Article

A targeted antioxidant reveals the importance of mitochondrial reactive oxygen species in the hypoxic signaling of HIF-1α

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Abstract Exposure to limiting oxygen in cells and tissues induces the stabilization and transcriptional activation of the hypoxia-inducible factor 1 alpha (HIF-1α) protein, a key regulator of the hypoxic response. Reactive oxygen species (ROS) generation has been implicated in the stabilization of HIF-1α during this response, but this is still a matter of some debate. In this study we utilize a mitochondria-targeted antioxidant, mitoquinone (MitoQ), and examine its effects on the hypoxic stabilization of HIF-1α. Our results show that under conditions of reduced oxygen (3% O2), MitoQ ablated the hypoxic induction of ROS generation and destabilized HIF-1α protein. This in turn led to an abrogation of HIF-1α transcriptional activity. Normoxic stabilization of HIF-1α, on the other hand, was unchanged in the presence of MitoQ suggesting that ROS were not involved. This study strongly suggests that mitochondrial ROS contribute to the hypoxic stabilization of HIF-1α.

Keywords: Hypoxia; Antioxidant; Reactive oxygen species; Hypoxia-inducible factor-1α; Mitochondrion

1. Introduction

The transcription factor HIF-1 (hypoxia inducible factor-1) occupies a central position in oxygen homeostasis and is considered to be a key regulator in cellular responses to reduced oxygen concentrations. The practicalities of the HIF system have been the subject of regular reviews (for recent additions see [1,2]). In brief, HIF-1 is a heterodimer composed of an oxygen sensitive α subunit and a constitutively active β subunit [3]. In normoxic conditions for cell culture (ca. 21% oxygen) the HIF-1α protein subunit is targeted for ubiquitination and subsequent proteasomal degradation following hydroxylation of proline [4,5] and asparagine residues [6]. Under conditions of reduced oxygen (from 0 to 5%) this post-translational degradation is inhibited, possibly due to reduced binding of oxygen to HIF-prolyl hydroxylase (PHD) enzymes that require oxygen for their function. Thus hypoxic conditions lead to a stabilization of HIF-1α protein which can then transactivate downstream HIF-target genes. Recently, it has been described that PHD gene products, themselves, are subject to hypoxic regulation via HIF-1 [7] and thus contribute to a growing understanding of this central pathway [8].

There is accumulating evidence for the importance of reactive oxygen species (ROS) as secondary messengers in a variety of cellular situations [9]. Redox signaling of HIF-1α is an emotive subject in the literature [10] and the argument for ROS signaling to this protein is not entirely clear. Even more so, the contribution of mitochondrial-derived ROS is debated [11,12] with evidence for [13–15] and against [16] a mitochondrial-ROS signal. Often these contradictory reports result from studies utilizing different broad-range antioxidants and also the employment of rho (0) cell lines devoid of mitochondrial DNA [17]. These rho (0) cells which have been depleted of mitochondrial DNA by long term incubation with the teratogen ethidium bromide lack a functioning electron transport chain [18], the major site for ROS production. It is clear, however, that loss of mitochondrial DNA can change the expression of a number of genes [19] and influence cross talk between mitochondria and the nucleus [20]. This, in turn, may have ROS-independent effects on HIF-1α expression [16] and also oxygen consumption [15]. Two commonly used generic antioxidants for the study of HIF-1 stabilization are N-acetyl-L-cysteine (NAC) and pyrrolidine carbonate (PDTC). NAC, apart from its direct role as an antioxidant, can raise intracellular concentrations of cysteine and thus increase available glutathione (GSH). GSH can then detoxify ROS via the glutathione-peroxidase coupled reaction. NAC is, however, a rather poor scavenger of superoxide [21] and the reaction between NAC and hydrogen peroxide is slow and may not be significant. Also, at the rather high concentrations of NAC normally used in studies (in the millimolar range) NAC has the potential to inhibit multiple MAP kinase pathways [22]. PDTC possesses both antioxidant and pro-oxidant characteristics [23], and can directly increase GSH oxidation, thus

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Abbreviations: HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase; PHD3, prolyl hydroxylase 3; RT-PCR, reverse-transcription PCR; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; HRE, hypoxia responsive element; pVHL, von Hippel-Lindau protein; MitoQ, mito-quinone
lowering the GSH/GSSG ratio [24]. PDTC, however, is also reported to have several distinct targets in cells [22] and any PDTC-mediated effect on HIF-1α expression may not be directly linked to its role as an antioxidant.

Recently, a ubiquinone derivative, mitouqubiquinone (MitoQ), [25] has been developed in which ubiquinone is covalently linked to a triphenylphosphonium (TPP) cation, allowing a several-hundred fold accumulation within mitochondria. The active antioxidant form of MitoQ is the reduced ubiquinol form and this is regenerated by the electron transport chain, and can selectively block mitochondrial oxidative damage by detoxifying ROS [26–29]. This selective accumulation within mitochondria thus makes MitoQ a useful tool to study mitochondrial-derived ROS and their potential signaling properties during hypoxia.

In an attempt to resolve the issue of whether or not mitochondrial-derived ROS are involved in hypoxic signaling of HIF-1α, we took advantage of this selective antioxidant, and here we describe that indeed ROS are required for the post-transcriptional stabilization of HIF-1α during hypoxic incubation of cells. We also demonstrate that the downstream activation of HIF-1α target genes is also perturbed in the presence of MitoQ. Normoxic stabilization of HIF-1α, on the other hand, does not appear to require ROS. To our knowledge this is the first presentation on the use of a selective antioxidant in studying the hypoxia-response pathway, and underscores the contention that mitochondrial-derived ROS are important signals during the cellular response to limiting oxygen environments.

2. Materials and methods

2.1. Reagents and cell culture

Culture media and fetal bovine serum were from Invitrogen Life Technologies (Carlsbad, CA). MitoQ was synthesized as described [25]. The proteosomal inhibitor MG132 was purchased from Calbiochem–Novabiochem Corp. (La Jolla, CA). All remaining chemicals, unless otherwise noted, were from Sigma Chemical Co. (St. Louis, MO). Specific cell treatment conditions are cited in the figure legends. A human hepatoblastoma cell line Hep2B (ATCC HB-8064) was routinely maintained in MEM supplemented with 10% fetal bovine serum, and 2 mM sodium pyruvate (1 mM, at 37°C in a humidified environment of 5% CO2–95% air. The fibrosarcoma cell line HT1080 (ATCC CCL-121) and the pVHL deficient renal clear cell carcinoma cell lines 786-0 were cultured in DMEM supplemented with 10% fetal bovine serum and 2 mM sodium pyruvate. The 786-0 cells also received G418 at a concentration of 500 μg/ml. The fibrosarcoma cell line HT1080 (ATCC CCL-121) and the pVHL deficient renal clear cell carcinoma cell lines 786-0 were cultured in DMEM supplemented with 10% fetal bovine serum and 2 mM sodium pyruvate. The 786-0 cells were lysed and cleared by centrifugation. Protein concentrations were determined with the Pierce BCA protein assay kit (Pierce Chemicals, Boulder, CO) using BSA to generate a standard curve. An equal amount of each protein sample (30 μg) was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and probed with anti-HIF-1α monoclonal Ab (BD Transduction Labs, Lexington, KY), anti-PHD3 polyclonal Ab (Novus Biologicals, Littleton, CO), and anti-tubulin monoclonal Ab (Sigma). Peroxidase-coupled secondary antibodies were detected by the enhanced Chemiluminescence (ECL) method from Amersham (Amersham Pharmacia Biotech, UK).

2.2. RNA extraction and RT-PCR

RNA extraction and cDNA synthesis were performed using thepeqDNA® kit (peqLab, UK). The reaction was carried out in a volume of 20 μl containing 1 μg of total RNA, 0.5 μM of each primer and 200 pmol of dNTPs. After amplification, the PCR products were separated on a 1% agarose gel and stained with ethidium bromide. The primers used were: for human PHD3 5’-GACAGGTATCCTCTGGTGCAT-3’ and 5’-TCAAGGTGTCGAGG-3’ (GenBank NM_022073); for human HIF-1α 5’-

2.3. Western blotting analysis

Preparation of total protein extracts was carried out as described [32]. Briefly, after treatments, cells were quickly washed in ice-cold PBS supplemented with phosphatase inhibitors (10 mM NaF, 10 mM β-glycerophosphate, 10 mM p-nitrophosphophosphate, and 1 mM NaVO4) and a protease-inhibitor cocktail (Roche Diagnostics). Cells were lysed and cleared by centrifugation. Protein concentrations were determined with the Pierce BCA protein assay kit (Pierce Chemicals, Boulder, CO) using BSA to generate a standard curve. An equal amount of each protein sample (30 μg) was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and probed with anti-HIF-1α monoclonal Ab (BD Transduction Labs, Lexington, KY), anti-PHD3 polyclonal Ab (Novus Biologicals, Littleton, CO), and anti-tubulin monoclonal Ab (Sigma). Peroxidase-coupled secondary antibodies were detected by the enhanced Chemiluminescence (ECL) method from Amersham (Amersham Pharmacia Biotech, UK).

2.4. Reporter gene assays

An HRE vector (pHRE-1), containing three copies of a synthetic oligonucleotide of the minimal PGK-1 HRE cloned upstream of an SV40 minimal promoter (in the pG3L vector, Promega) was a kind gift from Dr. Kaye Williams (University of Manchester, UK). Transient co-transfection assays of this reporter (200 ng) plus pHTR-RL (containing a thymidine kinase promoter upstream of Renilla reiformis, 75 ng) was carried out in 24-well plates containing 1 × 105 cells. Twenty four hours after transfection, cells were placed in hypoxia for 16 h. After treatment, the cells were harvested and luminescence was measured in a 96-well luminometer (Thermo Labsystems) using the STOP AND GLOW™ reporter assay system (Promega, Madison, WI).

2.5. Measurement of reactive oxygen species

For quantitative assessment of cellular ROS, more specifically hydrogen peroxide (H2O2), we used the Amplex Red® Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes, Eugene, OR), which uses horseradish peroxidase to react with H2O2 in a 1:1 stoichiometry to produce resorufin. We measured resorufin levels with a Multiskan plate reader (Thermo Labsystems). A standard curve was generated using H2O2, and results were expressed as nanomoles H2O2 per 5 × 104 cells.

2.6. Oxygen consumption

Endogenous respiration in whole cells was measured as described previously [32]. Briefly, exponentially growing cells were cultured in the presence or absence of 1 μM MitoQ for 16 h, washed with PBS and collected by trypsinization. After resuspension in Hanks balanced salts solution (HBSS) the cells (3 × 106 cells/ml) were transferred to a chamber equipped with an Oxygen electrode (Rank Bros., Bottisham, UK). Oxygen consumption rates were measured over a 30 min period and mitochondrial specificity was confirmed by addition of 1 mM KCN.

3. Results and discussion

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3. Results and discussion

3.1. MitoQ destabilizes HIF-1α protein during hypoxia

It is well established that incubation of cells at reduced oxygen concentrations leads to an increased level of HIF-1α protein due to decreased turnover, and consequently to an upregulation of PHD3, an HIF-1α target gene (Fig. 1A). To determine the effects of MitoQ on HIF-1α expression, we first conducted a dose–response assay to examine the ability of
Hep3B and HT1080 cell lines to express HIF-1α protein during hypoxia in the presence of the antioxidant. Increasing concentrations of MitoQ from 1 to 5 μM dose-dependently decreased the steady state level of HIF-1α during incubation at 3% oxygen for 4 h, in both the Hep3B (Fig. 1B) and HT1080 (Fig. 1C) cell lines, but was without effect on α-tubulin levels. Protein levels of PHD3 were also decreased in a concentration-dependent fashion (Fig. 1B). Importantly, control experiments using the lipophilic cation linker TPP, responsible for targeting MitoQ to mitochondria, up to concentrations of 5 μM had no effect on HIF-1α expression during hypoxia, thus excluding a non-specific effect of the TPP group (Fig. 1D). Further dose–response assays showed that the optimal MitoQ concentration in these experiments was 1 μM (data not shown). As MitoQ is specifically taken up by energized mitochondria, its accumulation in these organelles increases with time until a steady-state distribution is reached. We therefore next determined if pre-incubation with this compound, followed by a medium change before exposure to hypoxia, would mimic the results obtained when the antioxidant was added during the hypoxic episode. Cells were thus “loaded” with either 1 or 2 μM MitoQ for 6 h, followed by a medium change, and then exposed to 3% oxygen for 3 h. Cell death during this period, as assessed using the XTT assay was less than 10% (data not shown). As revealed in Fig. 1E the hypoxic expression of HIF-1α was blocked after pre-treatment with MitoQ, thus showing that the antioxidant was well retained within the cells.

Next, to test whether the inhibition of hypoxia-dependent HIF-1α expression with MitoQ occurred at the level of protein stabilization or protein synthesis, we employed the proteosomal inhibitor MG132. In the presence of MG132 the continuously synthesized HIF-1α accumulates during normoxia as proteosomal destruction is halted. Thus, if MitoQ was acting at the post-synthesis level it would have no effect on the accumulation of HIF-1α. The results shown in Fig. 1F demonstrate that Hep3B cells incubated with MG132 accumulate HIF-1α and the addition of MitoQ has no effect in this process, revealing that MitoQ does not act at the level of protein synthesis. Finally, to further confirm this finding, we used the von Hippel-Lindau E3 ubiquitin ligase (pVHL) negative cell line 786-0, in which HIF-1α is stabilized in normoxia [33]. As shown in Fig. 1G, addition of MitoQ to the culture medium has no effect on the expression of HIF-1α protein in this cell line. Overall, these results indicate that loss of HIF-1α during hypoxia, in the presence of MitoQ, is not due to a reduction in protein synthesis, but rather point towards the destabilization of the protein through disruption of mitochondrial-ROS.

3.2. Loss of HIF-1α transcriptional activity with MitoQ
Hypoxic stabilization of HIF-1α results in the activation of a host of downstream hypoxia-responsive genes [2]. To ascertain whether these genes were downregulated in the presence of MitoQ, we repeated the dose–response experiment described above, in the Hep3B cell line, and collected cells for RNA analysis. As seen in Fig. 2A, there was no change in HIF-1α mRNA levels during incubation with MitoQ, suggesting that the loss of HIF-1α protein was also not due to decreased transcription of the gene. On the other hand, both known target genes for HIF-1α, PHD3 and Aldolase, were downregulated, in a concentration-dependent manner, upon incubation with the antioxidant (Fig. 2A) with no effect on actin mRNA. To confirm that the decrease in transcriptional activation of the HIF-1 target genes was indeed the result of the absence of HIF-1, we used a luciferase reporter vector utilizing a hypoxia responsive element (HRE). Transient transfections of this reporter construct in Hep3B cells resulted in an increase in luciferase protein upon incubation at 3% oxygen (Fig. 2B) compared to normoxia, consistent with hypoxic activity. The addition of 1 μM MitoQ during this hypoxic episode decreased luciferase activity to basal levels, thus supporting the contention that HIF-1α mediated transcription is absent after blockade of ROS.

3.3. MitoQ does not impair respiration, but decreases ROS in hypoxia
Recently, it has been described that loss of mitochondrial electron transport chain (ETC) activity can result in the degradation of HIF-1α during incubation of cells at reduced oxygen
levels [32]. The probable mechanism is the redistribution of intracellular oxygen after “disruption” of the ETC by mitochondrial poisons or nitric oxide (NO), which in turn might lead to oxygen availability for PHD activity [34]. As MitoQ is targeted to the mitochondrion we wanted to assess whether oxidative phosphorylation was perturbed in this situation. To this end we measured oxygen consumption rates in whole cells which had been pre-incubated for 16 h with 1 lM MitoQ. As shown in Fig. 3A, there was no change in oxygen consumption under these conditions, suggesting that MitoQ does not obstruct the ETC. To address the potential involvement of NO, we measured oxygen consumption in Hep3B cells in the presence of the NO-synthase inhibitor L-NAME (N-nitro-L-arginine methyl ester). Our results showed that oxygen consumption in Hep3B cells was identical in the presence or absence of 500 lM L-NAME (Fig. 3B), suggesting that NO was not participating in this system. Lastly, using a quantitative enzymatic assay for ROS [35] we measured the cumulative cellular levels of H2O2 during incubation of cells at 3% oxygen for 4 h (a time chosen to match HIF-1α stabilization studies) with or without the addition of MitoQ. In line with a previous report [27], addition of MitoQ blocked the hypoxic increase of ROS in a dose-dependent manner (Fig. 3C). Fluorescence staining of cells in similar experiments, using the ROS-reactive dye 2′,7′-dichlorodihydrofluorescein diacetate revealed similar results (data not shown).

Taken together, the results presented here are consistent with the hypothesis that hypoxia increases the amount of mitochondrial-generated ROS, which in turns leads to a stabilization of HIF-1α protein and activation of the hypoxia response pathway. Scavenging of ROS, at the source, with a mitochondrial-targeted antioxidant leads to the suppression of HIF-1α stabilization, a loss in transcriptional activity, and an arrest of the hypoxia pathway.

Fig. 2. Effect of MitoQ on HIF-1α and target gene expression during hypoxia. (A) Hep3B cells were incubated in the presence of vehicle or MitoQ (1–5 μM) for 6 h at 3% O2 before collection and RNA isolation. mRNA species were quantified by RT-PCR and DNA products were visualized with ethidium bromide on 2% agarose gels. This experiment was performed three times and representative data are shown. (B) Analysis of HIF-1α mediated reporter gene transcription. Hep3B cells were co-transfected with pHTK-RL and pHRE-1 reporter vectors. Twenty-four hours after transfection, cells were incubated for a further 16 h at 21% or 3% O2 in the presence of vehicle or 1 μM MitoQ. The ratio of firefly to Renilla luciferase expression was then determined. Results are representative of three independent experiments performed in triplicate.

Fig. 3. MitoQ does not impede oxidative phosphorylation but removes mitochondrial ROS. (A) Whole cell oxygen consumption in Hep3B cells. Cells (3 x 10⁶/ml) were re-suspended in HBSS after a pre-incubation period of 16 h in medium with the addition of either 1 μM MitoQ or vehicle. Oxygen levels were monitored over a 30 min period. Graph shown is representative from three different experiments. Oxygen consumption rates were reported as 2.008 ± 0.06 and 1.97 ± 0.04 (nmol/min/10⁶ cells) for vehicle and MitoQ respectively. (B) Oxygen consumption was measured in Hep3B cells in the presence or absence of 500 μM L-NAME (added 30 min before start of the experiment). The result shown is representative from duplicate experiments. (C) Cellular ROS, measured by H2O2, during a 4 h incubation at 21% or 3% oxygen, plus or minus the addition of 0.5 or 1 lM MitoQ. MitoQ was added 15 min before the start of the experiment.

3.4. Normoxic stabilization of HIF-1α is unaffected by MitoQ

Having shown that MitoQ could blockade the hypoxic stabilization of HIF-1α, it was of interest to examine its effects on non-hypoxic HIF-1α stabilization. To this end, the commonly used pharmacological agents for oxygen-independent stabilization of HIF-1α, cobalt chloride (CoCl2) and desferroxamine mesylate (DFO), were examined for a possible association with mitochondrial-ROS production. We designed an experiment wherein Hep3B cells were pre-incubated for 12 h with MitoQ or vehicle followed by a medium change and addition of either DFO or CoCl2 (final concentration 100 μM) to
the culture medium. Cells were then incubated for a subsequent 6 h in the presence of these agents. The results, presented in Fig. 4, show that the presence of either DFO or CoCl2 resulted in the stabilization of HIF-1α after a 6-hour period. In the same experiment, cells which had been pre-incubated with 1 μM MitoQ before addition of either DFO or CoCl2 still retained the ability to express HIF-1α during this time. Thus, MitoQ had no effect on the ability of DFO and CoCl2 to stabilize HIF-1α under these conditions, and we conclude that mitochondrial ROS are not involved in this mechanism. This result, in the context of CoCl2, is in line with recent reports showing that CoCl2 can deplete intracellular ascorbate stores resulting in a decreased PHD activity [36], and can also inhibit the interaction between hydroxylated HIF-1 and pVHL [37]. The mechanism of DFO action on HIF-1 has not been investigated in detail, but there is a general consensus that DFO, by accumulating intracellular iron, inhibits the activity of iron-requiring PHD enzymes.

3.5. Conclusions

In the present study, we have shown that the application of a targeted mitochondrial antioxidant eliminates the hypoxic generation of ROS and concurrently reduces HIF-1α levels by destabilization of the protein, resulting in the loss of transcriptional activity. Evidence that these two processes are linked arises from our findings that the destabilizing effect of MitoQ on HIF-1α only occurred during hypoxic increases in ROS, as the normoxic levels of HIF-1α in a VHL mutant cell line (which produces constitutively stabilized HIF-1α) were unchanged in the presence of MitoQ. We further corroborated this by showing that pharmacological intervention using DFO and CoCl2, which are not thought to involve mitochondrial ROS, stabilized HIF-1α independently of MitoQ. Recently, a novel pathway of HIF-1α degradation has been highlighted in which the disruption of mitochondrial function can lead to oxygen re-location for non-mitochondrial purposes during hypoxic episodes [34]. We tested whether MitoQ could be participating in this pathway by measuring oxygen consumption in whole cells in the presence of this antioxidant, but found that the respiration rate was unchanged. Another possible mechanism for the MitoQ-induced loss of HIF-1α in hypoxia could be that, by stabilizing endogenous NO (by blocking ROS which react with it), MitoQ addition could lead to the availability of NO to inhibit complex IV; this could result in an increase in local concentrations of oxygen for PHD activity. However, oxygen consumption in our cells was not affected by the NO-synthase inhibitor L-NAME and so we have no evidence for NO involvement in our system. Undoubtedly, there is the potential for NO and ROS to interact via mechanisms distinct from oxygen redistribution, for example peroxynitrite generation leading to nitrotyrosine formation (reviewed in [38]). Future work will make use of MitoQ to define these possible contributions.

Considerable data indicate that ROS are an important means of cellular signaling [39], although the specific mechanisms whereby ROS accomplish this are still under study. Many reports in the literature support the notion that an elevation of (mitochondrial?) ROS, during hypoxia, can control the activation of HIF-1α [11,15,27,40], although there is not universal agreement [16,17,41]. Our work with a novel targeted antioxidant strongly suggests that mitochondrial-ROS are intrinsically linked to HIF-1α expression during hypoxia. Although we have not investigated the mechanism(s) by which the ROS signal is transduced to HIF-1α, it is evident from the literature that multiple protein kinases have the potential to be involved [42,43]. In light of our study, a potentially instructive line of analysis, to address signal transduction pathways leading from mitochondrial ROS, could be to carry out gene expression analysis in a chosen cell line, under hypoxia, in the presence or absence of this antioxidant. Clearly, a caveat of this approach would be that any ROS effects could be by way of kinase/phosphatase modifications, and not at the level of transcription.

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