Response of mitochondrial reactive oxygen species generation to steady-state oxygen tension: implications for hypoxic cell signaling

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Oxygen limitation at complex IV could lead to cytochrome c reduction, abrogating its ability to scavenge $O_2^-$, thus enhancing mitochondrial ROS leakage. However, an overriding paradox exists in the above models because the availability of the critical substrate for $O_2^-$ generation, $O_2$, is limited in hypoxia. This raises an important question: how can $[O_2]$ be limited enough to inhibit complex IV yet still be sufficient to afford $O_2^-$ generation? In response to this paradox, an alternative hypothesis has been proposed wherein mitochondrial ROS generation decreases during hypoxia (4, 59, 87), and this decrease acts as a hypoxic signal. This is in agreement with studies showing that several cellular sources of ROS decrease their output as $[O_2]$ falls (34, 47, 49, 60).

An important tenet of all the models described above is that the machinery for both $O_2$ sensing and ROS generation is contained within the mitochondrial respiratory chain itself; i.e., the mitochondrion comprises an autonomous signaling unit. If this is the case, it follows that mitochondria in isolation should behave like those inside cells and adjust their ROS generation (up or down) in response to hypoxia. Given the potential importance of mitochondrial ROS generation to hypoxic signaling, it is therefore interesting to note that the response of isolated mitochondrial
ROS generation to low [O₂] within the physiological/hypoxic range has not been measured.

Isolated mitochondrial ROS generation has been measured at 10 μM-1 mM [O₂] (11, 25, 82), but this is far above the Kₘ of complex IV (27, 38) for O₂. Therefore, such experiments cannot directly test the hypothesis that O₂ limitation at complex IV enhances ROS generation, since the complex never becomes O₂ limited. Furthermore, the use of nonphysiological inhibitors, such as antimycin A, has been necessary to observe increased ROS generation under hypoxia by isolated mitochondria (25). Simply put, the functionality of isolated mitochondria as autonomous hypoxic signaling units has not been adequately tested. Furthermore, whereas mitochondrial function has been previously examined at <10 μM O₂ using high-resolution respirometry (35, 36, 38), ROS cannot be measured in such systems because this technique requires a low mitochondrial concentration (<0.05 mg protein/ml) that is not compatible with the sensitivity of fluorescent ROS probes. Similarly, the high concentrations of mitochondria required for ROS measurements are not compatible with high-resolution respirometry, because rapid mitochondrial respiration in a closed chamber with a finite amount of O₂ would result in rapid anoxia.

To circumvent these technical limitations, an open-flow respirometry system (15, 26) was employed in this study to examine mitochondrial ROS generation at O₂ levels within the physiological/hypoxic range. In this system mitochondria were maintained at defined steady-state [O₂] as low as 0.1 μM, and the coupling of this system with a fluorimeter and spectrophotometer allowed for parallel measurements of respiration, ROS generation, and cytochrome spectra. The specific hypothesis tested was that O₂ limitation at complex IV causes a backup of electrons in the respiratory chain and enhanced ROS generation at complex III. Using this system, we show that, under all respiratory conditions studied, mitochondrial ROS generation decreased as [O₂] decreased.

MATERIALS AND METHODS

Mitochondrial isolation and reagents. All chemicals were from Sigma (St. Louis, MO) with the exception of Amplex red, which was from Molecular Probes (Eugene, OR). Adult male Sprague-Dawley rats, 200–250 g body mass, were from Harlan (Indianapolis, IN) and were housed in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the University Committee on Animal Research of the University of Rochester (protocol no. 2003–111). Liver mitochondria were isolated by differential centrifugation as previously described (17). Mitochondria were depleted of Ca²⁺ by incubation at 10 mg protein/ml in buffer comprising (in mM) 195 mannitol, 25 sucrose, 40 HEPES, 10 NaCl, 1 EGTA, and 5 succinate (pH 7.2) for 15 min at 25°C. Several wash steps were subsequently performed in buffer comprising (in mM) 195 mannitol, 25 sucrose, and 40 HEPES (pH 7.2), and mitochondria were finally suspended in this buffer at >50 mg protein/ml and kept on ice until use. The Ca²⁺ depletion step and subsequent washes were found to be necessary to measure any ROS generation by liver mitochondria. It is presumed that this was due to the use of Ca²⁺ to disrupt interactions between mitochondria and cellular membranes, as well as removing any contaminating catalase and peroxidases. The effects of Ca²⁺ on mitochondrial ROS generation are extremely complex (18) and are beyond the scope of this investigation.

Open-flow respirometry and ROS measurements. Open-flow respirometry was performed as previously described (15, 26) by using a custom-built open-flow respirometry chamber, in an Aviv Cary model 14 UV/VIS spectrophotometer (Aviv Biomedical, Lakewood, NJ), with the addition of a fiber optic spectrophotometer (Ocean Optics, Dunedin, FL). The head-space flow of humidified N₂, O₂, and air was 100 ml/min total and was controlled by mass-flow regulators (Sierra Instruments, Monterey, CA). The equation \( Q = \frac{m(C^* - C)}{K_m + C} \) was used to calculate respiration rate (Q) under steady-state conditions (15, 26), wherein \( C^* \) is the predicted liquid phase [O₂] and \( C \) is the measured liquid phase [O₂]. Typical values for the mass transfer coefficient \( m \) were 0.002/s. ROS generation was measured using Amplex red (60, 74). Mitochondria (2.5 mg protein) were suspended in 2.5 ml of buffer comprising 125 mM KCl, 25 mM sucrose, 5 mM MgCl₂, 5 mM KH₂PO₄, 0.05% (wt/vol) fat-free BSA, 2 μM rotenone, 10 μM Amplex red, and 1 U/ml of type II horseradish peroxidase, pH 7.3 at 37°C. Rotenone was present to inhibit ROS generation due to reverse electron flow through complex I (73), and BSA was present to chelate free fatty acids that may cause mitochondrial uncoupling. State 4 respiration was imposed by adding 10 mM succinate plus 1 μM oligomycin to inhibit ATP synthase. State 3 respiration was imposed by adding 10 mM succinate and 4 mM ADP. The use of complex I substrates, such as glutamate/malate, had no effect on the response of ROS generation to hypoxia in this system (data not shown). Once the O₂ concentration in the liquid phase had reached a steady state, fluorescence was monitored for a minimum of 3 min to establish the rate of ROS generation, which was then calibrated by the addition of 0.25 μM H₂O₂. It was found unnecessary to add SOD to maximize H₂O₂ measurement in these incubations because the rate was the same regardless of whether SOD (80 U/ml of Cu/Zn-SOD) was present. This is probably due to the high endogenous rate of spontaneous dismutation of O₂⁻ (30) or possibly some residual SOD in the liver mitochondrial preparation.

Visible spectrophotometry. Visible wavelength experiments were performed under the same conditions as ROS experiments; however, due to conflicting absorbance spectra, Amplex red was not included. All absorbance spectra were recorded at steady-state [O₂] using a wavelength scan between 400 and 650 nm. A nominal maximally reduced spectrum was recorded at the end of each run in the presence of sodium dithionite, and the percent reduction was calculated relative to this.

Nitrotyrosine Western blot analysis. To investigate the possibility of mitochondrial peroxynitrite (ONOO⁻) formation during hypoxia (76), isolated rat liver mitochondria were exposed to steady-state [O₂] conditions nominally referred to as normoxia (air saturation) or hypoxia (1.0 μM O₂), using the open-flow system as described in Open-flow respirometry and ROS measurements. A positive control was also prepared by adding a bolus of 250 μM ONOO⁻ in the normoxic condition. Western blotting procedures were as previously described (80), with the exception of the primary antibody being a mouse monoclonal anti-nitrotyrosine (Cayman, Ann Arbor, MI) at a dilution of 1:2,000.

Statistics and curve fitting. In an open-flow respirometry system, the final steady state [O₂], which is reached by the system, varies between runs, because the desired final [O₂] can only be approximated by the user (by varying the Po₅ of the inflowing gas) and is essentially “decided” by the activity of the mitochondria. Therefore, to obtain graphs of [O₂] versus ROS generation and [O₂] versus respiration with error bars only on the y-axis, it was necessary to apply curve fitting to extrapolate values at common O₂ concentrations. Steady-state values of [O₂] and either ROS generation or respiration were entered into Prism software (GraphPad, San Diego, CA), and curve fitting was performed by using the equation \( y = (B_{max} - x)/(K_a + x) \), where \( B_{max} \) is the rate of maximal binding of a substrate (O₂) to an enzyme (cytochrome oxidase) and x and y are x- and y-axes, respectively. For each individual data set, y values were then extrapolated at common values of [O₂], and the averages (± SE) are presented in Figs. 2–6. Since mitochondrial respiration and ROS generation are complex multienzyme systems, \( K_{m} \) is not applicable, and therefore the
RESULTS

With the use of the open-flow respirometry system (see MATERIALS AND METHODS), measurements of respiration and ROS generation by isolated rat liver mitochondria were obtained at various steady-state [O\textsubscript{2}] levels. Figure 1 shows the response of isolated mitochondrial respiration to [O\textsubscript{2}], indicating that respiration either in state 3 (ATP turnover, Fig. 1A) or state 4 (quiescent, Fig. 1B) decreased with [O\textsubscript{2}]. The p50 values (i.e., [O\textsubscript{2}] at which respiration was 50% inhibited) for states 3 and 4 were 2.7 ± 1.0 and 3.6 ± 0.7 μM O\textsubscript{2}, respectively. These p50 values are in agreement with those we previously reported in an open-flow respirometry system using isolated rat liver mitochondria (15), although they are slightly higher than those reported by other authors using either open-flow (26, 29) or closed-chamber respirometry systems (90). The reason for these discrepancies is currently unknown but could be due to differences in both tissue and animal sources of mitochondria, respiratory substrates, or incubation conditions, such as mitochondrial protein concentration and temperature. Nevertheless, for the purposes of this investigation into mitochondrial ROS generation, the data in Fig. 1 serve to validate the utility of the open-flow respirometry system as a means to manipulate mitochondrial respiratory activity using steady-state [O\textsubscript{2}] as an independent variable.

Having established the validity of this system for regulating mitochondrial respiratory activity by [O\textsubscript{2}] (15, 26), we next sought to investigate the effects of this manipulation on ROS generation. Figure 2 shows the response of isolated mitochondrial ROS generation to [O\textsubscript{2}]. These data clearly indicate that mitochondrial ROS generation decreases with [O\textsubscript{2}] in a manner similar to that seen for respiration, yielding p50 values of 0.34 ± 0.04 μM for state 3 and 0.8 ± 0.3 μM O\textsubscript{2} for state 4 (P = 0.24, ANOVA). Under no conditions was an increase in ROS generation ever seen at low [O\textsubscript{2}]. The maximal rates of ROS generation observed in these experiments under normoxic conditions (plateau at >50 μM O\textsubscript{2}) were in agreement with previously published values for rat liver mitochondria (66). In further agreement with previous studies (74), mitochondria in state 4 exhibited a higher rate of ROS generation than those in state 3.

Since the hypothesis being tested in this study was that O\textsubscript{2} limitation at complex IV causes a backup of electrons in the respiratory chain and enhanced ROS generation at complex III, it was important to verify that the O\textsubscript{2} levels attained in this experimental system were low enough to elicit reduction of the
respiratory chain. Therefore, the redox state of cytochromes \(a/a_3\) was monitored at varying steady-state \(O_2\) levels in conjunction with ROS generation and respiration measurements. The results of this analysis (Fig. 3) show that the respiratory chain does indeed become reduced at the \(O_2\) levels attained in this study. Therefore, the reason we did not observe increased ROS generation at low \([O_2]\) was not simply because the \(O_2\) levels were too high to permit respiratory chain reduction.

The percentage of total mitochondrial electron flux diverted to ROS (i.e., pmols of \(H_2O_2\) generated per nmol \(O_2\) consumed) was calculated from the data in Figs. 1 and 2 and is presented in Fig. 4. These data show, that in both respiratory states, as \([O_2]\) decreased, the percentage of electrons diverted to ROS generation increased. A potential factor that may govern mitochondrial ROS generation is the degree of uncoupling of oxidative phosphorylation. Respiratory control ratio (RCR, state 3 divided by state 4) is a commonly used measure of mitochondrial coupling and has previously been shown to correlate both directly and indirectly with \([O_2]\) (37, 53). Therefore, RCR was calculated from the current data set, and the results (Fig. 5) indicate that, in this experimental system, RCR decreases at low \([O_2]\) (i.e., mitochondria become uncoupled).

Another potential factor that may govern the release of ROS from mitochondria under hypoxic conditions is nitric oxide \((NO^-)\) (75), either from the cytosol or from the somewhat controversial mitochondrial \(NO^-\) synthase (13). The reaction of \(NO^-\) with \(O_2^-\) occurs at almost diffusion-limited rates and can effectively compete with SOD for \(O_2^-\) to form \(ONOO^-\). It was hypothesized that if \(ONOO^-\) was formed during hypoxia, this would result in nitrination of tyrosine residues. Therefore, the presence of nitro-tyrosine in mitochondrial proteins exposed to hypoxia was examined by Western blot analysis. The results in Fig. 6 show that hypoxic exposure of mitochondria under the experimental conditions used in Figs. 1–5 did not lead to any difference in the detectable levels of nitrination versus a normoxic control.

**DISCUSSION**

In addition to the now classical HIF-1\(\alpha\) (70) hypoxic signaling paradigm, involving \(O_2\)-sensitive prolyl-hydroxylases (43), an emerging paradigm is that mitochondrially derived ROS can act as intermediates in both hypoxic and nonhypoxic signaling pathways, including a direct action of these ROS on HIF-1\(\alpha\) itself (7, 10, 25, 32, 40, 67, 85, 86, 88, 89). The notion of increased mitochondrial ROS under hypoxic conditions is based on the hypothesis that \(O_2\) limitation at complex IV triggers ROS generation at upstream respiratory complexes. From such a hypothesis, it follows that the signaling machinery for increased mitochondrial ROS in response to hypoxia is...
intrinsic to the mitochondrial respiratory chain. Thus, it should be possible to recreate this phenomenon in isolated mitochondria. We directly tested this hypothesis using an open-flow respirometry system, which permits examination of isolated mitochondrial function at tightly controlled steady-state [O2] levels (15, 26). Our main finding was that in this isolated mitochondrial system, free from extramitochondrial signaling pathways, ROS generation decreased in hypoxia.

From this result, it is concluded that the biochemical and/or signaling machinery for ROS generation in response to hypoxia is not an intrinsic part of the respiratory chain that is present in isolated mitochondria. It is important to emphasize that this conclusion does not preclude the possibility that mitochondrial ROS generation can increase in response to hypoxia in intact cells; it merely posits that the mechanism by which mitochondria may generate ROS in hypoxia is more complicated than purely O2 limitation at complex IV and backup of electrons in the respiratory chain.

There are several possible reasons for the apparent divergence between the current data and previous studies that have demonstrated increased apparent mitochondrial ROS in hypoxic cells. One possibility is that hypoxia may elicit ROS generation from nonmitochondrial sources, including NADPH oxidases, xanthine oxidase, or cytosolic peroxidases (86). Indeed, a recent study (3) in endothelial cells concluded that ROS from both NADPH oxidases and mitochondria are equally important in hypoxia-induced Ca2+ mobilization.

Another potential variance regarding the origin of ROS in hypoxic cells may be the specificity of fluorescent indicators for either mitochondria or ROS (2, 6, 9, 20, 63, 66, 79). One example is the NO'-sensitive probe diaminofluorescein, the fluorescent product of which accumulates in mitochondria even if NO' is added extracellularly (28). Thus a mitochondrial fluorescent signal does not necessarily indicate a mitochondrial origin for ROS. Regarding specificity for particular reactive species, the widely used ROS probe dichlorofluorescein can generate as well as report ROS (9, 66). Accordingly, recent experiments have shown that exposure of cells to hypoxia resulted in decreased signals from the chemiluminescent ROS probe lucigenin, several ROS electron-paramagnetic resonance probes, and the fluorescent ROS probe Amplex red. However, the same hypoxic exposure caused an increased dichlorofluorescein signal (50, 55). Clearly, the specificity of ROS probes in intact cells remains an obstacle to progression in this field, and, moreover, it is anticipated that novel mitochondria-specific ROS probes (e.g., MitoSOX red) may complicate this issue even further, since their mitochondrial accumulation depends on mitochondrial membrane potential (∆ψm), which may change during hypoxia (37, 53).

To overcome these technical issues, the current investigation used a well-defined biochemical system, i.e., isolated mitochondria. Since H2O2 is membrane permeable, in an isolated mitochondrial system, Amplex red assay is specific for mitochondrially derived H2O2. Amplex red was also utilized for its ability to measure H2O2 generation as a kinetic rate. This overcomes a limitation of ethidium-based dyes (dihydroethidium, MitoSOX red, etc.), which, although specific for superoxide, exhibit maximal fluorescence in the presence of DNA (91). Thus the use of such dyes may be a problem where DNA is limiting (e.g., mitochondria), although recent methodological developments with dihydroethidium-based dyes may overcome such limitations (45).

Another possible reason for discrepancy between previous cellular results and our current mitochondrial data is the use of classical mitochondrial inhibitors such as rotenone and antimycin A in cell systems. Such compounds are often used to manipulate ROS generation, but they act on the respiratory chain and thus also impact mitochondrial parameters, such as ATP synthesis and Ca2+ handling, which may be important for cytosolic ROS generation. Thus an effect of a classical mitochondrial inhibitor on cellular ROS generation does not necessarily indicate mitochondria are the source of ROS; it merely suggests that some aspect of mitochondrial function is required for cellular ROS generation (2, 46). In agreement with this, it was recently shown that the activation of ERK, previously attributed to mitochondrial ROS generation, actually depends more on mitochondrial ATP synthesis than it does on ROS (1, 8). Furthermore, many classical mitochondrial inhibitors are known to affect other cellular proteins. For example, antimycin A mimics the BH3 domain of Bcl-family proteins (83), whereas rotenone is a microtubule dissociator (12). Notably, microtubule disruption can affect HIF-1α expression levels (33), and it is known that HIF regulates the expression of several mitochondrial proteins (69). Thus, in long-term use, agents such as rotenone may have unexpected effects on mitochondrial proteins. Novel methods of inhibiting mitochondria, such as small-interfering RNA, are more specific (39) but may still cause secondary effects on cellular bioenergetics. As described earlier in the introduction, we chose to examine isolated mitochondria in the current study since they represent a well-defined biochemical system that lacks many of the technical issues mentioned here and are a viable model to directly test the hypothesis that O2 limitation at complex IV can elicit ROS generation.

Clearly, when differences between cells and mitochondria are examined, it is impossible to ignore that isolated mitochondria are removed from their intracellular signaling environment and may therefore be missing posttranslational protein modifications that are important for ROS signaling responses. For
example, it has been shown that association of the Rieske iron-sulfur protein (RISP) with complex III is phosphorylation dependent (41), and, notably, small-interfering RNA knock-down of this same protein diminishes the hypoxic ROS signaling response (39). Therefore, it might be predicted that, in mitochondria isolated without phosphatase inhibitors, the RISP would be dephosphorylated during the isolation procedure, leading to its dissociation from complex III, resulting in a loss of hypoxia-induced ROS generation. Experiments are needed to test this hypothesis.

Isolated mitochondria are also deprived of small signaling molecules that can impact on ROS generation (16, 17). One such molecule is NO, a potent inhibitor of mitochondrial complex IV (15, 17, 27). Complex IV inhibition by NO increases its $K_M$ for O$_2$, such that ROS generation by upstream complexes would be activated at higher O$_2$ levels (27, 61). Whereas such a mechanism may partially explain the reported activation of HIF-1α in response to NO (57), these reports conflict with others showing that NO suppresses HIF-1α (72). Furthermore, other complex IV inhibitors (e.g., cyanide) do not stimulate mitochondrial ROS generation (14, 81). Whereas NO may inhibit additional sites in the respiratory chain (17, 21), the effects of this on ROS generation and hypoxic signaling are unknown. It should be noted that nitrite was absent from the current experiments, thus ruling out the possibility for NO formation from nitrite by complex IV under hypoxia (23). Furthermore, if NO was being formed in hypoxia and was acting to increase mitochondrial ROS generation, this would lead of ONOO$^-$ formation, and the data in Fig. 6 showing no formation of nitro-tyrosine in hypoxia suggest that this is not the case.

An alternative model, which may reconcile the apparently conflicting observations between isolated mitochondria and cellular systems, proposes that a decrease in mitochondrial ROS generation may act as a hypoxic signal (4, 47, 49, 59, 87). In this model, signaling pathways constitutively activated by the “tonal” generation of mitochondrial ROS under normoxic conditions would be downregulated in hypoxia (4). This model is supported by data from this investigation showing that isolated mitochondrial ROS generation decreases in hypoxia (Fig. 3). Further support for this model is also seen in 1) recent data from whole cells using fluorescent ROS probes (50, 55); 2) the observation that inhibiting complex IV per se (e.g., by cyanide) inhibits mitochondrial ROS generation (14, 81); and 3) the observations of Chance (24), who reported that inhibiting complex IV with cyanide did not lead to the reduction of upstream complexes due to a “cushioning effect” in the respiratory chain.

The data in Fig. 5 also offer some insight into the proposal that mitochondrial ROS generation may decrease in hypoxia. These data show that RCR decreases with [O$_2$]; i.e., mitochondria become uncoupled in hypoxia. It is generally agreed that mild mitochondrial uncoupling (e.g., via activation of uncoupling proteins) may decrease mitochondrial ROS generation (14, 58, 74), and thus such uncoupling may be an underlying mechanism of decreased ROS generation at low O$_2$. Together, the current and previous data all suggest that the intrinsic response of the isolated mitochondrial respiratory chain to low [O$_2$] is to decrease its ROS generation.

In addition to the two contrasting models of hypoxic signaling outlined above, the data in Fig. 4 present a third possibility. These data show that the percentage of mitochondrial electron flux diverted toward ROS generation increases in hypoxia. Such a change in the fractional diversion of electrons toward ROS may be a hypoxic signal, but currently it is difficult to envisage the molecular basis by which cells could integrate this information to mediate downstream hypoxic signaling. It is also very important to emphasize that these data (Fig. 4) in no way indicate an increase in the absolute amount of ROS at low O$_2$. Even though the fractional electron flux diverted to ROS increases, the total electron flux decreases more, such that ROS still goes down at low O$_2$.

In summary, the current investigation exposed isolated mitochondria to a steady-state controlled [O$_2$] environment to test the hypothesis that O$_2$ limitation at complex IV causes an increase in ROS generation. From the ensuing refutation of this hypothesis at the isolated mitochondrial level, it is concluded that the machinery for O$_2$ sensing and hypoxic signaling within cells is not entirely contained within the mitochondrial respiratory chain alone. This conclusion does not preclude the possibility that mitochondrial ROS generation and/or O$_2$ sensing by complex IV are involved in hypoxic cell signaling. It merely posits that in intact cells, the mitochondria→ROS→hypoxic signaling paradigm may involve additional cell signaling factors that are not present in the isolated mitochondrial system. The search for these factors is ongoing.

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REFERENCES


HYPOXIA AND MITOCHONDRIAL ROS

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