Indeed, it is more likely that these free radicals initiate signaling via S-nitrosylation, or tyrosine nitration and that these modifications activate the signaling pathway between the mitochondrion and HIF-1 in the nucleus. Attempts to identify these proteins are currently underway.

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## Highlights

- HEK293 mammalian cells, which lack a NOS isoform, make nitric oxide under hypoxia
- Cytochrome c oxidase produces nitric oxide in hypoxic HEK293 cells
- Nitric oxide from cytochrome c oxidase stabilizes HIF-1α in hypoxic cells
- A new mode of mitochondrial involvement in hypoxic signaling in mammals is proposed

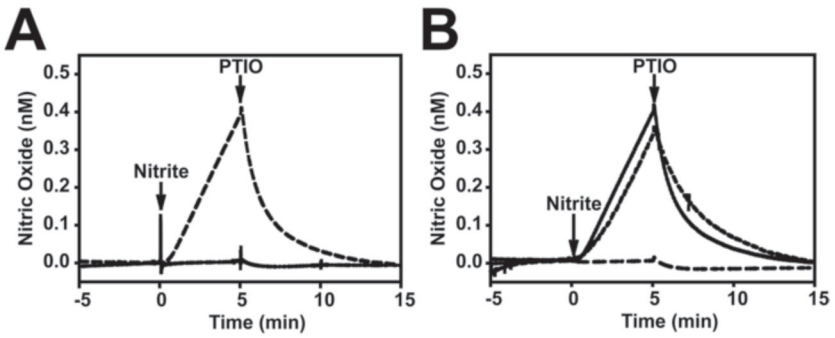


**Figure 1. HEK293 cells have Cco/NO activity**

(A) HEK293 cell lysates were prepared either with (dashed line) or without (solid line) phosphatase inhibitor. NO production was measured under anoxic conditions with the Innovative Instruments inNOII system as described in Materials and Methods using 3 mg/mL cell lysate. 1 mM $NaNO_2$ was added as indicated. The NO scavenger, PTIO, was added at the time indicated to confirm the specificity of the observed signal. (B) The effects of sodium azide (5 mM; long dashed line) or L-NAME (1 mM; short dashed line) on Cco/NO activity in phosphatase inhibitor- treated HEK293 cell lysates were measured as described in Panel (A).

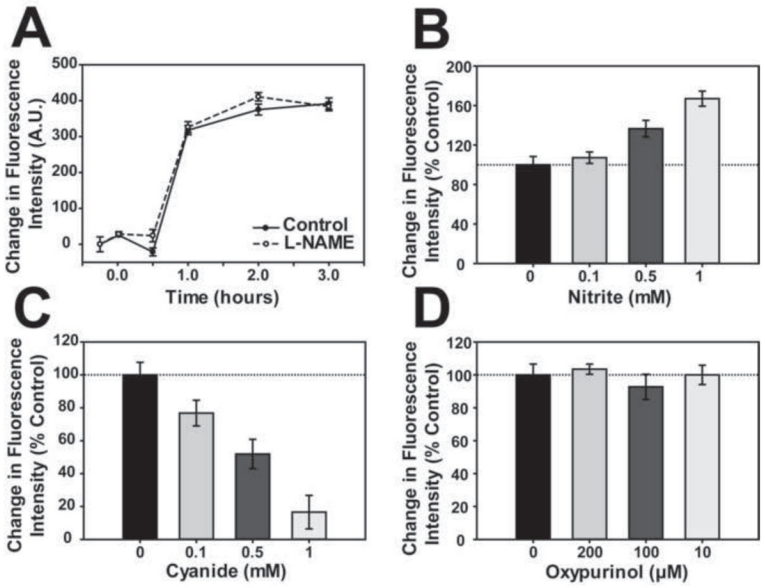


**Figure 2. Effect of hypoxia on NO levels in HEK293 cells**

(A) NO levels were measured in HEK293 cells at different times after exposure to hypoxia (1% $O_2$) using the NO-scavenging fluorescent probe, DAF-2DA, in the presence (open circles; solid line) or absence (closed circles; dashed line) of 1 mM L-NAME. (B-D) NO levels were measured in HEK293 cells using DAF-2DA as described in above, following 3 hours of hypoxic incubation (1% $O_2$) in the presence of the indicated concentrations of (B) $NaNO_2$, (C) potassium cyanide, or (D) oxypurinol. For all assays, the change in fluorescence intensity is represented as the percent of signal compared to the untreated hypoxic control.

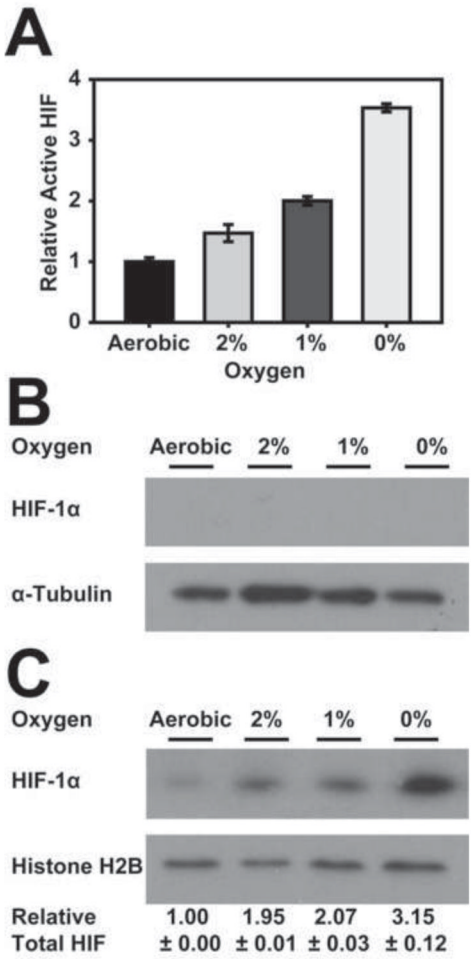


**Figure 3. HIF-1 expression levels increase as $O_2$ concentrations decrease**

HEK293 cells were shifted to 0%, 1%, or 2% $O_2$ for 3 hours, as described in Materials and Methods or were left at atmospheric $O_2$ levels. An Active Motif® nuclear extraction kit was used to produce cytosolic and nuclear extracts. (A) The nuclear extract was examined using an Active Motif® HIF-1 ELISA assay for active HIF-1 levels. (B) The cytosolic fraction was analyzed by immunoblotting using anti-HIF-1α and anti-α-Tubulin antibodies. (C) The nuclear fraction was analyzed by immunoblotting using anti-HIF-1α and anti-Histone H2B antibodies. Quantitation was as described in Materials and Methods.

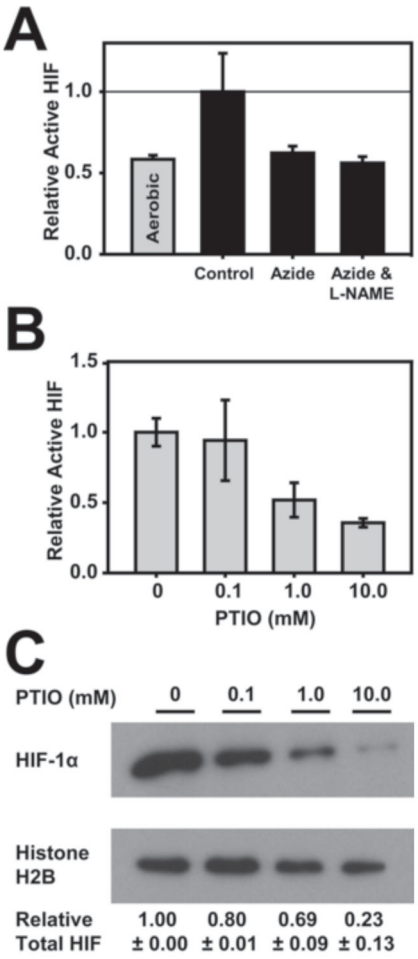


**Figure 4. Azide and PTIO prevent the accumulation of HIF-1 in hypoxic cells**

HEK293 cells were shifted to 1% $O_2$ for 3 hours as described in Materials and Methods or were left at atmospheric $O_2$ levels (A) in the presence or absence sodium azide (5 mM) and/or L-NAME (1 mM), as indicated or (B) in the presence of PTIO at the indicated concentrations. Active HIF-1 levels were measured as described in the legend to Figure 3. (C) Nuclear HIF-1α levels in the PTIO samples were evaluated by immunoblot analysis using anti-HIF-1α and anti-Histone H2B antibodies. Quantitation was as described in Materials and Methods.