Oligomycin inhibits HIF-1α expression in hypoxic tumor cells

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Gong, Yanqing, and Faton H. Agani. Oligomycin inhibits HIF-1α expression in hypoxic tumor cells. Am J Physiol Cell Physiol 288: C1023–C1029, 2005; doi:10.1152/ajpcell.00443.2004.—Hypoxia-inducible factor-1 (HIF-1) is a key regulator of cellular responses to reduced oxygen availability. The contribution of mitochondria in regulation of HIF-1α in hypoxic cells has received recent attention. We demonstrate that inhibition of electron transport complexes I, III, and IV diminished hypoxic HIF-1α accumulation in different tumor cell lines. Hypoxia-induced HIF-1α accumulation was not prevented by the antioxidants Trolox and N-acetyl-cysteine. Oligomycin, inhibitor of FoF1-ATPase, prevented hypoxia-induced HIF-1α protein accumulation and had no effect on HIF-1α induction by hypoxia-mimicking agents desferrioxamine or dimethylglyoxal-glycine. The inhibitory effect of mitochondrial respiratory chain inhibitors and oligomycin on hypoxic HIF-1α content was pronounced in cells exposed to hypoxia (1.5% O2) but decreased markedly when cells were exposed to severe oxygen deprivation (anoxia). Taken together, these results do not support the role for mitochondrial reactive oxygen species in HIF-1α regulation, but rather suggest that inhibition of electron transport chain and impaired oxygen consumption affect HIF-1α accumulation in hypoxic cells indirectly via effects on prolyl hydroxylase function.

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by 10 min of incubation in the same buffer on ice and homogenization in a glass Dounce homogenizer. Nuclei were pelleted by centrifugation at 10,000 g for 10 min, the supernatant was discarded, and nuclei resuspended in a buffer (0.42 M KCl, 20 mM Tris-HCl [pH 7.5], 20% (vol/vol) glycerol, 1.5 mM MgCl₂) freshly supplemented with dithiothreitol, sodium vanadate, PMSF, leupeptin, and aprotinin. The suspension was rotated at 4°C for 30 min and then centrifuged for 30 min at 14,000 rpm. The supernatant containing the nuclear proteins was collected.

ROS measurements in cells. Cells were cultured in 24-well plates to 80–90% confluency and, before exposure, loaded with 10 µM 5- and 6-chloromethyl-2,7-dichlorodihydrofluorescein diacetate for 30 min in the culture medium without phenol red. Cellular esterases cleave the acetoxymethyl group, forming a charged molecule that becomes highly fluorescent when oxidized to dichlorofluorescein (DCF). Baseline DCF fluorescence was measured with a CytoFluor 4000 plate reader (450 nm excitation, 530 nm emission), followed by addition of inhibitors in the medium. Subsequently, the cells were placed in hypoxia chambers or kept in the normoxic incubator. After 3 h of incubation, DCF fluorescence was measured. All experiments were performed in quadruplicate.

Cell viability analysis. Cells were treated with oligomycin under either normoxic or anoxic conditions for 4 and 8 h, followed by cell viability assay using Trypan blue exclusion (0.4% Trypan blue, light microscopy).

ATP measurements in cells. ATP measurement in cells was performed as described previously (2). Briefly, cells were washed with PBS and lysed by the addition of 0.1 M NaOH. The cells and media were collected, rapidly frozen, and stored at −80°C. ATP intracellular concentrations were analyzed with a luciferase bioluminescence procedure (2). A total of 10 µl of cell extract were mixed with 50 µl of ATP reagent composed of 0.5 ml of imidazole-HCl buffer [1 M], 0.02 ml of MgCl₂ [1 M], 0.750 ml of KCl [1 M], and vortexed. To measure ATP content, 10 µl of cell lysate were mixed with luciferin-luciferase reagent (250 µl) and the light emission was measured using a Lumi-Vette luminometer (model S900, Chrono-log, Havertown, PA). All experiments were performed in triplicate, and the ATP content was normalized for cell protein. The protein content was measured using a standard Bradford assay (Bio-Rad, Hercules, CA).

RESULTS

Antioxidants do not prevent HIF-1α accumulation in hypoxic cells. To investigate the suggested role of mitochondria-derived ROS on HIF-1α accumulation during hypoxia (6, 7), cells were exposed to hypoxia in the presence or absence of antioxidants NAC and Trolox. HIF-1α accumulation in hypoxic Mum2B, Hep3B, and U87 cells was not affected by antioxidant treatment (Fig. 1A). To demonstrate cellular effects of Trolox, we investigated its effects on cobalt-induced HIF-1α. Figure 1A shows that Trolox effectively inhibited cobalt-induced HIF-1α in Mum2B cells. To confirm that the concentration of Trolox used in our experiments attenuated ROS production, DCF fluorescence was measured in cells exposed to normoxia or hypoxia. Hypoxia resulted in a 1.9-fold increase in ROS production, in agreement with other studies (7, 26) (Fig. 1B). Trolox dramatically decreased ROS production under either normoxic (89% decrease in DCF fluorescence) or hypoxic (97% decrease in DCF fluorescence) conditions (Fig. 1B), although it did not affect HIF-1α accumulation. These results suggested that mitochondrial ROS is not involved in HIF-1α regulation during hypoxia and prompted us to investigate further how inhibition of the respiratory chain down-stream of the superoxide-generating site on complex III affects HIF-1α accumulation in hypoxic cells.

Inhibition of complex III with antimycin A and inhibition of complex IV prevents HIF-1α protein stabilization during hypoxia. It has been reported that inhibition of mitochondrial complex I (1, 2, 6, 7, 9) and complex III activity with myxothiazol (6, 7, 9), which blocks ROS generation from complex III; upstream complex III inhibitor myxothiazol, but not downstream complex III inhibitor antimycin A, prevents ROS formation (6, 7). The ability of both inhibitors to prevent an increase in HIF-1α further supports the notion that ROS generated from complex III is not required for HIF-1α stabilization under hypoxic conditions.
Hypoxic accumulation of HIF-1α was also prevented by azide, a complex IV inhibitor (Fig. 2, A and B). Together, these results show that inhibition of the respiratory chain at the level of complexes I, III, and IV prevents hypoxic accumulation of HIF-1α protein and that mechanisms other than complex III-generated ROS must account for HIF-1α inhibition in hypoxic cells.

Inhibitory effects of mitochondrial respiratory chain blockers on HIF-1α accumulation depend on O2 concentration. HIF-1α protein levels vary proportionally over a physiologically relevant range of O2 tension. We investigated whether mitochondrial inhibitors are equally effective in preventing HIF-1α accumulation in cells under moderate hypoxia (1.5% O2) and severe hypoxia (anoxia). To achieve near-anoxic conditions, cells were exposed to a gas mixture (5% CO2-95% N2) that still contained residual 0.2–0.3% oxygen and designated here as anoxia. Respiratory chain inhibitors markedly prevented HIF-1α protein increase under hypoxic conditions (Fig. 3, A and B) but were less effective in inhibiting anoxic HIF-1α increase (Fig. 3, A and B). ROS generation is higher under hypoxic than anoxic conditions (26), and this has been used to explain differential effects of respiratory chain inhibitors on HIF-1α accumulation under hypoxic and anoxic conditions (26). However, our findings (Figs. 1 and 2) demonstrate that ROS is not involved in hypoxic HIF-1α regulation. Taken together, these results indicate that the molecular mechanism of differential effects of mitochondrial inhibitors on HIF-1α accumulation under hypoxic and anoxic conditions is distinct from mitochondrial ROS generation.

Mitochondrial inhibitors did not affect significantly ATP levels under normoxic or anoxic conditions (Fig. 3C), consistent with the notion that tumor cells have high glycolytic capacity, and short-term decrease in oxidative phosphorylation did not alter cellular ATP. This indicates that potential changes in the cellular ATP are not the underlying cause of the effect of respiratory chain inhibitors on HIF-1α.

Oligomycin prevents hypoxia-induced HIF-1α accumulation. Mitochondrial respiratory chain constitutes part of a larger functional entity that is affected by coupling of electron transport and oxidative phosphorylation in living cells (21). This prompted us to investigate whether inhibition of F0F1-ATPase activity might affect HIF-1α regulation. We demonstrate that oligomycin, an F0F1-ATPase inhibitor, suppressed HIF-1α accumulation in hypoxic Mum2B (Fig. 4A) and U87 cells (Fig. 4E) without altering HIF-1α content during normoxia (Fig. 4D). Oligomycin did not affect HIF-1α upregulation by hypoxia-mimicking agents DFO and DMO (Fig. 4, B and C, respectively), which directly inhibit the prolyl hydroxylation step of HIF-1α (13, 14). To ascertain that cell response...
Oligomycin treatment compared with normoxic control; Fig. 5B). This is in agreement with the notion that tumor cells have high glycolytic capacity, and short-term decrease in oxidative phosphorylation does not markedly alter cellular ATP. This result indicates that changes in the cellular ATP are not the underlying cause of the effect of oligomycin on HIF-1α.

To determine whether cell treatments resulted in any significant cytotoxicity, we performed a Trypan blue viability test. MUM2B and U87 cells did not show any significant decrease in viability during an 8-h exposure period to oligomycin under oxic, hypoxic, or anoxic conditions, significantly longer than the 3- to 4-h duration of our experiments (data not shown).

**Effect of oligomycin on HIF-1α accumulation is also dependent on O2 concentration.** The inhibitory effect of oligomycin on HIF-1α was also dependent on the degree of oxygen concentration; the effect was small under anoxic conditions and marked during hypoxia (1.5% O2) (Fig. 6). Treatment with oligomycin does not decrease, but rather increases, mitochondrial ROS production (21); hence, this result further supports the notion that inhibition of FoF1-ATP synthase prevents HIF-1α accumulation via a mechanism that is distinct from ROS generation during hypoxia.

To further investigate whether HIF-1α destabilization by oligomycin is dependent on the O2-dependent degradation domain of HIF-1α by a prolyl hydroxylase-dependent mechanism, we used a deletion construct containing residues 521 to 652 of HIF-1α (fused to GFP-V5 protein), which confers O2 dependence (9) (Fig. 7A). Immunoblotting with anti-V5 antibodies showed stabilization of the construct by hypoxia (1.5% O2) and destabilization in the presence of oligomycin (Fig. 7A). Cell treatment with DFO, used as a positive control, resulted in the stabilization of the deletion fragment as predicted. There-

![Fig. 4. Oligomycin prevents hypoxia-induced HIF-1α accumulation but does not affect desferrioxamine (DFO)- or dimethyloxalyl glycine (DMO)-induced HIF-1α.](#)

![Fig. 5. DFO and DMO reversed oligomycin suppression of HIF-1α in hypoxic cells.](#)
protein accumulation in hypoxic cells. Nitric oxide (NO) inhibits HIF-1α accumulation during hypoxia (2, 9, 18, 29); effects on mitochondrial complex I (2) as well as complex IV (9), have been implicated as underlying mechanisms. Notably, NO under certain conditions has been reported (20) to induce HIF-1α in normoxic cells (reviewed in Ref. 4) and possibly affect prolyl hydroxylase function. Here we show that inhibition of respiratory complex III by either myxothiazol or antimycin A (Fig. 2, A and B) and inhibition of complex IV also inhibited HIF-1α accumulation (Fig. 2, A and B), in agreement with the report by Hagen et al. (9), and that the use of antioxidants did not inhibit hypoxia-induced accumulation, although it inhibited cobalt-induced HIF-1α (Fig. 1A). In agreement with our findings, the inhibitory effect of antioxidants on cobalt-induced HIF-1α expression was recently reported by others (5). Interestingly, Chachami et al. (5) showed that the effect of cobalt on HIF-1α is mediated via phosphatidylinositol 3-kinase, rather than by interference of cobalt with iron availability for prolyl hydroxylase function, as suggested earlier (14). These results further support the notion that mitochondrial ROS does not regulate HIF-1α in hypoxic cells; instead, mechanisms other than complex III-generated ROS must account for inhibition of HIF-1α accumulation by respiratory chain inhibitors.

The mitochondrial respiratory chain constitutes part of a larger functional entity that is affected by coupling of electron transport and oxidative phosphorylation in living cells. Oligomycin, an inhibitor of FoF1-ATPase, also suppressed accumulation of HIF-1α in hypoxic tumor cells (Fig. 4A), and this effect is specific for oxygen deprivation because oligomycin inhibitory effects of oligomycin on HIF-1α hypoxic induction depend on cellular oxygen concentration. Mum2B cells were exposed to normoxia, anoxia, or 1.5% O2 hypoxia for 3 h in the absence or presence of 5 μg/ml (+) or 10 μg/ml (++) oligomycin (Oli). Whole cell lysates were prepared to determine HIF-1α and actin protein content, followed by scanning densitometry of autoradiogram signals for HIF-1α and actin. Changes are expressed as percentage decrease relative to HIF-1α maximum induction in anoxia or hypoxia (n = 2 independent experiments).

fore, inhibition of hypoxic HIF-1α accumulation by oligomycin is due to protein destabilization that involves a prolyl hydroxylase-dependent mechanism.

We next compared the stability of full-length wild-type (WT) HIF-1α (WT-HIF-1α) and HIF-1α with two mutated prolines (HIF-P402A/P564A) that render HIF-1α resistant to prolyl hydroxylase-mediated degradation. Cells were exposed to hypoxia (1.5% O2) in the presence or absence of oligomycin (Fig. 7B). Hypoxia resulted in increased accumulation of WT-HIF-1α (Fig. 7B, lane 2), which was markedly reduced by oligomycin (Fig. 7B, lane 3). In contrast, HIF-P402A/P564A was expressed in normoxic cells (Fig. 7B, lane 4), confirming resistance to prolyl hydroxylase-mediated degradation, and expression was not increased during hypoxia (Fig. 7B, lane 5). Treatment with oligomycin did not cause any decrease in HIF-1α accumulation (Fig. 7B, lane 6). This result is consistent with the notion that oligomycin causes HIF-1α protein destabilization that is dependent on prolyl hydroxylation of Pro402 and Pro564.

**DISCUSSION**

HIF-1α cellular content is regulated by pVHL and prolyl hydroxylases, a group of enzymes that hydroxylate HIF-1α on specific proline residues (13, 14, 19). The regulation of HIF-1α expression is, however, more complex and dependent on other factors besides oxygen concentration. This is not surprising, considering that particular cell types in different tissues have different roles, perform different functions, and respond differently to various forms and degrees of stress and therefore have retained the ability to integrate multiple signals that converge into HIF-1α activation. There is evidence that glucose metabolism (15), tricarboxylic acid (TCA) cycle intermediates (8), and the function of the respiratory chain (1, 2, 6, 7, 9) affect HIF-1α regulation.

Earlier reports indicated that mitochondrial respiratory chain activity is required for accumulation of HIF-1α during hypoxia (6, 7), whereas others have not found evidence to support a conclusive role for mitochondria in regulating HIF-1α (30, 31). Inhibition of electron transport chain by rotenone and myxothiazol (1, 2, 6, 7, 9, 26) has been shown to prevent HIF-1α accumulation during hypoxia (1.5% O2) in the presence or absence of 10 μg/ml oligomycin (Oli). Whole cells were prepared, followed by SDS-PAGE and immunoblotting with anti-V5 antibody. DFO (200 μM) treatment was used as positive control. Actin bands indicate uniformity of loading. A: U87 cells were transfected with either 5 μg of wild-type (wt) HIF-1α plasmid or 5 μg of mutated mHIF-1α (lane 7 contains untransfected control cell lysate). After 24 h of recovery, cells were exposed to 1.5% O2 hypoxia for 3 h in the presence or absence of 10 μg/ml oligomycin (Oli). Cell lysates were prepared, followed by SDS-PAGE and immunoblotting using anti-V5 antibody. Probing for actin indicates uniformity of loading.
had no effect on DFO- or DMO-induced HIF-1α (Fig. 4, B and C). Other respiratory chain inhibitors (rotenone, myxothiazol) similarly inhibit only hypoxia-induced HIF-1α expression, but not HIF-1α induction by hypoxia-mimicking agents DFO and cobalt (6, 7), further supporting a specific oxygen-dependent effect on HIF-1α regulation due to respiratory chain inhibition.

These findings, as well as previously reported results regarding HIF-1α inhibition by mitochondrial inhibitors (1, 6, 7, 9, 26), could be explained by the model suggested by Hagen et al. (9), based on the notion that inhibition of mitochondrial respiratory chain complexes I, III, and IV decrease oxygen consumption, resulting in redistribution of oxygen to cytosol, which in turn helps to maintain prolyl hydroxylase activity, resulting in HIF-1α protein degradation. Here we demonstrate that oligomycin causes destabilization of HIF-1α via a prolyl hydroxylase-dependent degradation (Fig. 7, A and B), and this could also be explained by the same model (9). Inhibition of ATPase would prevent the proton flux back through the mitochondrial inner membrane via the ATP synthase proton channel, keeping the electrochemical gradient at its maximum potential difference (21). This would inhibit proton pumping by complexes I, III, and IV, cause stalling of electron flow through the electron transport chain, and result in impaired oxygen consumption, which would explain the effect of oligomycin. Taken together, implications of these results as well as implications of the model proposed by Hagen et al. (9) would be that inhibition of respiratory chain during moderate hypoxia (1.5% O2) causes redistribution of oxygen to cytosol, thereby maintaining the activity of prolyl hydroxylases, a necessary step for HIF-1α protein degradation. However, under conditions of near-anoxia when oxygen concentration is nearly diminished, any significant oxygen redistribution is not possible. Consequently, inhibition of the respiratory chain under anoxic conditions should not be able to prevent hypoxic accumulation of HIF-1α. Indeed, our results show that HIF-1α accumulation during anoxia is relatively insensitive to respiratory chain inhibition, in contrast to hypoxia (Fig. 3, A and B; Fig. 6).

An alternative mechanism that could incorporate the observed effects of respiratory chain inhibitors on hypoxic HIF-1α regulation emerged from a recent study (8). Impaired electron transport might have a secondary effect on TCA cycle, resulting in changes in cellular concentration of intermediate products. Some TCA cycle intermediate products have the ability to affect prolyl hydroxylase activity and consequently HIF-1α accumulation, as shown in the study by Verma and colleagues (8). This appears intriguing, because it could reconcile the role of respiratory chain, TCA cycle, and prolyl hydroxylase function on HIF-1α expression.

Hypoxia is an important component of the tumor microenvironment and HIF-1α is considered to be a positive factor for tumor progression by virtue of stimulating tumor angiogenesis, mediating metabolic adaptations of tumor cells to hypoxia, and in many cases by increasing resistance to hypoxia-induced cell death (23, 27). In addition to implications for understanding mitochondrial mechanisms of HIF-1α regulation, the ability of oligomycin to interfere with HIF-1α regulation could have other potential applications. Recently, a family of F0F1-ATPase inhibitors, including oligomycin, was shown to have selective cytotoxic effects on human tumor cell lines (25). These agents selectively sensitized tumor cells to apoptosis (25) and characteristically altered gene expression profiles of the 60 human cancer cell lines of the National Cancer Institute (25). The exact mechanisms for induction of apoptosis by this strategy remain to be established. In this study, we chose to use two human cell lines that are highly resistant to apoptosis, Mum2B and U87, to investigate how oligomycin affects HIF-1α expression during short-term hypoxia. The results point toward an additional pathway through which agents like oligomycin might enhance tumor cell killing by preventing HIF-1α protein accumulation during hypoxia. This sets the stage for further investigation of the effects of this class of agents on sensitization of hypoxic tumor cells using as a model cells such as Mum2B and U87 that are very resistant to apoptosis. The ability of oligomycin to suppress HIF-1α during hypoxia suggests new possibilities to exploit this and other F0F1-ATPase inhibitory agents in designing new strategies aimed at increasing cytotoxic effects on tumor cells.

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REFERENCES

11. Huang LE, Arany Z, Livingston DM, and Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-

12. Huang LE, Gu J, Schau M, and Bunn HF. Regulation of hypoxia-inducible factor 1α is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 95: 7987–7992, 1998.


