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invited review

Cellular oxygen sensing by mitochondria: old questions, new insight

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Chandel, Navdeep S., and Paul T. Schumacker. Cellular oxygen sensing by mitochondria: old questions, new insight. *J Appl Physiol* 88: 1880–1889, 2000.—Hypoxia elicits a variety of adaptive responses at the tissue level, at the cellular level, and at the molecular level. A physiological response to hypoxia requires the existence of an O_2 sensor coupled to a signal transduction system, which in turn activates the functional response. Although much has been learned about the signaling systems activated by hypoxia, no consensus exists regarding the nature of the underlying O_2 sensor or whether multiple sensors exist. Among previously considered mechanisms, heme proteins have been suggested to undergo allosteric modification in response to O_2 binding or release at different P_{O_2} levels. Other studies suggest that ion channels may change conductance as a function of P_{O_2}, allowing them to signal the onset of hypoxia. Still other studies suggest that NADPH oxidase may decrease its generation of reactive O_2 species (ROS) during hypoxia. Recent data suggest that mitochondria may function as O_2 sensors by increasing their generation of ROS during hypoxia. These oxidant signals appear to act as second messengers in the adaptive responses to hypoxia in a variety of cell types. Such observations contribute to a growing awareness that mitochondria do more than just generate ATP, in that they initiate signaling cascades involved in adaptive responses to hypoxia and that they participate in the control of cell death pathways.

hypoxia; reactive oxygen species; NADPH; erythropoietin; hypoxia-inducible factor-1

ADAPTATION TO HYPOXIA INVOLVES a wide range of responses that occur at different organizational levels in the body. At the organismal level, for example, the increase in alveolar ventilation that occurs during environmental hypoxia involves the interaction of chemoreceptors, the respiratory control centers in the medulla, and the respiratory muscles and lung/chest wall systems. At the tissue level, for example, pulmonary vascular smooth muscle cells constrict during alveolar hypoxia in a response referred to as hypoxic pulmonary vasoconstriction. By increasing vascular resistance, this response tends to divert blood away from the lung during in utero development and tends to optimize the matching of blood flow to alveolar ventilation after birth and during adulthood. At the cellular level, a number of well-documented responses to hypoxia can be seen. These include the release of neurotransmitters by the glomus cells of the carotid body, secretion of the hormone erythropoietin by cells of the kidney and liver, and the release of vascular growth factors by parenchymal cells in many tissues. In each of these examples, the initiation of the response to hypoxia requires the existence of a fundamental cellular O_2-sensing mechanism that detects the fall in P_{O_2} and initiates a signal transduction sequence that culminates in the activation of the particular functional response. The molecular identity of the cellular O_2 sensor in each of the above responses has long been sought, and a large number of putative O_2-sensing mechanisms have been proposed. However, despite considerable progress in our understanding of the pathways that are activated during cellular hypoxia, no consensus has been reached regarding the mechanism by which O_2 sensing is achieved. Moreover, debate continues regarding the possible overlap in sensing mechanisms that are involved among the diverse func-

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tional responses in different cell types. Recent reviews have provided an excellent comprehensive summary of evidence supporting different mechanisms of O_2 sensing in systems ranging in complexity from gram-negative bacteria up to mammalian cells (1, 8, 31, 49, 54). The diversity of viewpoints presented in these reviews is a testament to the absence of a consensus on the mechanisms of cellular O_2 sensing in mammalian cells. This study prolongs this trend by providing a brief overview of a number of putative O_2-sensing mechanisms and then proposing a novel mechanism based on work in our own laboratory.

THE HEME-PROTEIN HYPOTHESIS FOR OXYGEN SENSING

Much has been learned about the signal transduction sequences that are activated during hypoxia and about the mechanisms responsible for effecting the target responses. In the case of the erythropoietin gene, our understanding of the transcriptional activation response to hypoxia was advanced significantly with the identification of hypoxia-inducible factor (HIF)-1 as the transactivator (61, 62). HIF-1 is a basic helix-loop-helix member of the PAS family of transcription factors, and its active form is a heterodimer comprised of α- and β-subunits (60). Although mRNA for both subunits can be detected under normoxic conditions, the α-subunit is degraded rapidly by the ubiquitin/proteasome breakdown pathway (29, 30). Consequently, Western blotting analysis cannot detect the α-subunit during normoxia, whereas the β-subunit is constitutively present (67). Under hypoxic conditions, the α-subunit becomes stabilized, leading to dimer formation, nuclear translocation, and DNA binding. These discoveries have catalyzed a rapidly expanding understanding of additional HIF family members, which appear to participate in a wide range of cellular hypoxic responses. However, despite this growing understanding, the mechanism responsible for the posttranslational stabilization of the HIF-1 α-subunit during hypoxia has not been established.

One long-held view has been that the O_2 sensor responsible for initiating the erythropoietin transcriptional response to hypoxia involves a heme protein. According to that theory, hypoxia would be detected by an allosteric shift in a protein capable of reversibly binding O_2 at a heme site. The idea is based on the observation that injection of cobalt chloride ($CoCl_2$) induces erythropoietin secretion and polycythemia in rats (23) and that incubation of certain hepatoma cell lines (Hep 3B or Hep G2) with $CoCl_2$ leads to a dose-dependent enhancement of erythropoietin mRNA levels under normoxic conditions (22). Goldberg and colleagues (22) proposed that Co^{2+} is incorporated into newly synthesized heme, thereby locking the O_2 sensor into a deoxy configuration and so mimicking the effects of hypoxia. CO (10%), a physiologically stable molecule known to interact with heme groups, was found to inhibit the induction of erythropoietin induced during 1% O_2. This was taken as further evidence for the involvement of a heme in the O_2-sensing mechanism, presumably because CO shifted the protein to the oxy configuration. Finally, CO did not disrupt the induction of erythropoietin by Co^{2+}, which was interpreted as further evidence for the heme hypothesis (22). One concern regarding the heme protein hypothesis arose with the report by Srinivas et al. (55), who found that inhibitors of heme synthesis failed to abolish the response to hypoxia in a reporter cell system. These observations do not rule out the possible involvement of a heme protein in the O_2-sensing mechanism. However, they do raise questions about the possible role of Co^{2+} incorporation into newly synthesized heme. Conceivably, the O_2 sensor could involve a heme-containing protein, but Co^{2+} could be acting on the signal transduction pathway downstream from that site.

THE O_2-SENSITIVE ION CHANNEL HYPOTHESIS

In recent years, a number of reports have emerged suggesting that ion channels may be affected by local O_2 levels, raising the possibility that these could function as O_2 sensors in certain cell types. For example, in type I cells from the adult rabbit carotid body, Lopez-Barneo et al. (39) found that hypoxia could inhibit a K^+ current. Subsequent studies have identified a wide range of different O_2-sensitive ion channels that have been identified in different cell types (4, 40, 46).

Although it is now well established that certain ion channels adjust their conductance properties in response to changes in P_{O_2}, it is less certain whether the channels are themselves responsive to O_2 or whether the changes in conductance reflect a secondary response activated by a separate, possibly nearby, O_2 sensor. This possibility is appealing because it could explain why such a diverse collection of ion channels exhibit a sensitivity to O_2, without the need to postulate that a variety of O_2-responsive subunits are intrinsic to the channels. The notion that a nearby O_2 sensor may be involved is supported by the observations that O_2-sensitive channels are frequently responsive to oxidizing and reducing agents. In this regard, Fearon et al. (20) expressed an L-type Ca^{2+} channel α_{1c}-subunit in HEK-293 cells and found that whole cell patch-clamp recordings of Ca^{2+} currents were inhibited by hypoxia. The oxidizing agent *p*-chloromercuribenzenesulfonic acid abolished the response to hypoxia, suggesting that hypoxia may affect channel activity via redox modulation of specific thiol residues (20). The ability to demonstrate O_2-dependent channel gating in experiments with isolated membrane patches tends to suggest the involvement of a membrane-associated process (21). For example, Perez-Garcia et al. (47) found that expression of Kv4.2 channels plus KvB1.2 subunits in HEK-293 cells conferred sensitivity to redox modulation and to low P_{O_2}. Interestingly, membrane patches also demonstrated a change in conductivity during 100% N_2 exposure, which could indicate either a direct effect of P_{O_2} on the channel or the involvement of a nearby membrane-associated O_2 sensor (47). Patel et al. (45) cotransfected COS cells with Kv2.1 channels and the Kv9.3 subunit and found that channel activity was inhibited by hypoxia but only in a subset of cells

studied. They concluded that the O_2-sensing function was not intrinsic to the Kv2.1-Kv9.4 complex, on the basis of the fact that only some transfected cells responded to low O_2 concentration (45). However, an alternative interpretation is based on a recent report by Rustenbeck et al. (52), who found that mitochondria were present in excised membrane patches from pancreatic β-cells. The possibility that mitochondria may remain associated with ATP-sensitive K^+ (K_{ATP}) channels by being dragged into the pipette during inside-out excised patch experiments raises the possibility that such organelles could be involved in the O_2-sensing response. Because some membrane patches might not contain these organelles, the hypoxic response might not be observed in all experiments. This hypothesis is further fueled by their observation that mitochondrial inhibitors can affect the K_{ATP} channel behavior in those studies. Collectively, these results are intriguing but will require further testing to determine *1*) whether O_2-sensitive channels respond in the physiological range of hypoxia, *2*) whether the modest changes in gating seen at low P_{O_2} can fully explain the physiological response to hypoxia, and *3*) whether the O_2 sensitivity is intrinsic to the channel or whether the O_2 sensor resides in organelles such as mitochondria that may remain attached in excised patch experiments. In regard to this last point, it would be useful to determine if a cloned O_2-sensitive channel protein retains its response to hypoxia when inserted into a lipid bilayer, as that system would permit a direct evaluation of the effects of hypoxia on the channel in the absence of other potential O_2-sensing systems.

THE NAD(P)H OXIDASE HYPOTHESIS

A number of investigators have suggested that a potential mechanism of O_2 sensing could incorporate redox-dependent reactions involving members the NADPH oxidase family. This oxidase system is comprised of a multisubunit assembly consisting of a membrane-bound catalytic complex of $gp91^{phox}$ and $p22^{phox}$ subunits, which together form a flavo-cytochrome b_{558}, and a cytosolic regulatory component consisting of $p47^{phox}$, $p67^{phox}$, and other regulatory subunits such as the GTPase proteins Rac-1 or Rac-2. At least in neutrophils, activation of the oxidase is regulated by the cytosolic components, which translocate to the membrane where they associate with cytochrome b_{558} and activate catalytic activity (15). According to the NADPH model of O_2 sensing (24), electrons derived from NADPH are shuttled to O_2 by the oxidase at a rapid rate during normoxia, generating superoxide. This would create a relatively oxidized redox state in the cytosol, caused by the relatively rapid rate of formation of reactive O_2 species (ROS). Dismutation of superoxide would generate H_2O_2, which could be involved in local Fenton reactions at sites where ferrous iron is bound (18, 55), thereby inducing a site-specific oxidation of a regulatory protein. In either case, this model would require the catalytic subunits of the NADPH oxidase system to remain continuously activated during normoxia. During hypoxia, a decrease in the availability of O_2 would lead to a slowed rate of electron transport through this system, resulting in a slowed rate of superoxide generation and a consequent shift in the cytosol redox to a more reduced state. Because the rate of ROS generation presumably would vary with the availability of O_2, this system could effectively function as an O_2 sensor. By generating ROS levels at a rate related to O_2 concentration, such a system could also explain the changes in conductance of "O_2-sensitive K^+ channels" without the need to invoke a direct effect of O_2 on the channel itself. In an O_2-sensing system involving a membrane oxidase, hydrogen peroxide or superoxide could function as the second messenger linking the O_2 sensor (the oxidase) to the target (nearby K^+ channels) (59).

Evidence has emerged suggesting that subunit components of the NADPH oxidase system are expressed in a number of cells known to be O_2 responsive. These include neuroepithelial bodies in the lung (68), type I cells of the carotid body (36), and pulmonary vascular myocytes (41). However, few studies directly implicate these systems in the O_2-sensing function, and a number of studies point against their involvement. Patients with chronic granulomatous disease (CGD) suffer from a genetic defect in one or more of the subunits of NADPH oxidase. Loss of a subunit of that oxidase would abolish its activity and thereby abrogate the adaptive functions that depend on its participation. Patients with CGD are still able to maintain normal erythropoietin levels and can still respond to hypoxia, which suggests that NADPH oxidase function is not required for the O_2 sensing underlying erythropoietin expression. Wenger et al. (65) showed that CGD-derived cell lines deficient in either the $p22^{phox}$ or $gp91^{phox}$ subunits were still able to express vascular endothelial growth factor (VEGF) and aldolase mRNA during 1% O_2 compared with wild-type controls (65). These results further suggest that the NADPH oxidase system is not involved in the O_2 sensing underlying erythropoietin expression. It could be argued that the O_2 sensing in that response involves a different isoform of the NADPH oxidase, one not disrupted by CGD. However, a special concern relates to the effects of diphenyleneiodonium (DPI), an inhibitor of electron transport in a wide range of flavoprotein oxidases including NADPH oxidase. If hypoxia activates cellular responses via decreased ROS generation by an NADPH oxidase system, DPI should mimic the effects of hypoxia by suppressing ROS generation during normoxia. Although DPI has been shown to abolish the response to hypoxia, it does not mimic the hypoxic response during normoxia.

The NADPH oxidase model has also been invoked to explain the O_2 sensing underlying hypoxic pulmonary vasoconstriction in the lung (41). According to that hypothesis, alveolar hypoxia should decrease the production of ROS by the NADPH oxidase system, causing a shift in cytosolic redox to a more reduced state in pulmonary artery smooth muscle cells. This redox shift would then lead to the inactivation of redox-dependent membrane K^+ channels (2). The resulting membrane

depolarization would then lead to opening of voltage-dependent Ca^{2+} channels, entry of Ca^{2+}, and subsequent smooth muscle cell contraction. The current paradigm points to the probable involvement of one or more voltage-dependent K^+ channels, based in part on the observation that nonspecific K^+ channel inhibitors block the constriction elicited by hypoxia. In this system, the NADPH oxidase would function as the primary O_2 sensor, whereas the membrane ion channels would function as the effectors of the vasoconstriction response (64).

Recently, Archer et al. (3) found that transgenic mice lacking the $gp91^{phox}$ subunit of the NADPH oxidase showed a decrease in the generation of ROS detected by lucigenin chemilumenescence at the lung surface. However, the whole cell K^+ current response to hypoxia and the whole lung vasoconstrictor response to hypoxia were not inhibited. According to the NADPH oxidase model, the lack of a normally functioning oxidase system in knockout animals should mimic the redox changes normally associated with hypoxia. However, the pressure-flow relationships of the mutant mouse lungs were normal, and the responses to hypoxia were preserved, which does not fit with the model. Of course, it is possible that adaptive changes in the antioxidant systems of the knockout animal prevented such a response. However, as describe above, it is troublesome that DPI abolishes the response to hypoxia in lungs of normal animals (25, 69). Again, inhibition of the NADPH oxidase system by DPI should mimic the effects of hypoxia by limiting electron transfer through that oxidase. This would attenuate the ROS generation and associated redox changes during normoxia, resulting in a strong vasoconstrictor response. Although DPI blocks the vasopressor response to hypoxia, it does not cause sustained vasoconstriction during normoxia. Therefore, the findings of Archer et al. (3) would tend to rule out an NADPH-based mechanism as an O_2 sensor underlying hypoxic pulmonary vasoconstriction.

MITOCHONDRIAL HYPOTHESIS OF O_2 SENSING

Mitochondria have long been considered as potential sites of O_2 sensing, based on the fact that they bind O_2 and are responsible for the lion's share of O_2 utilization in the cell. Detection of anoxia ($P_{O_2} = 0$) by mitochondria is conceptually simple because electron transport and oxidative phosphorylation cease in the absence of O_2. The resulting effects on cell function are profound, and one could argue that this represents an "O_2 sensor-functional response" sequence. One might even argue that anoxic cell death represents an extreme case of this response pathway. However, a more difficult question relates to how mitochondria could detect differences in O_2 tensions within the physiological range. One possible way to signal changes in O_2 supply would be through changes in the redox state of the electron transport system or through effects on the earlier steps involved in the generation of reducing equivalents (i.e., NADH) in the Krebs cycle. Classic studies have shown that cellular respiration does not become O_2 supply limited until the extracellular P_{O_2} falls below 5–7 Torr (32, 33, 63, 66). The apparent Michaelis-Menten constant (K_m) for O_2 of cytochrome-*c* oxidase has been reported to be <1 μM, allowing state 3 respiration by mitochondria to remain independent of P_{O_2} down to <2 Torr (12, 66). If electron transport is not limited by the O_2 supply under hypoxia ($P_{O_2} = 5–50$ Torr), it is difficult to understand how mitochondrial redox could be affected by O_2 concentration. One possibility is that the "peri-mitochondrial P_{O_2}" in intact cells could be much lower than the extracellular P_{O_2}, due to O_2 gradients between the plasma membrane and mitochondria. It has also been suggested that tissue-specific isoforms of the oxidase could exist with much higher apparent K_m values (42); experimental evidence of this possibility has never emerged. In the case of the erythropoietin response, mitochondrial inhibitors such as cyanide have failed to abolish specific responses to hypoxia (43, 48), which suggests that mitochondrial electron transport must not be involved. However, in the case of the carotid body, it has long been known that cyanide can produce a potent stimulation. This and other observations have led some investigators to hypothesize that mitochondria are involved in the O_2 transduction process in that organ (7). However, the apparent differences in responses to mitochondrial inhibitors in different O_2-sensing systems have led to controversy regarding the role(s) that mitochondria play in the O_2 sensing among different cell types (8). Of course, it is possible that mitochondria function in an O_2-sensing capacity in some systems but not in others. In either case, an attractive feature of a mitochondrial role is that, by virtue of their existence in virtually all cells, they could potentially confer a universal sensitivity to O_2 and explain the many similarities in the responses to low P_{O_2} seen among many diverse cell types.

Recent studies from our laboratory point to a possible role of cytochrome oxidase in the response to hypoxia (5, 6, 10–12). Data suggest that, during hypoxia, the V_{max} of the oxidase is reversibly decreased, whereas the apparent K_m remains unchanged. The decrease in V_{max} (~50%) does not directly limit normal respiration but does affect mitochondrial redox by requiring that the electron carriers operate in a more reduced state. This is manifested by a significant increase in the NADH concentration of the cell during moderate hypoxia (53). This adaptation in enzyme function appears to develop over 1–2 h in some cell types (11), whereas other cells undergo the change more rapidly (6). Interestingly, return of the enzyme to its "normoxic" state appears to occur rapidly on reoxygenation in all cell types. If this change in cytochrome oxidase function represents the primary O_2 sensor, how is this signal transmitted throughout the cell? More recent evidence points to ROS as important elements in the signal transduction process.

THE MITOCHONDRIA-DERIVED ROS HYPOTHESIS

During mitochondrial respiration, O_2 is chemically reduced to water by the transfer of four electrons at cytochrome oxidase. The resulting free energy change

is conserved in the form of ATP synthesis. It has been estimated that 2–3% of the O_2 consumed by mitochondria is incompletely reduced, yielding ROS (9). Univalent electron transfer to O_2 generates superoxide, a modestly stable free radical anion. Superoxide can potentially be generated at a number of different sites, including complex I, the ubisemiquinone site of complex III, and other electron transfer proteins (56). ROS do not appear to be generated by cytochrome oxidase itself (19), due to the high-affinity kinetic trapping of O_2 at the binuclear center, which occurs while the four electrons are sequentially transferred (58). The tendency for the electron transport chain to generate superoxide through promiscuous electron transfer to O_2 depends on factors that include the availability of O_2, the reduction state of the electron carriers, and the mitochondrial membrane potential (16, 35, 56, 57). It is conceivable that the decrease in cytochrome oxidase V_{max} during hypoxia (11, 12) (see above) is responsible for an increase in mitochondrial redox state and that this in turn accelerates ROS generation during hypoxia. However, definitive evidence of this hypothesis has not been presented.

Recent data do suggest that ROS generated by mitochondria play a physiological role in the cellular responses to hypoxia (13, 17). Like nitric oxide, superoxide and hydrogen peroxide are moderately stable reactants that are potentially useful as intracellular or even intercellular signaling molecules. Using fluorescent dyes to detect an oxidative signal, Duranteau and colleagues (17) studied the oxidation of 2′,7′-dichlorofluorescin (DCFH) in cardiomyocytes under controlled O_2 conditions (17). The cells were studied in a flow-through chamber (0.5 ml) maintained at 37°C on an inverted microscope. The perfusate (1 ml/min) was bubbled with different O_2 concentrations in a water-jacketed equilibration column mounted above the microscope stage and was delivered to the chamber via a short length of tubing. The reduced diacetate form of the dye DCFH was continually present in the medium (5 μM). Oxidation of the dye within the cell yields the fluorescent compound 2′,7′-dichlorofluorescein (DCF), which was detected with a 12-bit digital cooled charge-coupled device camera. In the absence of dye, no fluorescence can be detected. After addition of dye to the medium, evidence of cell fluorescence is detected within a few minutes. Dye oxidized outside of the cell, or dye oxidized within the cell that leaks out, is carried away in the perfusate flow. Under steady-state conditions, cellular fluorescence reflects a balance between the rate of oxidation of DCFH in the cell and the rate at

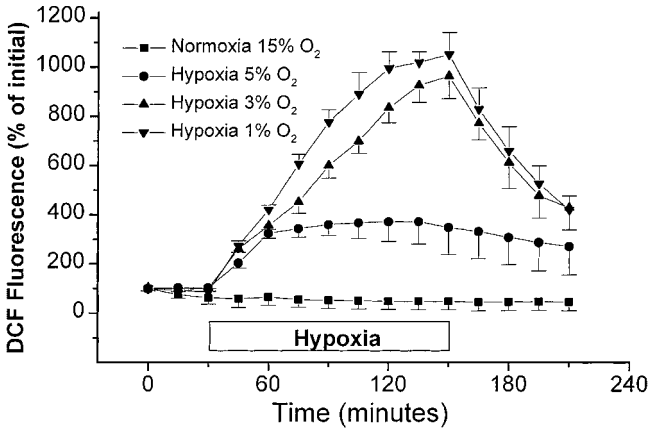

Fig. 1. Increases in 2′,7′-dichlorofluorescein (DCF) fluorescence during hypoxia in embryonic cultured cardiomyocytes studied in a flow-through chamber. Lower levels of P_{O_2} were associated with greater increases in 2′,7′-dichlorofluorescin (DCFH) oxidation. Period of hypoxic exposure is indicated by the box. [From Ref. 17, with permission.]

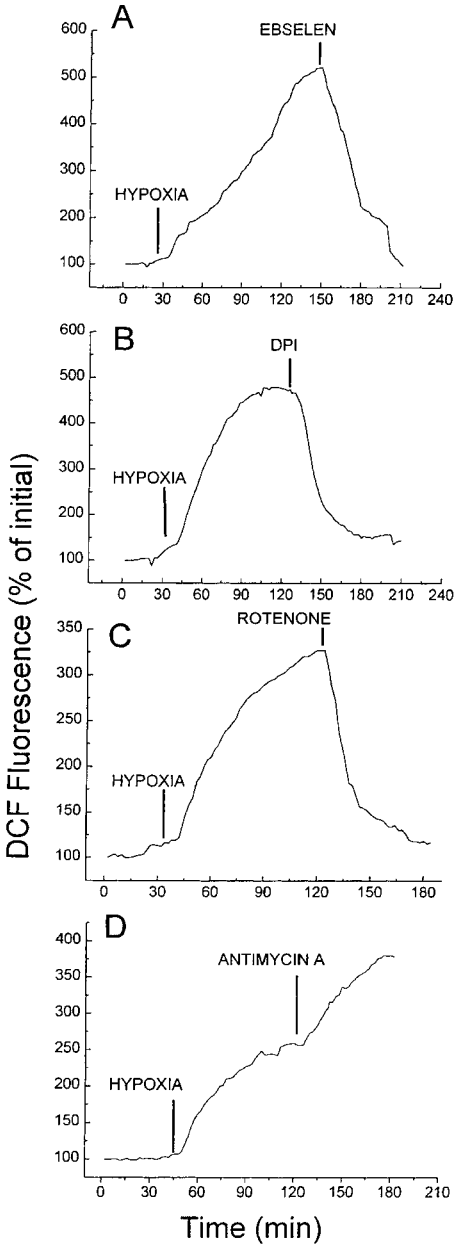

Fig. 2. *A*: effects of the antioxidant ebselen on oxidant signaling during hypoxia in Hep 3B cells. *B*: effects of the flavoprotein inhibitor diphenyleneiodonium (DPI) on oxidant signaling during hypoxia. *C*: effects of the mitochondrial complex I inhibitor rotenone on oxidant signaling during hypoxia. *D*: effects of the mitochondrial inhibitor antimycin A on oxidant signaling during hypoxia. [From Ref. 13, with permission.]

which oxidized dye escapes and is carried away. Paradoxically, oxidation of DCFH increased as the O_2 concentration was decreased from normoxic levels (16% O_2), with minimal effects seen at 8% and a maximal effect observed at 1% O_2 (Fig. 1). Within minutes of decreasing the O_2 concentration, intracellular fluorescence increased, reflecting an increase in the rate of dye oxidation. On return to normoxia, fluorescence decreased as the rate of escape of oxidized dye exceeds the rate of dye oxidation in the cell.

What causes oxidation of the dye? In vitro studies of DCFH suggest that H_2O_2 cannot directly oxidize the dye, whereas H_2O_2 addition to intact cells leads to rapid oxidation (27, 37, 50, 51). Studies indicate that DCFH is oxidized enzymatically in the cells via peroxidases that require H_2O_2 for the process (27). This suggests that DCFH oxidation can provide a sensitive measure of H_2O_2 generation in cells. There is no evidence to suggest that intracellular reductases can reduce the dye once it has been oxidized. Superoxide itself is not effective at oxidizing DCFH (37), although H_2O_2 generated by the dismutation of superoxide in cells can lead to rapid oxidation. Hydroxyl radical produced by H_2O_2 in the presence of ferrous iron can also oxidize the dye (37), as can nitric oxide (26). Regardless of the precise mechanism of dye oxidation, the appearance of cellular fluorescence reflects the rate of accumulation of DCF, which is a rather indirect measure of the rate of ROS generation. In a cell type that responds rapidly to hypoxia such as the carotid body, an immediate increase in DCF fluorescence would not be predicted even if ROS generation increased rapidly, due to the need to accumulate sufficient levels of oxidized dye to be able to detect a change in fluorescence. Because of its potential oxidation by a number of different mechanisms, the DCFH oxidation during hypoxia is, by itself, insufficient to demonstrate that the O_2-sensing pathway involves ROS generation. Ultimately, the role of mitochondrial oxidants in the signaling activated by hypoxia needs to be tested by other approaches, to determine whether the functional responses to hypoxia also appear to depend on oxidant signaling pathways originating in the mitochondria.

The observation that DCFH oxidation increases during hypoxia suggested that an oxidant stress is generated at low P_{O_2}, although the source of the ROS was not initially clear. To determine whether mitochondria were responsible for the oxidant signal seen during hypoxia in Hep 3B cells, which secrete erythropoietin during hypoxia, studies were carried out using inhibitors of electron transfer (13). As seen with cardiomyocytes, hypoxia increased DCFH oxidation in Hep 3B cells, with the greatest increases seen at the lowest P_{O_2} levels (1% O_2) (13). The antioxidant ebselen, a glutathione peroxidase mimetic, abolished the DCFH response to hypoxia, presumably by enhancing the scavenging of H_2O_2. (Fig. 2*A*) The antioxidant pyrrolidinedithiocarbamate (PDTC), a thiol reductant, similarly attenuated oxidant signaling. Addition of DPI to Hep 3B cells also led to an attenuation of fluorescence, suggesting that a flavin-containing electron carrier was required (Fig. 2*B*). However, a large number of oxiodo-reductase systems utilize flavin groups, including mitochondrial complex I, NADPH oxidase, and nitric oxide synthase (28, 38, 44). Hence, DPI is not useful in identifying the source of the oxidant signal. Rotenone, a selective inhibitor of mitochondrial complex I, caused a decrease in DCF fluorescence, suggesting that the rate of oxidation had decreased (Fig. 2*C*) (13, 17). From this, it can be inferred that the oxidation of DCFH requires electron transport in the proximal region of the mitochondrial electron transport chain. Myxothiazol is an inhibitor of electron transfer to the Rieske iron-sulfur center of the mitochondrial bc1 complex, and this compound similarly attenuated DCFH oxidation during hypoxia.

These data are compatible with the O_2-sensing model depicted in Fig. 3. During normal mitochondrial respiration, reducing equivalents generated in aerobic glycolysis or in the Krebs cycle are passed along a chain of electron acceptors with progressively greater oxidation-reduction potentials. At complexes I, III, and IV, the free energy released is captured and utilized to extrude a proton from the mitochondrial matrix into the intermembrane space. This process generates an electrochemical potential across the inner mitochondrial membrane, which is used by the F_0F_1 ATP synthase for the

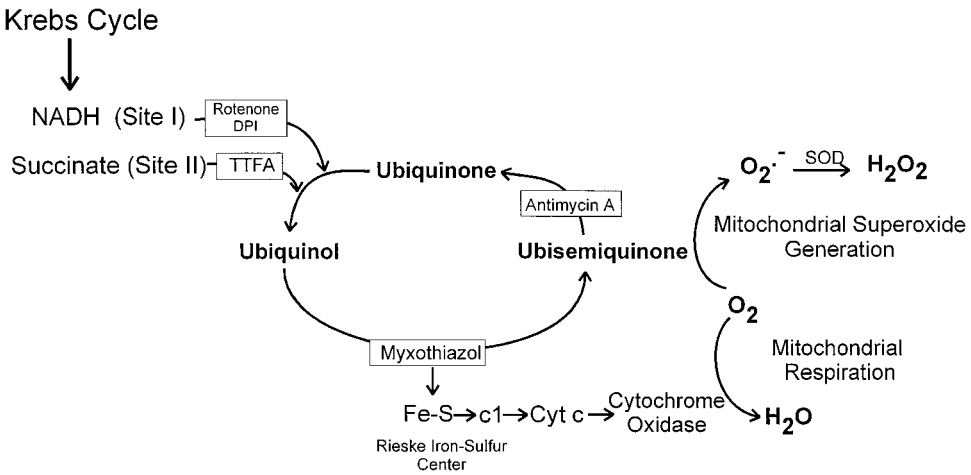

Fig. 3. Proposed model of mitochondrial oxidant signal generation involved in O_2 sensing. Mitochondrial respiration involves transport of reducing equivalents generated in the electron transport chain; through the electron transport chain to cytochrome oxidase, which transfers 4 electrons to O_2. Ubisemiquinone, a free radical intermediate in the electron transport chain, can potentially generate superoxide via univalent electron transfer to O_2. Boxes show sites of inhibition. SOD, superoxide dismutase; cyc c, cytochrome *c*.

generation of ATP from ADP and P_i. In the bc1 complex, ubiquinol becomes oxidized to ubisemiquinone as it passes an electron to the Rieske iron-sulfur center. Ubisemiquinone is a free radical that can transfer an electron to molecular O_2, yielding superoxide. The generation of superoxide likely represents a balance between the availability of O_2 and the size of the ubisemiquinone pool (56). Inhibitors that block electron transfer upstream from that site (DPI, rotenone, myxothiazol, Amytal) tend to prevent the formation of ubisemiquinone and thereby diminish ROS generation. Mitochondrial inhibitors that act at more downstream sites tend to augment ROS generation by increasing the generation of ubisemiquinone. For example, cyanide and azide, which inhibit electron transfer by cytochrome oxidase, tend to augment ROS generation by mitochondria. Likewise, antimycin A augments ROS generation by inhibiting electron transfer at the downstream end of the bc1 complex (Fig. 2*D*). Thus, although a number of different mitochondrial inhibitors all block electron transport, they exhibit differential effects on ROS generation depending on their site of action relative to the ubisemiquinone step. To the extent that cytochrome oxidase functions as the ultimate O_2 transducer in this response (12), the hypoxia-mediated changes in its function could explain the changes in mitochondrial redox responsible for changes in super-

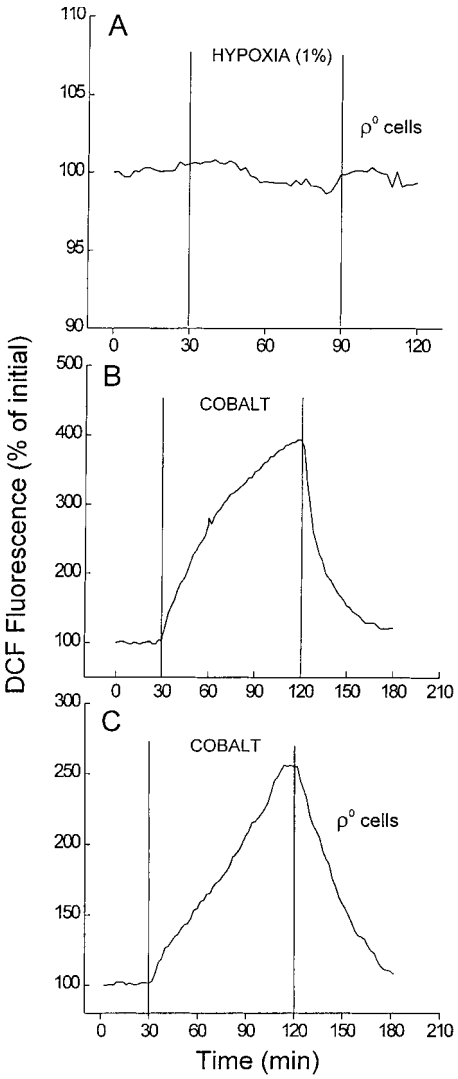

Fig. 4. *A*: DCFH oxidation in mutant ρ^0 Hep 3B cells under controlled O_2 conditions. Unlike wild-type cells, increased DCFH oxidation was not detected during hypoxia. *B*: effects of $CoCl_2$ on oxidant DCFH oxidation during normoxia in wild-type Hep 3B cells. Increased DCFH oxidation suggests increased reactive O_2 species (ROS) generation. *C*: effects of $CoCl_2$ on oxidant DCFH oxidation during normoxia in ρ^0 Hep 3B cells. Increased DCFH oxidation suggests increased ROS generation despite the absence of mitochondrial electron transport. [From Ref. 13, with permission.]

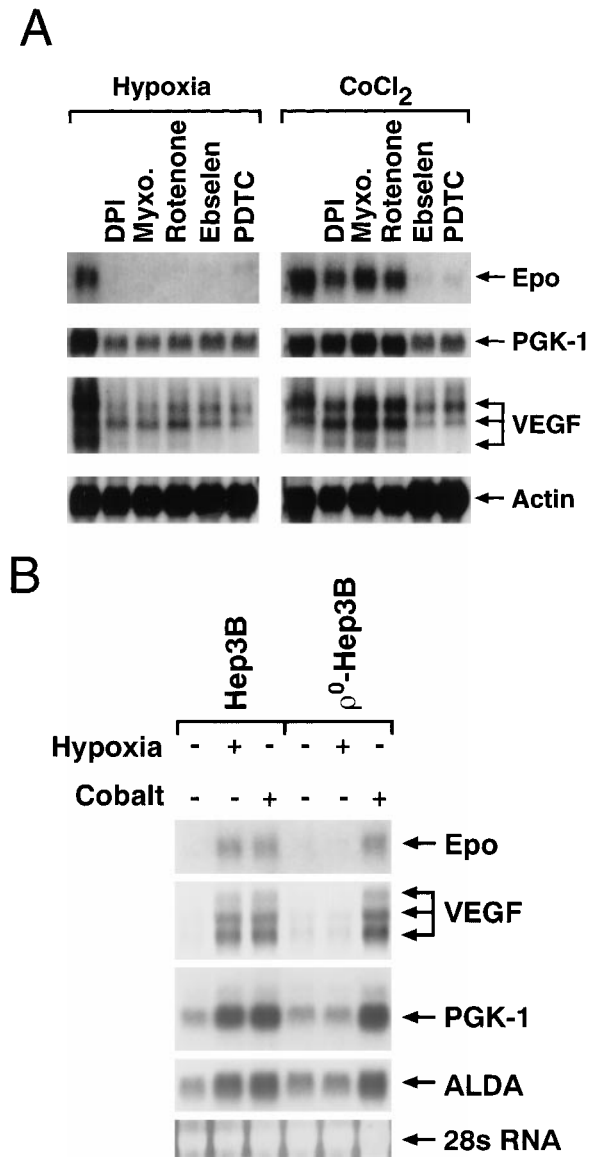

Fig. 5. *A*: Northern blots of mRNA expression for erythropoietin (Epo) and vascular endothelial growth factor (VEGF) in Hep 3B cells during hypoxia or $CoCl_2$ administration. Mitochondrial inhibitors DPI, myxothiazol (myxo), and rotenone abolished the response to hypoxia only, whereas antioxidants ebselen and pyrrolidinedithiocarbamate (PDTC) abolished expression in response to both hypoxia and $CoCl_2$. *B*: Northern blots of mRNA expression for Epo and VEGF in wild-type and ρ^0 Hep 3B cells. The ρ^0 Hep 3B cells did not respond to hypoxia but retained the ability to activate expression in response to $CoCl_2$. ALDA, aldolase A. [From Ref. 13, with permission.]

oxide generation at the ubisemiquinone step. However, it is also possible that superoxide generation by ubisemiquinone is regulated directly by O_2 concentration.

To approach this problem using a genetic tool, we generated ρ^0 cells, a functional mitochondria-deficient cell line. Rapidly dividing cells can be mutated into a ρ^0 state by incubation with ethidium bromide, which inhibits the replication of mitochondrial DNA that is required for critical subunits of certain mitochondrial electron transport complexes and for part of the F_0F_1 ATP synthase. The ρ^0 cells are therefore incapable of supporting mitochondrial respiration or oxidative phosphorylation. Survival and growth of ρ^0 cells requires glycolytically derived ATP (34). Because ρ^0 cells lack mitochondrial electron transport, they provide insight into mechanisms requiring mitochondrial redox changes or requiring the generation of mitochondrial ROS (14). In ρ^0 Hep 3B cells, hypoxia failed to stimulate oxidative signaling, as evidenced by an absence of DCFH oxidation (Fig. 4*A*) (13). How does $CoCl_2$ mimic the effects of hypoxia? Studies with DCFH dye suggest that Co^{2+} augment ROS generation to an extent that mimics the response during hypoxia (Fig. 4*B*). Interestingly, although ρ^0 Hep 3B cells failed to increase ROS generation during hypoxia, they retained the ability to generate ROS during Co^{2+} treatment, suggesting that $CoCl_2$ mimics hypoxia by stimulating ROS generation via a nonmitochondrial pathway (Fig. 4*C*). Ebselen and PDTC blocked the response to Co^{2+} treatment in ρ^0 cells, suggesting that Co^{2+} was generating an oxidant signal.

The physiological significance of the above signaling system can be seen in the regulation of gene expression of erythropoietin and VEGF during hypoxia. Wild-type Hep 3B cells activate mRNA expression during hypoxia or in response to $CoCl_2$ (100 μM) during normoxia (Fig. 5*A*) (13). The mitochondrial inhibitors DPI, myxothiazol, and rotenone abolished the mRNA message and DNA binding of HIF-1 in response to hypoxia, consistent with a role of mitochondria in the response (13). The antioxidant ebselen, a glutathione peroxidase mimetic, and PDTC, a metal chelator, both abolished the response to hypoxia, presumably by enhancing the scavenging of H_2O_2. During normoxia, $CoCl_2$ treatment activated the erythropoietin mRNA message and antioxidants abolished this response. However, mitochondrial inhibitors were ineffective in blocking the response to Co^{2+}, which is consistent with the involvement of a nonmitochondrial ROS generating mechanism with Co^{2+}. In ρ^0 cells, the response to hypoxia was absent but the response to $CoCl_2$ was retained, which again was consistent with a mitochondrial sensing mechanism (Fig. 5*B*). Collectively, these results suggest that hypoxia-induced increases in mitochondrial ROS are required for the activation of HIF-1.

Clearly, many questions remain regarding the mechanisms by which mitochondria contribute to the process of O_2 sensing. The story of how mitochondrial oxidants may participate in the O_2-sensing response is not yet complete, and additional work must be done to clarify the underlying mechanisms of that system and the extent to which it may function in diverse systems ranging from the carotid body to the Hep 3B cell. A recent resurgence of interest in mitochondria has occurred over the past several years with the realization that, in addition to being the major supplier of ATP, mitochondria play an important role in signal transduction, including the early events of apoptosis, and in glucose sensing in pancreatic islet cells. The possibility that mitochondria may also function as a site of O_2 sensing is an exciting one, for it opens the door to the suggestion that many of the diverse cellular responses to hypoxia may involve this interesting organelle.

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