FORUM ORIGINAL RESEARCH COMMUNICATION

Transcriptional Regulation of Yeast Oxidative Phosphorylation Hypoxic Genes by Oxidative Stress

Jingjing Liu¹ and Antoni Barrientos^{1,2}

Abstract

Aims: Mitochondrial cytochrome *c* oxidase (COX) subunit 5 and cytochrome *c* (Cyc) exist in two isoforms, transcriptionally regulated by oxygen in yeast. The gene pair *COX5a/CYC1* encodes the normoxic isoforms (Cox5a and iso1-Cyc) and the gene pair *COX5b/CYC7* encodes the hypoxic isoforms (Cox5b and iso2-Cyc). Rox1 is a transcriptional repressor of *COX5b/CYC7* in normoxia. *COX5b* is additionally repressed by Ord1. Here, we investigated whether these pathways respond to environmental and mitochondria-generated oxidative stress. **Results:** The superoxide inducer menadione triggered a significant de-repression of *COX5b* and *CYC7*. Hydrogen peroxide elicited milder de-repression effects that were enhanced in the absence of Yap1, a key determinant in oxidative stress resistance. *COX5b/CYC7* was also de-repressed in wild-type cells treated with antimycin A, a mitochondrial *bc*₁ complex inhibitor that increases superoxide production. Exposure to menadione and H₂O₂ enhanced both, Hap1-independent expression of *ROX1* and Rox1 steady-state levels without affecting Ord1. However, oxidative stress lowered the occupancy of Rox1 on *COX5b* and *CYC7* promoters, thus inducing their de-repression. **Innovation:** Reactive oxygen species (ROS)-induced hypoxic gene expression in normoxia involves the oxygen-responding Rox1 transcriptional machinery. Contrary to what occurs in hypoxia, ROS enhances Rox1 accumulation. However, its transcriptional repression capacity is compromised. **Conclusion:** ROS induce expression of hypoxic *COX5b* and *CYC7* genes through an Ord1- and Hap1-independent mechanism that promotes the release of Rox1 from or limits the access of Rox1 to its hypoxic gene promoter targets. *Antioxid. Redox Signal.* 19, 1916–1927.

Introduction

OXYGEN IS THE terminal electron acceptor during mitochondrial oxidative phosphorylation (OXPHOS), which is the major biochemical reaction that provides energy in the form of ATP to sustain aerobic life of eukaryotic organisms (37). As byproducts, unstable reactive oxygen and nitrogen species can form during respiration (10, 38) and act as signaling molecules (38) or, when in excess, can be harmful to the cells (10).

In all eukaryotes, cells, tissues, and organisms have acquired programs to sense oxygen levels and take action accordingly with short-term and long-term responses (2, 37). These programs require sensors and dedicated transcriptional factors that will help reprogramming gene expression (20, 37). In the yeast *Saccharomyces cerevisiae*, adaptation to hypoxia/anoxia involves the induction or de-repression of a multiplicity of genes co-regulated by specific transcriptional factors, including the *heme*-dependent general aerobic repressor Rox1 (31); the *heme*-independent aerobic repressor Ord1, which is specific to a few hypoxic genes (29); as well as several gene-specific activators (1).

One of the ways in which cells adapt to hypoxic conditions is by building a more efficient mitochondrial respiratory chain (MRC). A key MRC enzyme is cytochrome *c* oxidase (COX), which catalyzes electron transfer from ferrocytochrome *c* to

Innovation

Our study highlights the mechanism by which oxidative phosphorylation hypoxic genes are transcriptionally de-repressed by oxidative stress in the yeast *Saccharomyces cerevisiae*. Under oxidative stress, the general repressor of hypoxic gene Rox1 is overexpressed in a Hap1-independent manner. However, expression of hypoxic *COX5b* and *CYC7* genes is significantly induced through a mechanism that involves the release of Rox1 from their target promoters independently of other transcriptional factors, such as Ord1. Thus, this study will impact biology by adding new dimensions in understanding how overlapping programs exist to sense oxygen deprivation and oxidative stress and adapt to respond to these challenges.

Departments of ¹Biochemistry & Molecular Biology and ²Neurology, University of Miami Miller School of Medicine, Miami, Florida.

molecular oxygen *via* the four redox active metal cofactors present in its catalytic core. Electrons enter COX through the dinuclear Cu_A site, located in subunit 2, and are sequentially transferred to a low-spin *heme a* located in subunit 1 and then intramolecularly to the active site where a high-spin binuclear *heme a*₃-Cu_B forms a center for oxygen binding and reduction. The electron transfer reaction is coupled to proton translocation from the matrix to the intermembrane space thus contributing to the generation of the proton gradient used to drive ATP synthesis. COX is a hetero-oligomeric protein complex formed by 11 subunits in yeast. Three mitochondrial DNA-encoded subunits form the catalytic core of the enzyme. The remaining subunits are encoded in the nuclear DNA, and act as a protective shield surrounding the catalytic core (19). Importantly, COX regulates the overall electron flow through the MRC, and contains oxygen-regulated subunit isoforms in yeast and mammals.

In yeast, COX subunit 5 exists in two interchangeable isoforms encoded in the nuclear genes *COX5a* and *COX5b*. Their expression is inversely regulated transcriptionally by oxygen concentration and *heme* (25, 37). Noticeably, cytochrome *c* (*Cyc*) also exists in two oxygen-regulated isoforms, iso1 (encoded by *CYC1*) and iso2 (encoded by *CYC7*) (9). *COX5a* and *CYC1* are expressed in normoxia whereas *COX5b* and *CYC7* are expressed under hypoxia. In normoxia, in the presence of *heme*, the transcriptional complex Hap2/3/4/5, which controls the expression of most yeast nuclear genes involved in mitochondrial biogenesis, activates *COX5a* and *CYC1* transcription. Simultaneously, the *heme*-induced transcriptional activator Hap1 activates the expression of Rox1 (43), a transcriptional repressor that inhibits the aerobic transcription of *COX5b* and other hypoxic genes, such as *CYC7* (31). Interestingly, the *heme*-independent transcriptional repressor, Ixr1/Ord1, specifically represses *COX5b* transcription under normoxia (29). Under hypoxia, *heme* levels are low thus limiting the activity of Hap1 and the Hap2/3/4/5 complex. Consequently, *COX5a* expression is not induced, while *COX5b* expression is de-repressed, which enables the assembly of COX containing the hypoxic isoform. Mutations in *rox1* and *ord1* are known to increase normoxic Cox5b levels enough to allow respiratory growth of a yeast strain carrying a null *cox5a* allele (29, 43). However, the existence of two repressors of aerobic *COX5b* expression is intriguing and it remains to be explored whether they act independently on the *COX5b* promoter and whether they respond to different stimuli.

The two Cox5 isoforms confer different kinetic properties to the COX holoenzyme (3, 44). The hypoxic Cox5b isoform, which acts synergistically with the hypoxic iso-2 *cyc* isoform, modifies an internal step in electron transport between *heme a* and the binuclear *heme a*₃-Cu_B reaction center that alters COX kinetics, enhancing the catalytic constant (3). As a result, by regulating the proportion of each isoform assembled into the holoenzyme, the catalytic activity of COX is modulated thus resulting in the adjustment of the MRC electron transfer rate to changes in environmental oxygen tension. The recent discovery that, in hypoxic conditions, hypoxic COX produces higher amounts of nitric oxide (NO) than the normoxic enzyme further emphasizes the role of COX in hypoxic signaling, since NO serves as an additional signal to activate expression of several hypoxic genes (11).

Recent evidences have linked hypoxic signaling to oxidative stress: (i) the expression of hypoxic genes in both yeast and mammalian cells requires mitochondria-generated re-

active oxygen species (ROS) (22, 27); (ii) the redox state of *heme* is important for the hypoxic response in yeast (28); (iii) when shifted from normoxia to hypoxia, cells undergo a transient increase in ROS levels, which could act as a signal that induces hypoxic gene expression both in yeast (18) and mammalian tissues (7, 14).

Thus, it is reasonable to hypothesize that the very same program that evolved to maximize energy production and O₂ utilization in hypoxia may be also used to control the response to oxidative stress. Here, we have investigated how oxidative stress could induce hypoxic gene expression in yeast cells, with a focus on the hypoxic gene pair *COX5b* and *CYC7*. We found that full aerobic *COX5b* repression requires both Rox1 and Ord1 to act synergistically on the *COX5b* promoter. However, in oxidative stress conditions, only Rox1 plays an important role in *COX5b* and *CYC7* de-repression *via* a mechanism distinct from hypoxic signaling. Oxidative stress unexpectedly enhances *ROX1* expression but prevents Rox1 occupancy on its target gene promoters, thus inducing hypoxic gene de-repression.

Results

Rox1 and Ord1 play complementary but nonoverlapping roles on COX5b expression

To explore the regulation of *COX5b* expression, we started by analyzing whether the known *COX5b* normoxic repressors, Ord1 and Rox1, play the same role on the *COX5b* promoter. We observed that in normoxia, *COX5b* expression is increased similarly in either *rox1Δ* or *ord1Δ* mutant cells, approximately threefold of wild-type (WT) levels (Fig. 1A). However, *COX5b* expression is further induced (about sixfold) in a double-mutant *rox1Δord1Δ* strain (Fig. 1A), suggesting that Rox1 and Ord1 independently contribute to *COX5b* repression. A similar pattern of *COX5b* expression was observed when the cells were grown in hypoxia (0.5% oxygen) (Fig. 1B), probably because residual amounts of Rox1 and normoxic levels of Ord1 accumulate (Fig. 1C) and act on the *COX5b* promoter in these conditions.

In contrast, when the experiments were performed in anoxia, *COX5b* expression was similar in WT, *Δrox1*, *Δord1*, and in *Δrox1Δord1* cells (approximately six- to sevenfold higher than in normoxia) (Fig. 1D), indicating a full *COX5b* de-repression in these conditions. Analysis of *ORD1* indicated that unlike *ROX1*, whose expression is regulated by oxygen levels (45), *ORD1* expression is oxygen independent and Ord1 accumulates at similar levels both in normoxia and hypoxia (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars). However in anoxia, Ord1 steady-state levels are increased (Fig. S1B) and, further, chromatin immunoprecipitation (CHIP) analyses showed that *COX5b* promoter occupancy by Ord1 was enhanced by approximately fivefold in anoxia (Supplementary Fig. S1C), thus suggesting basic differences in the mechanism of action of Rox1 and Ord1.

The Northern blot analyses presented in Figure 1C showed that the expression of *ROX1* and *ORD1* in the yeast genetic background used here (W303) is not cross-regulated as previously proposed for BY4741 cells (13). This was further substantiated by quantitative polymerase chain reaction (PCR) analyses that showed that expression of *ROX1* and *ORD1* both in normoxia and in anoxia is independent of their respective protein products (Supplementary Fig. S1D).

FIG. 1. Synergistic repression of *COX5b* by *Ord1* and *Rox1*. (A) Northern blot analyses of *COX5b* mRNA levels in normoxia (cultures exposed to atmospheric 21% oxygen levels) and (B) in hypoxia (cultures exposed to 0.5% O₂) and (C) mRNA levels of *ROX1* and *ORD1* in hypoxia. Cells were exposed to hypoxia by incubation in a hypoxic glove box (Coy Laboratory Products, Inc., Grass Lake, MI). A probe against actin (*ACT1*) was used as loading control. The graphs represent the quantification of the *COX5b/ACT1*, *ROX1/ACT1*, and *ORD1/ACT1* signals. To quantify the signals, the images were digitalized and densitometric analyses were performed using the histogram function of the Adobe Photoshop program. All Northern blot analyses were performed in duplicate using independent samples. The quantification values obtained in both repetitions did not differ by more than 5%. (D) Quantitative RT-PCR analyses of *COX5b* expression on the indicated strains following 4 h in anoxia (0% oxygen). For these studies, yeast cultures were supplemented with 0.5% Tween80 and 12 μ g/ml ergosterol to maintain cell integrity/viability in the absence of *heme* while they were exposed to an oxygen-free environment (90% nitrogen, 5% hydrogen, and 5% CO₂, 37°C) using a COY anaerobic chamber (COY Laboratory Products, Inc.). Following 4 h of incubation, the cells were placed on ice, immediately cold washed with 1% DEPC-treated water, and frozen at -80°C before processing. Error bars represent the mean $\pm$ SD of three independent experiments. COX, cytochrome *c* oxidase; DEPC, diethyl pyrocarbonate; RT-PCR, reverse transcriptase-polymerase chain reaction; SD, standard deviation; WT, wild-type.

Taken together, our results show that *ORD1* and *ROX1* expression is regulated differently and that they play complementary but nonoverlapping roles in the regulation of *COX5b* expression.

Superoxide triggers induction of *COX5b/CYC7* expression

The different roles of *Rox1* and *Ord1* on *COX5b* repression suggested the hypothesis that maybe *ORD1* expression and function could be regulated by environmental or cell intrinsic factors other than oxygen levels, such as oxidative stress. Gene expression profiles obtained by microarray analysis have previously shown that *COX5b* is upregulated although at different levels in response to various ROS-generating compounds, including H₂O₂, menadione, and diamide (21).

We have explored in our system whether menadione, a superoxide-generating compound, affects *COX5b* expression. When WT cells were challenged with a mild menadione stress (0.5 mM), we observed a time-dependent induction of *COX5b* expression (Fig. 2A) as well as an increase in the expression of both *ROX1* and *ORD1* (Fig. 2B). The effect of menadione was not exclusive on *COX5b* because expression of at least two additional hypoxic genes, *CYC7* and *ANB1*, which are regulated by *Rox1* but not by *Ord1* (31), was also found to be induced (Fig. 2C). *COX5b* expression triggered by menadione was similar in *rox1Δ*, *ord1Δ*, and *rox1Δord1Δ* cells and significantly higher than in WT cells (Fig. 2D).

These results suggest that superoxide induces a cell response involving expression of at least some *Rox1*-regulated hypoxic genes.

FIG. 2. Menadione induces expression of hypoxic genes. **(A)** Quantitative RT-PCR analysis of *COX5b* expression in WT cells incubated for up to 3 h in the presence of the indicated concentrations of menadione. **(B)** Quantitative RT-PCR analysis for the expression of the transcriptional factors *ROX1* and *ORD1*, and **(C)** the hypoxic genes *CYC7* and *ANB1* (coding for the hypoxic isoform of the translation elongation factor eIF-5A) in cells incubated in the presence of menadione. **(D)** Quantitative RT-PCR analysis of *COX5b* expression in WT *rox1* Δ , *ord1* Δ , and *rox1* Δ *ord1* Δ cells incubated during 2 h in the presence of the 0.5 mM menadione. Error bars represent the mean $\pm$ SD with *p*-values from comparisons to untreated cells denoted by **p* < 0.05. *Cyc*, cytochrome *c*.

Hydrogen peroxide enhances expression of *COX5b* and *CYC7*

To understand the specificity of the menadione effect, we challenged WT cells with a different kind of ROS, namely, H_2O_2 . Northern blot analyses showed a H_2O_2 -dose-dependent increase in *ROX1* expression, while no significant change was observed in the expression of *ORD1* (Fig. 3A). These results were confirmed by quantitative reverse transcriptase-PCR (RT-PCR) analyses (Fig. 3B). Unexpectedly, the increase in *ROX1* expression was shown to be Hap1 independent (Supplementary Fig. S2). Quantitative RT-PCR analyses additionally showed that H_2O_2 treatment of WT cells triggered a modest but consistent increase in *COX5b* expression, while the expression of *CYC7* was significantly increased by more than twofold (Fig. 3C). We tested the effect of H_2O_2 on *COX5b* expression in the absence of each of its two normoxic repressors. H_2O_2 treatment of *rox1* Δ and *rox1* Δ *ord1* Δ strains did not induce any significant change on *COX5b* expression on these strains, while treatment of the *ord1* Δ strain triggered a modest increase in *COX5b* expression (Fig. 3D).

Next, we asked whether the H_2O_2 effect on hypoxic genes was reproduced in a *yap1* Δ strain. Yap1 is a transcription factor, major regulator of the defense response to oxidative stress, and *yap1* Δ cells are known to have limited H_2O_2 tolerance and higher intracellular ROS levels (39). The *yap1* Δ mutation produced minor effects on the expression of *ROX1* and *ORD1* and just a slight increase in *COX5b* expression, similar to H_2O_2 -treated WT cells (Fig. 3E). Exposure to H_2O_2 induced a similar pattern of expression of *ROX1* and *ORD1* in *yap1* Δ and WT cells (Fig. 3E). However, *COX5b* expression

was significantly increased in *yap1* Δ cells (Fig. 3E), similar to the increase observed in menadione-treated WT cells. Exposure of *yap1* Δ to H_2O_2 could secondarily generate additional ROS, including superoxide, which would contribute to further increase in *COX5b* expression.

ROS target *Rox1* to facilitate induction of *COX5b* and *CYC7* expression

The induction of hypoxic gene repressors and their targets by oxidative stress is intriguing. To understand the mechanism behind this observation, we proceeded to analyze the steady-state protein level of *Rox1* and *Ord1* upon oxidative stress treatment.

Rox1 and *Ord1* steady-state protein levels were analyzed in cells exposed to either H_2O_2 or menadione. *Ord1* levels were found unchanged in WT and *yap1* Δ cells exposed to either menadione or H_2O_2 (Fig. 4A, B). On the contrary, and consistently with *ROX1* mRNA levels, *Rox1* was increased 1.5–2-fold in WT cells treated with each compound (Fig. 4C, D). The increased accumulation of *Rox1* in these conditions was found to depend upon the presence of Yap1 (Fig. 4D). These results indicate that the mechanism of hypoxic gene de-repression does not result from a failure of transcriptional repressors to accumulate.

Fast adaptation to different stresses is often achieved through the translocation of transcriptional factors to/out of the nucleus and the binding or not of these transcriptional factors to their target genes. We thus hypothesized that the induction of either *COX5b* or *CYC7* may be achieved through the absence of their normoxic repressor/s from their promoter

FIG. 3. Hydrogen peroxide induces expression of hypoxic genes. (A) Northern blot analyses of ROX1 and ORD1 expression in WT cells incubated in the presence of increasing H_2O_2 concentrations during 1 h. To quantify the signals in the lower panel, the images were digitalized and densitometric analyses were performed using the histogram function of the Adobe Photoshop program. The analysis of two independent experiments did not differ by more than 5%. (B) Quantitative RT-PCR analysis for the expression of the transcriptional factors ROX1 and ORD1 in WT cells incubated in the presence or absence of 1 mM H_2O_2 during 1 h. (C) Quantitative RT-PCR analysis for the expression of the hypoxic genes COX5b and CYC7 in WT cells incubated in the presence or absence of 1 mM H_2O_2 during 3 h. (D) Quantitative RT-PCR analysis of COX5b expression in WT, *rox1Δ*, *ord1Δ*, and *rox1Δ ord1Δ* cells incubated in the presence or absence of 1 mM H_2O_2 during 3 h. Error bars represent the mean $\pm$ SD with p -values from comparisons to untreated cells denoted by * p < 0.05. (E) Quantitative RT-PCR analysis for the expression of ROX1, ORD1, and COX5b in WT and *yap1Δ* cells incubated in the presence or absence of 1 mM H_2O_2 during 3 h. Error bars represent the mean $\pm$ SD with p -values from comparisons to untreated cells denoted by * p < 0.05.

region. To test this hypothesis, we analyzed the presence of Rox1 and Ord1 on the COX5b promoter upon menadione treatment by ChIP. Figure 5A schematically depicts the structure of COX5b and CYC7 promoters. Following exposition to menadione, the presence of Rox1 in the COX5b and CYC7 promoters was lowered significantly (Fig. 5B, C). In contrast, no change in COX5b promoter occupancy by Ord1 was detected in these conditions (Fig. 5D).

H_2O_2 triggered an increased COX5b promoter occupancy by Ord1 in both WT and *yap1Δ* cells (Fig. 6A), while the COX5b occupancy of Rox1 was barely altered in WT cells but significantly lowered in *yap1Δ* cells (Fig. 6B). The CYC7 occupancy of Rox1 was instead already significantly diminished in WT cells (Fig. 6C).

These results suggest that ROS could directly or indirectly alter the accessibility, prevent the binding and/or induce the release of Rox1 to/from its target hypoxic genes.

Effect of mitochondrial superoxide on hypoxic gene expression

Finally, we asked whether MRC-generated superoxide could directly induce a similar effect on hypoxic gene ex-

pression. For this purpose, we treated WT cells with 10 μ M antimycin A, a respiratory chain complex III inhibitor, which completely inhibits cell respiration and generates a significant amount of superoxide (41) (data not shown). Antimycin A-treated cells displayed increased expression levels of both COX5b and CYC7 (Fig. 7).

Taken together, our results suggest that environmental and mitochondria-generated ROS induce the expression of hypoxic genes.

Discussion

Eukaryotic organisms rely on OXPHOS for aerobic production of ATP. In yeast, remodeling of the OXPHOS system, including switching of normoxic to hypoxic isoforms of COX subunits and Cyp, represents a homeostatic mechanism to maintain optimal efficiency of mitochondrial respiration in response to changes in cellular oxygenation. An intrinsic feature of cellular respiration is the generation of ROS that can play signaling roles or produce deleterious effects depending on their concentrations. Several studies have shown increased ROS levels during the transitions from normoxia to hypoxia (18, 22, 23). Here, we have identified overlapping

FIG. 4. Steady-state levels of Rox1 and Ord1 in cells cultured in the presence of menadione or H₂O₂. Whole cell extracts from WT (A, B, C, and D) and *yap1Δ* cells (B, D) expressing 2HA-tagged Ord1 and 9Myc-tagged Rox1 and cultured in the presence or absence of either 0.5 mM menadione (A, C) or 1 mM H₂O₂ (B, D) for the indicated amount of times were prepared as described in the Materials and Methods section. Cell extracts were subsequently used for western blot analyses using anti-HA and anti-Myc antibodies. An antibody that recognizes Pgk1 was used as a loading control. The images were digitalized and used by densitometric analyses with the histogram function of the Adobe Photoshop program. Quantification analysis in lower panels includes data from at least three independent experiments. Error bars represent the mean $\pm$ SD with *p*-values from comparisons to untreated cells denoted by **p* < 0.05. Pgk1, phosphoglycerate kinase 1.

transcriptional programs that exist to sense oxygen deprivation and oxidative stress to adapt to these challenges in yeast. The mechanism disclosed here involves the oxidative-stress-induced inactivation of Rox1, a general transcriptional repressor of hypoxic genes (Fig. 8).

Several lines of evidence allowed us to reach this conclusion. First, the expression of the hypoxic genes *COX5b* and *CYC7*, whose expression is controlled by Rox1, is enhanced in cells cultured in the presence of either menadione, a superoxide generating compound, or H₂O₂. Although the effect of

FIG. 5. ChIP analysis of promoter occupancy by Rox1 and Ord1 upon menadione treatment. (A) Schematic representation of *COX5b* and *CYC7* genes and the location of Rox1 and Ord1 binding sites in their promoters. Two consensus Rox1 binding sites on the *CYC7* promoter had been previously identified and spanned from position -105 to -121 and -495 to -490 although the second one does not seem to exert repression (30) and is not depicted here. To predict the Rox1 binding site on the *COX5b* promoter, we took an *in silico* approach using the SPGD promoter analysis program for the Rox1 binding site, which confirmed its location previously inferred based on promoter reporter activity assays (26). It spans from positions -239 to -195. The Ord1 binding site on the *COX5b* promoter was analyzed through pairwise alignment of the promoter sequence against the known Ord1 binding sequence on the *TIR1* gene promoter [not shown, (8)]. Two contiguous Ord1 binding boxes were identified and spanned from position -418 to -532 and from position -567 to -666. (B) WT cells cultured in the presence or absence of 0.5 mM menadione for 3 h were tested for occupancy of Rox1 on *COX5b* promoter region, (C) Rox1 occupancy on *CYC7* promoter, and (D) Ord1 occupancy on *COX5b* promoter. Cells were grown to mid-log phase (OD₆₀₀ = 1) before subjected to oxidative stress (0.5 mM menadione). ChIP analyses were carried out as described in the Materials and Methods section. DNA fragments containing the predicted Ord1 and Rox1 binding sites were amplified with oligonucleotides described in the Supplementary data (Supplementary Table S2). PCR products (typically, 100–200 bp) were separated on 2% agarose gels (left panels). Error bars represent the mean $\pm$ SD with *p*-values from comparisons to untreated cells denoted by **p* < 0.05. ChIP, chromatin immunoprecipitation.

the latter was minor on *COX5b*, it was significantly enhanced in a *yap1Δ* strain. These results suggest that superoxide is probably the preferred inducer of the response although other ROS could also initiate the response. For comparison, in hypoxia/anoxia, *ROX1* transcription is severely compromised because it cannot be activated in the absence of *heme*, which is required for Hap1 function. As a consequence, Rox1 is not available for hypoxic gene repression in these conditions. In contrast, our results show that oxidative stress does

not limit *ROX1* expression but rather restricts its interaction with its target promoters (Fig. 8). Oxidative stress induced *ROX1* expression in a Hap1-independent manner, probably as a compensatory effect for its diminished repression function on its target promoters.

The mechanism described here for oxidative stress induction of *COX5b* and *CYC7* is also effective on other hypoxic genes, such as the OXPHOS unrelated *ANB1*, coding for the hypoxic isoform of translation elongation factor eIF-5A.

FIG. 8. Mechanism of ROS-induced hypoxic gene expression. The proposed roles of Hap1 and Rox1 in hypoxia, normoxia, and oxidative stress are depicted. See explanation in the text. ROS, reactive oxygen species.

However, it may not be universal for all Rox1-regulated genes. It has been recently shown that Rox1, which is required for the transcriptional repression of ergosterol biosynthesis triggered by osmotic stress, may also contribute to the repression of this pathway elicited by oxidative stress (34). In general, it remains to be understood how Rox1 is released from its target promoters during hypoxia and stress. It is known, nonetheless, that during the transition from normoxia to hypoxia, Rox1 dissociates with different kinetics from the promoters of target genes, such as *ANB1* and *HEM13* (33). However, it needs to be taken into account that Rox1 oligomerizes *in vitro* (17), suggesting that multiple Rox1 monomers could possibly bind to the promoter region and act cooperatively or not to tune gene expression. Additionally, Rox1 is well known to recruit the general transcription co-repressors Tup1/Ssn6 to limit hypoxic gene expression (6, 33). Several mechanisms have been invoked to account for de-repression of Tup1/Ssn6-co-regulated genes. For example, following the recruitment model, phosphorylation of Mig1, involved in glucose repression, results in Mig1 nuclear export and subsequent relieve of glucose repression (40). In contrast, phosphorylation of Rfx1, involved in repression of DNA-damage-regulated genes, results in inactivation of its DNA binding capacity (40). Here, oxidative stress could induce specific modifications on either Rox1 or *COX5b/CYC7* promoters that may affect the binding.

The release of Rox1 from its target promoters could reasonably involve a redox posttranslational modification of this factor. For example, protein S-glutathiolation and mixed disulfide formation are known to play an important role in protein modifications and signaling pathways, including effects on redox-sensitive transcription factors (35, 42). However, because Rox1 does not contain cysteine residues, the response of Rox1 to changes in the cellular redox state should not involve thiol modification directly although oxidation of other residues remains plausible. The effect of Rox1 binding to its target promoters could be secondary to thiol redox modifications in additional factors although treatment of WT cells with the thiol crosslinking agent diamide (1.5 mM diamide for 1 h) did not induce *COX5b* expression (Supplementary Fig. S3).

Oxidative stress modestly induced *ORD1*, coding for the second major and more specific *COX5b* repressor. Ord1 does not act on *CYC7* but the possibility existed that the function of both repressors, Rox1 and Ord1, could be regulated by oxidative stress. Our results show that Ord1 accumulation is not

decreased but rather even enhanced in hypoxia/anoxia or under oxidative stress, and significantly increased its occupancy on the *COX5b* promoter in these conditions. Both Rox1 and Ord1 are known to contain high-mobility group domains that bind to and bend DNA (16, 32). We cannot discard the possibility that Ord1 accumulation saturating the two binding sites on the promoter and/or oligomerizing could contribute to Rox1 release, specifically from the *COX5b* promoter.

The physiological relevance of the ROS-induced hypoxic gene expression mechanism presented here was further enhanced by the observations that the *COX5b/CYC7* induction effect is achieved in antimycin A-treated WT cells, thus indicating that mitochondrial superoxide is able to induce hypoxic signaling. Previous studies reported that the mitochondrial complex *bc*₁ is required for hypoxia-induced ROS production and gene transcription in yeast (23). Similar observations have been reported in mammalian cells, where the hypoxia inducing factor 1 (HIF-1) complex, a transcriptional activator that functions as a master regulator of oxygen homeostasis in all metazoan species, has been shown to be activated by nonhypoxic stimuli involving mitochondria-generated ROS as essential signaling intermediates (22, 24, 27, 36). Noticeably, the mammalian homologue of Cox5, termed COX4, also exists in two isoforms encoded by *COXIV-1* and *COXIV-2* and COX subunit isoform switch is regulated by HIF-1(20).

In conclusion, we have established that in the yeast *S. cerevisiae*, oxidative stress induces the expression of at least some hypoxic genes, the OXPHOS-related *COX5b* and *CYC7* through a mechanism that involves the release from their promoters of Rox1, a general transcriptional repressor of hypoxic genes in normoxia (Fig. 8). Importantly, a similar response was triggered by environmental and mitochondria-generated ROS, thus highlighting the relevance of the mechanism described here.

Materials and Methods

Strains and growth conditions

The genotypes and sources of the *S. cerevisiae* strains used in this study are listed in Supplementary Table S1. The following media were used routinely to grow yeast: YPD (2% glucose, 1% yeast extract, and 2% peptone), WOGal (2% galactose and 0.67% W/O nitrogen base), and YPEG (2% ethanol, 2% glycerol, 1% yeast extract, and 2% peptone). The construction of strains carrying *ORD1-2HA* and *ROX1-9Myc* genomic tags

was performed as reported in the Supplementary Materials and Methods and Table S2.

Experimental design and treatment with ROS-generating compounds

For experiments involving treatments with ROS-generating compounds, cells were cultured in media other than glucose, usually YPEG media, to avoid catabolite-induced transcriptional gene repression. Cells were grown in YPEG media until mid-log phase was reached ($OD^{600}=1$). At this point, menadione or stabilized H_2O_2 (Sigma-Aldrich Corp., St Louis, MO) was added. Concentration titrations and time-course experiments were performed with both compounds. For H_2O_2 treatments, the concentration used in this study was chosen so as to achieve the maximum activation of *ROX1* while not inducing cell death as tested by propidium iodide staining and flow cytometry. Treatments of up to 3 h with 1 mM H_2O_2 were used in most experiments. A similar approach was used to select the conditions for menadione treatment. Menadione is known to be a superoxide-generating compound with a half-life of ~3 h (15). In agreement with previous studies (12, 21), we decided to culture our cells in the presence of 0.5 mM menadione for up to 3 h. At least two independent RNA extractions or whole cell extracts were analyzed.

RNA isolation and analysis

Total RNA was prepared from whole cells by a modified extraction with hot-acidic phenol (5) and used either for Northern blot or quantitative RT-PCR analyses as explained in the Supplementary Materials and Methods. At least two independent experiments with three replicas were performed for each analysis. Primers used to amplify the probe for the RT-PCR analysis are listed in Supplementary Table S2.

Chromatin immunoprecipitation

ChIP assays were performed essentially as described previously (4), with some minor modifications detailed in the Supplementary Methods.

Miscellaneous procedures

Standard procedures used for gene cloning, yeast transformation, preparation of yeast cell extracts, protein quantification, and western blot analyses are described in the Supplementary Methods.

Statistical analysis

At least two independent experiments with three replicas were performed for each analysis. Northern blot analyses were done in duplicate when the differences between the two replicas were within 5%. Data are presented as mean $\pm$ standard deviation of absolute values or percent of control. The values obtained for WT and drug-treated strains for the different parameters studied were compared by Student *t*-test. $p < 0.05$ was considered significant.

Supplementary data

Supplementary data include three supplementary figures, two supplementary tables, and Supplementary Materials and Methods.

Acknowledgments

The authors thank Dr. Myriam Bourens, Dr. Flavia Fontanesi, Dr. Alejandro Ocampo, and Dr. Iliana C. Soto for critically reading the article. Our study was supported by the Muscular Dystrophy Association to A.B. (grant number 186025), and by National Institutes of Health (NIH) grant GM071775 to A.B.

Author Disclosure Statement

No competing financial interests exist among the authors.

References

1. Abramova N, Sertil O, Mehta S, and Lowry, CV. Reciprocal regulation of anaerobic and aerobic cell wall mannoprotein gene expression in *Saccharomyces cerevisiae*. *J Bacteriol* 183: 2881–2887, 2001.
2. Acker T and Acker H. Cellular oxygen sensing need in CNS function: physiological and pathological implications. *J Exp Biol* 207: 3171–3188, 2004.
3. Allen LA, Zhao XJ, Caughey W, and Poyton RO. Isoforms of yeast cytochrome *c* oxidase subunit V affect the binuclear reaction center and alter the kinetics of interaction with the isoforms of yeast cytochrome *c*. *J Biol Chem* 270: 110–118, 1995.
4. Amberg D, Burke D, and Strathern J. Chromatin immunoprecipitation. In: *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*, edited by Amberg D, Burke D, and Strathern J. Woodbury, NY: Cold spring harbor laboratory press, 2005, pp. 169–174.
5. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K. (Eds). *Saccharomyces cerevisiae* In: *Current Protocols in Molecular Biology*. New York: Wiley, 1994, p. 13.
6. Balasubramanian B, Lowry CV, and Zitomer RS. The Rox1 repressor of the *Saccharomyces cerevisiae* hypoxic genes is a specific DNA-binding protein with a high-mobility-group motif. *Mol Cell Biol* 13: 6071–6078, 1993.
7. Bell EL and Chandel NS. Mitochondrial oxygen sensing: regulation of hypoxia-inducible factor by mitochondrial generated reactive oxygen species. *Essays Biochem* 43: 17–27, 2007.
8. Bourdineaud JP, De Sampaio G, and Lauquin GJ. A Rox1-independent hypoxic pathway in yeast. Antagonistic action of the repressor Ord1 and activator Yap1 for hypoxic expression of the *SRP1/TIR1* gene. *Mol Microbiol* 38: 879–890, 2000.
9. Burke PV, Raitt DC, Allen LA, Kellogg EA, and Poyton RO. Effects of oxygen concentration on the expression of cytochrome *c* and cytochrome *c* oxidase genes in yeast. *J Biol Chem* 272: 14705–14712, 1997.
10. Cadenas E. Biochemistry of oxygen toxicity. *Annu Rev Biochem* 58: 79–110, 1989.
11. Castello PR, David PS, McClure T, Crook Z, and Poyton RO. Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. *Cell Metab* 3: 277–287, 2006.
12. Castro FA, Mariani D, Panek AD, Eleutherio EC, and Pereira MD. Cytotoxicity mechanism of two naphthoquinones (menadione and plumbagin) in *Saccharomyces cerevisiae*. *PLoS One* 3: e3999, 2008.
13. Castro-Prego R, Lamas-Maceiras M, Soengas P, Carneiro I, Gonzalez-Siso I, and Cerdan ME. Regulatory factors controlling transcription of *Saccharomyces cerevisiae* *IXR1* by oxygen levels: a model of transcriptional adaptation from aerobiosis to hypoxia implicating *ROX1* and *IXR1* cross-regulation. *Biochem J* 425: 235–243, 2009.

14. Clanton TL. Hypoxia-induced reactive oxygen species formation in skeletal muscle. *J Appl Physiol* 102: 2379–2388, 2007.
15. Collins WR and Kirch ER. Determination and stability of menadione. *J Pharm Sci* 35:215–217, 1946.
16. Deckert J, Khalaf RA, Hwang SM, and Zitomer RS. Characterization of the DNA binding and bending HMG domain of the yeast hypoxic repressor Rox1. *Nucleic Acids Res* 27: 3518–3526, 1999.
17. Di Flumeri C, Liston P, Acheson NH, and Keng T. The HMG domain of the ROX1 protein mediates repression of HEM13 through overlapping DNA binding and oligomerization functions. *Nucleic Acids Res* 24: 808–815, 1996.
18. Dirmeier R, O'Brien KM, Engle M, Dodd A, Spears E, and Poyton RO. Exposure of yeast cells to anoxia induces transient oxidative stress. Implications for the induction of hypoxic genes. *J Biol Chem* 277: 34773–34784, 2002.
19. Fontanesi F, Soto IC, Horn D, and Barrientos A. Assembly of mitochondrial cytochrome *c* oxidase, a complicated and highly regulated cellular process. *Am J Physiol Cell Physiol* 291: C1129–C1147, 2006.
20. Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, and Semenza, GL. HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell* 129: 111–122, 2007.
21. Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, and Brown PO. Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell* 11: 4241–4257, 2000.
22. Guzy RD, Hoyos B, Robin E, Chen H, Liu L, Mansfield KD, Simon MC, Hammerling U, and Schumacker PT. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. *Cell Metab* 1: 401–408, 2005.
23. Guzy RD, Mack MM, and Schumacker PT. Mitochondrial complex III is required for hypoxia-induced ROS production and gene transcription in yeast. *Antioxid Redox Signal* 9: 1317–1328, 2007.
24. Guzy RD and Schumacker PT. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Exp Physiol* 91: 807–819, 2006.
25. Hodge MR, Kim G, Singh K, and Cumsy MG. Inverse regulation of the yeast COX5 genes by oxygen and heme. *Mol Cell Biol* 9: 1958–1964, 1989.
26. Hodge MR, Singh K, and Cumsy MG. Upstream activation and repression elements control transcription of the yeast COX5b gene. *Mol Cell Biol* 10: 5510–5520, 1990.
27. Klimova T and Chandel NS. Mitochondrial complex III regulates hypoxic activation of HIF. *Cell Death Differ* 15: 660–666, 2008.
28. Kwast KE, Burke PV, Staahl BT, Poyton RO, Raitt DC, Allen LA, and Kellogg EA. Oxygen sensing in yeast: evidence for the involvement of the respiratory chain in regulating the transcription of a subset of hypoxic genes. *Proc Natl Acad Sci U S A* 96: 5446–5451, 1999.
29. Lambert JR, Bilanchone VW, and Cumsy MG. The *ORD1* gene encodes a transcription factor involved in oxygen regulation and is identical to *IXR1*, a gene that confers cisplatin sensitivity to *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* 91: 7345–7349, 1994.
30. Lowry CV, Cerdan ME, and Zitomer RS. A hypoxic consensus operator and a constitutive activation region regulate the *ANB1* gene of *Saccharomyces cerevisiae*. *Mol Cell Biol* 10: 5921–5926, 1990.
31. Lowry CV and Zitomer RS. *ROX1* encodes a heme-induced repression factor regulating *ANB1* and *CYC7* of *Saccharomyces cerevisiae*. *Mol Cell Biol* 8: 4651–4658, 1988.
32. McA'Nulty MM, Whitehead JP, and Lippard SJ. Binding of Ixr1, a yeast HMG-domain protein, to cisplatin-DNA adducts *in vitro* and *in vivo*. *Biochemistry* 35: 6089–6099, 1996.
33. Mennella TA, Klinkenberg LG, and Zitomer RS. Recruitment of Tup1-Ssn6 by yeast hypoxic genes and chromatin-independent exclusion of TATA binding protein. *Eukaryot Cell* 2: 1288–1303, 2003.
34. Montanes FM, Pascual-Ahuir A, and Proft M. Repression of ergosterol biosynthesis is essential for stress resistance and is mediated by the Hog1 MAP kinase and the Mot3 and Rox1 transcription factors. *Mol Microbiol* 79: 1008–1023, 2011.
35. Paget MS and Buttner MJ. Thiol-based regulatory switches. *Annu Rev Genet* 37: 91–121, 2003.
36. Patten DA, Lafleur VN, Robitaille GA, Chan DA, Giaccia AJ, and Richard DE. Hypoxia-inducible factor-1 activation in nonhypoxic conditions: the essential role of mitochondrial-derived reactive oxygen species. *Mol Cell* 21: 3247–3257, 2010.
37. Poyton RO. Models for oxygen sensing in yeast: implications for oxygen-regulated gene expression in higher eucaryotes. *Respir Physiol* 115: 119–133, 1999.
38. Poyton RO, Ball KA, and Castello PR. Mitochondrial generation of free radicals and hypoxic signaling. *Trends Endocrinol Metab* 20: 332–340, 2009.
39. Rodrigues-Pousada C, Menezes RA, and Pimentel C. The Yap family and its role in stress response. *Yeast* 27: 245–258, 2010.
40. Smith RL and Johnson AD. Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends Biochem Sci* 25: 325–330, 2000.
41. Solans A, Zambrano A, Rodriguez M, and Barrientos A. Cytotoxicity of a mutant huntingtin fragment in yeast involves early alterations in mitochondrial OXPHOS complexes II and III. *Hum Mol Genet* 15: 3063–3081, 2006.
42. Sundar IK, Caito S, Yao H, and Rahman, I. Oxidative stress, thiol redox signaling methods in epigenetics. *Methods Enzymol* 474: 213–244, 2010.
43. Trueblood CE and Poyton RO. Identification of REO1, a gene involved in negative regulation of *COX5b* and *ANB1* in aerobically grown *Saccharomyces cerevisiae*. *Genetics* 120: 671–680, 1988.
44. Waterland RA, Basu A, Chance B, and Poyton RO. The isoforms of yeast cytochrome *c* oxidase subunit V alter the *in vivo* kinetic properties of the holoenzyme. *J Biol Chem* 266: 4180–4186, 1991.
45. Zitomer RS and Lowry, CV. Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. *Microbiol Rev* 56: 1–11, 1992.

Address correspondence to:

Prof. Antoni Barrientos

Departments of Neurology and Biochemistry & Molecular Biology
University of Miami Miller School of Medicine

1600 NW 10th Ave.

RMSB # 2067

Miami, FL-33136

E-mail: abarrientos@med.miami.edu

Date of first submission to ARS Central, February 28, 2012; date of final revised submission, June 2, 2012; date of acceptance, June 15, 2012.

Abbreviations Used

AA = antimycin A
bp = base pairs
ChIP = chromatin immunoprecipitation
COX = cytochrome *c* oxidase
Cyc = cytochrome *c*
DEPC = diethyl pyrocarbonate

MRC = mitochondrial respiratory chain
OXPHOS = oxidative phosphorylation
ROS = reactive oxygen species
RT-PCR = reverse transcriptase-polymerase chain
 reaction
SD = standard deviation
WT = wild-type