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Mitochondrial Complex III is Required for Hypoxia-Induced ROS Production and Gene Transcription in Yeast

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ABSTRACT

To survive, respiring organisms must sense and respond to changes in environmental oxygen levels. Complex III of the mitochondrial electron transport chain (ETC) has been implicated in the O_2 sensing pathway in mammals through its ability to increase production of reactive oxygen species (ROS) during hypoxia. The present study tested whether Complex III in yeast also contributes to O_2 sensing during hypoxia. Strains deficient in mitochondrial DNA (ρ^0), the Rieske iron—sulfur protein (ΔRip1) in Complex III, or an enzyme responsible for coenzyme Q biosynthesis (ΔCoq2) were studied to determine the importance of Complex III activity in the transcriptional response to hypoxia. Loss of Complex III function abrogated the hypoxia-induced increase in ROS in each strain. Northern analysis identified a set of genes that are activated by hypoxia in wild-type but not in ρ^0, ΔRip1, or ΔCoq2 strains. Yeast lacking the transcription factors Yap1p, Mga2p, and Msn2p were also deficient in hypoxic activation of gene transcription, suggesting the importance of redox regulation in hypoxic gene expression. The authors conclude that Complex III of the ETC is required for ROS production and for expression of a group of hypoxia-inducible genes in yeast. These findings indicate that the mitochondrial O_2 sensing mechanism is highly conserved throughout evolution. *Antioxid. Redox Signal.* 9, 1317–1328.

INTRODUCTION

MOST EUKARYOTIC ORGANISMS require molecular oxygen for biosynthetic processes and oxidative phosphorylation, making the availability of O_2 critically important for survival. Accordingly, organisms have evolved a complex set of responses activated by decreases in oxygen availability (hypoxia or anoxia) that function at the organismal, cellular, and molecular levels. These responses are adaptive because they augment the delivery of oxygen to cells and they enhance cellular survival during prolonged periods of oxygen deprivation.

In mammals, the ability to sense a decrease in O_2 tension is required for development, erythropoiesis, wound healing, survival in diverse environments during adulthood, and for life-threatening events such as tumor angiogenesis. Despite the importance of this process, the cellular mechanism of oxygen sensing remains elusive. We and others have proposed that mitochondria act as oxygen sensors by paradoxically increasing production of ROS during hypoxia (2, 8, 9, 21, 22, 45, 46). In mammalian systems, this increase in mitochondrial ROS signaling has been linked with a number of hypoxia-induced responses, including hypoxic pulmonary vasoconstriction (63), ischemic preconditioning in cardiomyocytes (18, 38), decreased Na/K-ATPase activity (12), hypoxia-induced glutathione depletion (46), and stabilization of hypoxia-inducible transcription factors (HIFs) (1, 8, 59). The stabilization of HIF-1a during hypoxia is regulated by a family of prolyl hydroxylases (PHDs) that use O_2 as a substrate to modify the oxygen-dependent degradation domain of the protein, thereby triggering interaction with the ubiquitin E3 ligase, vHL, resulting in subsequent proteasomal degradation (28, 66). This has led to the suggestion that PHDs may function as O_2 sensors, since their ability to trigger degradation of the a subunit is inhibited during anoxia (20, 25). However, their role as oxygen sensors during physiological hypoxia as opposed to anoxia is not fully understood. It is possible that hypoxia-induced mitochondrial

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ROS signals regulate PHD activity either directly or indirectly, although this mechanism has not been directly demonstrated.

Yeast respond to changes in O_2 availability by altering expression of a number of oxygen-responsive genes. Two classes of oxygen-sensitive genes have emerged, consisting of hypoxic genes that are transcriptionally activated during hypoxia, and aerobic genes that are transcribed only during normoxic conditions when oxygen is plentiful (36, 55). Among the genes induced by hypoxia, most are activated at microaerobic or anoxic conditions. A large number of hypoxic and aerobic genes are controlled by the transcription factors Rox1p and Hap1p, which repress hypoxic genes and activate aerobic genes, respectively, when oxygen levels are high (26, 36). Both of these factors are dependent upon heme biosynthesis, which is oxygen dependent, for their activity (26). Another putative oxygen-sensitive transcription factor is Mga2p, which is related to mammalian NF-kB and is regulated via the ubiquitin/proteasome pathway (27). Mga2p has been reported to activate transcription of the Δ9 fatty acid desaturase gene OLE1 during hypoxia (30, 49-51). Mga2p is normally membrane bound, and is activated by ubiquitin-mediated cleavage from the membrane, at which time it is proposed to act by stabilizing mRNA of target genes such as OLE1 (31). Since OLE1 transcription is induced by cobalt chloride and iron chelators (62), which stabilize HIF by inhibiting PHDs in normoxic mammalian cells, the factors regulating OLE1 such as Mga2 in yeast may be related to the factors that regulate HIF in mammalian cells.

A number of genes in yeast have also been shown to be regulated by oxidative stress via redox-sensitive transcription factors (11, 23, 39). Of particular interest is the transcription factor Yap1p (58), which responds to oxidative stress through Orp1p, which is activated in turn by H_2O_2 (14, 15). When oxidized by ROS such as H_2O_2, Orp1p reacts with and oxidizes Yap1p (15, 33). Oxidation of Yap1p in its cysteine-rich carboxy-terminal domain results in the formation of a disulfide bond-mediated interaction between an amino-terminal α-helix and its nuclear export signal, effectively masking that domain and trapping it in the nucleus (64, 65). Activated nuclear Yap1p then initiates transcription of important antioxidant genes such as thioredoxin II (TRX2) (34) and catalase (CTT1) (24), which feed back to counter the oxidative stress (15). Furthermore, Yap1p has been associated with hypoxic induction of SRP1 independently from the Rox1 pathway (6). The stress-responsive transcription factor Msn2p has also has been implicated in the induction of multiple genes by exogenous H_2O_2, including catalase (CTT1), HSP12, HSP104, and TSA1 (23). Msn2p is also known to be responsive to freezing stress (29), green tea polyphenol-mediated oxidative stress (44), and the toxic metabolite methylglyoxal (43). It is therefore likely that Msn2p, like Yap1p, is capable of being regulated by cellular ROS. This suggests that a hypoxia-induced oxidative signal could contribute to transcriptional regulation in yeast by activating ROS-sensitive transcription factors such as Mga2p, Yap1p, and Msn2p.

Mitochondrial structure and function are highly conserved among eukaryotes. Like mammalian cells, there is evidence that mitochondrial oxidative stress increases in yeast during hypoxia (8) and in response to severe oxygen deprivation (16). Furthermore, a link between mitochondria and hypoxia-induced gene transcription has been described (37). Given the similarity of these responses, we investigated the ability of yeast to generate mitochondrial ROS in response to hypoxia and then tested whether this response mediates the induction of a number of known hypoxia- and oxidative stress-induced genes in response to moderate or severe hypoxia. We find that yeast strains that are deficient in electron transport chain (ETC) function are impaired in terms of hypoxia-induced ROS production, and are also deficient in hypoxia-induced transcription of SOD1, HSP12, JEN1, and other genes. Furthermore, we find that yeast lacking the redox-sensitive transcription factors Yap1p and Msn2p, as well as the transcription factor Mga2p, lack hypoxia-induced expression of the above genes. These genes likely respond to hypoxia via hypoxia-induced ROS and redox signaling.

MATERIALS AND METHODS

Yeast strains and media

txWT W303-1a were obtained from Steve Kron (University of Chicago). Rho-zero yeast were generated by growing WT W303-1a yeast overnight in the presence of 50 ng/ml ethidium bromide. Colonies unable to grow on glycerol were designated as ρ^0. ΔRip1 yeast were generated from WT W303-1a via homologous recombination. ΔCoq2 yeast were obtained from Catherine Clarke (UCLA). ΔRip1 and ΔCoq2 strains were not deficient in mtDNA, since crosses with ρ^0 yeast produced viable growth on agar plates with glycerol as a carbon source. ΔYap1, ΔMga2, and ΔMsn2 yeast were obtained from Open Biosystems (Huntsville, AL). Yeast were grown in synthetic complete media (US Biologicals, Swampscott, MA) containing either 2% glucose or 3% glycerol as a carbon source. LacZ reporter constructs were provided by Mark Goldberg (Rutgers University).

Oxygen consumption

Yeast were grown overnight in glucose media, and diluted to an OD_{600} of 0.9 in either glucose or glycerol media and grown for 3 or 24 h. Yeast (1×10^8) were washed with PBS and resuspended in 2 ml of glucose or glycerol media and loaded into a sealed glass Warburg respirometer at room temperature. Changes in oxygen tension were measured with a Clark-type oxygen electrode, and oxygen consumption was calculated as a change in oxygen tension over time. Antimycin A (AA, 1 μg/ml) was used to block mitochondrial respiration, allowing us to quantify nonmitochondrial oxygen consumption.

ROS measurements

Yeast were grown overnight in glucose media, and diluted to an OD_{600} of 0.9 in either glucose or glycerol media and grown for 3 h. Dihydrodichlorofluorescin diacetate (DCFH-DA, 20 μM) was added for an additional 1 h to load the dye into cells. Yeast (0.5 ml) were added to 3 ml of gas-equilibrated buffer (PBS + 20 μM DCFH-DA). The yeast were incubated for 4 h under 21%, 21% + 400 μM CoCl2, 1% or 0% O_2, after which fluorescence was measured (ex. = 490 nm, em. = 530 nm) and normalized to cell number (OD_{600}). Data are presented as fold-

increase from normoxic (21% O_2) baseline. Optical measurements of O_2 tension in the incubation flask using a phosphorescence quenching method (40) revealed that bubbling samples with 0% O_2 (100% N_2) resulted in a final oxygen concentration of 0.1% O_2.

OLE1-LacZ reporter assay

Yeast were transformed with either a full-length OLE1-LacZ reporter gene (p62::-934 Ura+), or a construct containing a truncated reporter (p62::-255 Ura+) using a lithium acetate method and selected on Ura-media. Yeast strains transformed with the OLE1-LacZ reporter gene plasmid were grown to mid-log phase in either SC glucose or SC glycerol for 3 h under normoxic conditions. Cells were vigorously bubbled while shaking in a sealed container with 21%, 21% + 400 μM $CoCl_2$, 1% (hypoxia), or 0% (anoxia) O_2 mixtures for 6 h. Yeast then were lysed with glass beads, and beta-galactosidase levels were measured using the luminescent beta-galactosidase detection kit (Clontech, Mountain View, CA).

Northern blot analysis

Yeast were grown overnight in glucose media, washed in PBS, and diluted to an OD_{600} of 0.9 in either glucose or glycerol media and grown for 3 h. Cells were vigorously bubbled while shaking in a sealed container with 21%, 21% + 400 μM CoCl2, 1% (hypoxia), or 0% (final concentration of 0.1%) O_2 mixtures for 6 h. After 6 h of treatment with 21%, 1%, or 0% O_2 total RNA was purified using a hot acid-phenol RNA extraction method (62). Total RNA (3–4 μg) was electrophoresed using a MOPS/formaldehyde agarose gel and transferred to a nylon membrane (Hybond-XL, Amersham, Piscataway, NJ). Membranes were blotted with RT-PCR-generated probes for the genes Sod1, Hsp12, Hsp104, Ald2, Stf2, Ole1, Gsh1, Jen1, Ctt1, Tsa1, Adh2, Sut1, Trx2, Atf1, Ald3, Tir1, and Act1. Blots were quantified using densitometry and normalized to Act1 for loading control.

RESULTS

We compared mitochondrial respiration in wild-type and mitochondria-deficient yeast strains (ρ^0, ΔRip1, ΔCoq2). When grown in media containing 2% glucose as a carbon source, no significant difference in mitochondrial respiration rates were found between strains (Fig. 1). Previous reports have shown that when yeast are grown in nonfermentable carbon sources such as glycerol, the size and functionality of mitochondria is dramatically increased (19). We therefore grew wild-type and mitochondria-deficient yeast in media containing 3% glycerol as a carbon source for 3 h, and found a significant increase in wild-type respiration, with no increase in respiration in ρ^0, ΔRip1, or ΔCoq2 yeast strains (Fig. 1). Growth in glycerol media for 24 h did not produce a significant increase in respiration relative to rates achieved after growth for 3 h (data not shown). Furthermore, growth of mitochondria-deficient strains in glycerol media was not lethal, since induction of carbon source-dependent gene transcripts was maintained at 6 h (Table 1) and all strains maintained viability on glucose-containing

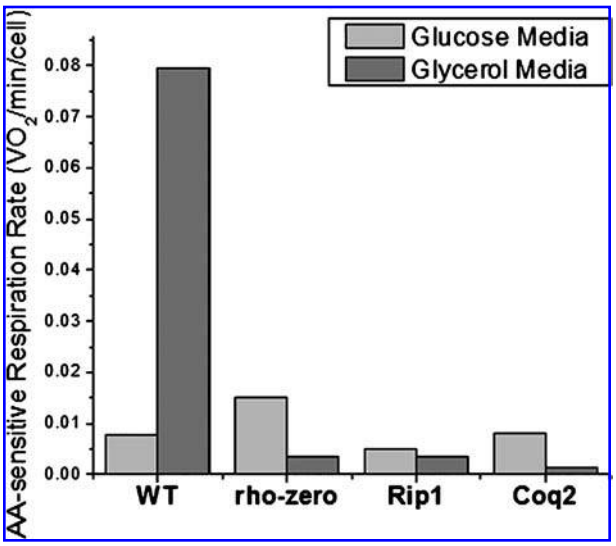


FIG. 1. Mitochondrial respiration is increased in yeast grown in media containing a nonfermentable carbon source. Yeast were grown overnight in media containing glucose, washed in PBS, then resuspended in media containing either glucose or glycerol for 3 h. 1×10^8 cells were collected and resuspended in 2 ml media, and oxygen consumption was measured over time using a Clarke-type electrode in a sealed container. Antimycin A (AA, 1 μg/ml) was administered to quantify nonmitochondrial oxygen consumption.

agar plates after growth in glycerol media for 24 h (data not shown). We therefore have two methods for assessing the importance of mitochondrial function on hypoxic responses: a) comparing wild-type cells grown in glucose and glycerol media; and b) comparing wild-type and mitochondria-deficient strains in glycerol media.

To determine whether ROS are produced during hypoxia, we employed the fluorescent probe DCFH-DA, which increases its fluorescence when oxidized by ROS such as hydrogen peroxide (H_2O_2). We first compared ROS production in response to hypoxia (1% O_2 and 0.1% O_2) in wild-type and mitochondria-deficient strains. When maintained in glucose media, no increases in ROS production were detected in any yeast strain during 4 h of hypoxia (Fig. 2A). Incubation with cobalt chloride (400 μM), which is postulated to generate ROS independently of mitochondria, oxidized DCFH in all strains tested (Fig. 2A). Therefore, yeast strains grown in glucose media are unable to generate ROS in response to hypoxia. Previous studies have also found a similar lack of responsiveness of DCFH when used with yeast grown in glucose-containing media (16). We then tested whether increasing the functionality of the mitochondrial ETC could enable hypoxia-dependent ROS production in wild-type yeast. Yeast preincubated in glycerol media for 3 or 24 h were able to increase ROS production in response to 4 h of hypoxia (Fig. 2B), indicating that functionality of the mitochondrial ETC, which can be obtained by growing yeast in respiratory media, is necessary for hypoxia-induced ROS generation. Prolonged incubation in glycerol media prior to hypoxic exposure did not significantly increase the magnitude of the hypoxic response (Fig. 2B), again indicating that the mitochondria were fully activated after only 3 h of growth in glycerol media.

Table 1. Gene Expression in Response to Glycerol Growth

Gene	*WT*	ρ^0	Δ*Rip1*	Δ*Coq2*	Δ*Yap1*	Δ*Mga2*	Δ*Msn2*
SOD1	1.79	3.136	1.556	1.978	1.935	1.805	1.562
HSP12	4.836	8.001	1.23	1.633	3.045	5.684	6.103
ALD2	5.664	5.346	4.107	2.341	5.001	6.144	3.208
OLE1	2.916	2.113	1.103	0.629	3.859	1.992	3.124
JEN1	16.726	81.161	12.794	21.558	17.7	24.25	20.11
CTT1	13.092	3.252	0.948	1.444	13.47	17.38	20.76
SUT1	0.665	2.468	0.865	0.767	0.663	0.532	0.896
TRX2	1.478	3.795	1.107	1.659	1.798	1.401	0.933

Yeast were grown overnight in synthetic media containing 2% glucose as a carbon source, after which they were washed in PBS and diluted to an absorbance of $OD_{600} = 0.7$ in media containing 2% glucose or 3% glycerol. Yeast were grown for 6 h, and total RNA was analyzed via slot blot and conventional Northern analysis, and quantified via densitometry. Data from at least three independent experiments were normalized to Act1 and presented as fold-increase from cells grown in glucose media.

We then tested whether the hypoxia-induced ROS production was attributable to the mitochondria or to growth conditions. Wild-type and mitochondria-deficient strains were incubated in glycerol media for 3 h, and then exposed to hypoxia for 4 h. Only wild-type yeast exhibited an increase in DCFH oxidation in response to hypoxia, whereas ρ^0, ΔRip1, and ΔCoq2 strains had either no change or a decrease in DCFH oxidation, compared to normoxic controls (Fig. 2C). The ETC-deficient strains used are still capable of inducing carbon-source dependent genes when switched to glycerol media (Table 1). Therefore these data indicate that activity of the mitochondrial electron transport chain is required for hypoxia-induced ROS production, since strains exhibiting a generalized loss of ETC function (ρ^0), loss of Complex III activity (ΔRip1), or loss of electron-shuttling activity into Complex III (ΔCoq2) are unable to increase ROS production in response to hypoxia.

Since yeast are able to generate ROS in response to hypoxia during conditions where mitochondrial function is required for growth [either growth on a non-fermentable carbon source or the diauxic shift (42, 54)], we hypothesized that this ROS is important for signaling, specifically for induction of hypoxia- and stress-induced genes. The yeast gene OLE1 has been implicated as a hypoxia-responsive gene (62), potentially due to ROS production since its transcription can be activated by cobalt chloride. OLE1 was not, however, activated in response to 1% O_2 in either glucose or glycerol media in wild-type yeast, and induction of OLE1 in response to 0.1% O_2 was present in both wild-type and mitochondria-deficient strains in glucose media (data not shown).

We then tested hypoxia-induced expression of a number of candidate genes, based on their known induction by oxidative stress and/or growth in glycerol media. Out of 16 candidate genes screened using slot-blot analysis, we found expression of a number of genes to be induced by a switch from glucose to glycerol media (Table 1), exogenous H_2O_2 (Table 2), and hypoxia. Curiously, almost every gene lacked induction by H_2O_2 when grown in glycerol media in all strains. Using a xylenol orange assay to directly measure H_2O_2 in solution, we found that, while a bolus of H_2O_2 given to yeast grown in glucose media remained stable over time, yeast grown in glycerol media for only 3 h were able to completely degrade H_2O_2 boluses of up to 400 μM within 30–40 min (data not shown). This is likely due to the large increase in transcription of antioxidant enzymes such as catalase when yeast are grown in glycerol media (Table 1), likely from increased ROS production produced as a normal byproduct of electron transport (47). While extracellular oxidants are scavenged from media in glycerol-grown yeast cultures, that does not rule out a possible role for compartmentalized signaling due to acute increases in oxidant production. Based on the findings of our screen, we decided to investigate the transcription of a number of these genes in depth via Northern blot analysis.

Transcription of the genes SOD1 (Cu/Zn-superoxide dismutase, Fig. 3A), HSP12 (Fig. 3B), ALD2 (Fig. 3C), JEN1 (Fig. 3D), SUT1 (Fig. 3E), and CTT1 (data not shown) all increased in response to hypoxia (1% and/or 0.1% O_2) in yeast grown in glycerol media. With the exception of SUT1, all of these genes also show transcriptional activation in response to both exogenous H_2O_2 and switching from growth in glucose media to glycerol media (Tables 1 and 2). With few exceptions, all of these genes also require a functional electron transport chain for their hypoxia-induced transcriptional activation (Fig. 3, data not shown for CTT1). A functional ETC, however, is not required for transcriptional activation of all genes mentioned above in response to both glucose-to-glycerol growth switch (Table 1) and exogenous H_2O_2 (Table 2), indicating that the capacity for transcription of these genes remain intact in ETC-deficient strains.

A few genes analyzed also showed that hypoxic induction was independent of a functional ETC. When grown in glucose media, TIR1 demonstrates a dramatic induction by 0.1% O_2 (Fig. 4A), as does TRX2, albeit to a lesser extent (Fig. 4B). These data, as well as data presented earlier regarding OLE1 transcription, demonstrate that not all hypoxic gene activation in yeast is dependent upon a functional and active electron transport chain, since induction was seen in glucose (nonrespiratory) media as well as in ETC mutants. Other pathways, such as heme-sensitive pathways and the Rox1p transcriptional repressor, likely play a role in these cases. Furthermore, the data show that ETC mutants are capable of responding to low oxygen in oxygen-sensitive pathways where oxygen levels are detected via a nonmitochondrial mechanism.

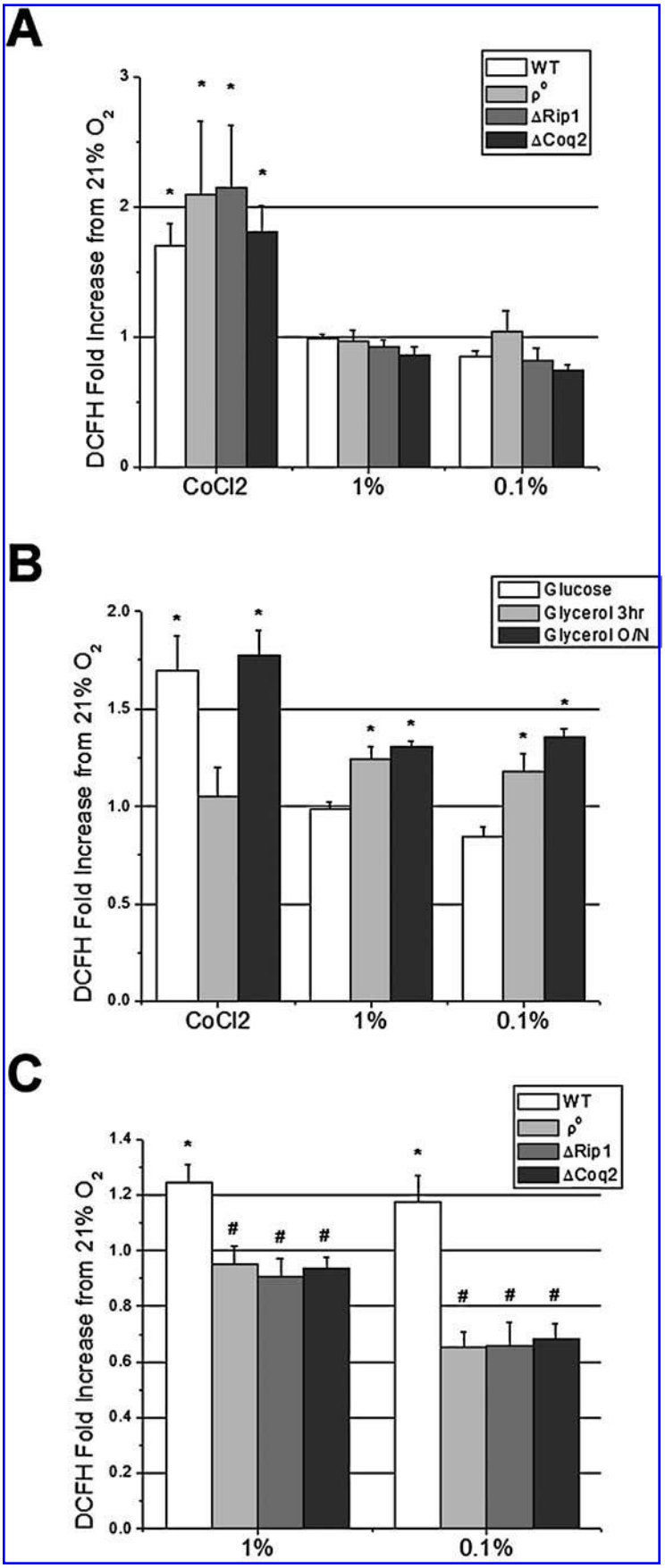


FIG. 2. Increased mitochondrial ROS production in response to hypoxia. Yeast were grown, loaded with DCFH-DA, and exposed to $CoCl_2$, 1%, or 0.1% O_2 as described in experimental procedures. (**A**) Wild-type and ETC-deficient yeast strains were grown for 3 h in glucose media prior to hypoxic exposure. (**B**) Wild-type yeast were grown in glycerol media either 3 h or overnight prior to hypoxic exposure. (**C**) Wild-type and ETC-deficient strains were grown in glycerol media for 3 h prior to hypoxic exposure. Data shown are averages of a minimum of 10 experiments. * indicates a significant change from normoxia ($p < 0.05$); # indicates a significant difference from WT values ($p < 0.05$).

Table 2. Gene Expression in Response to Exogenous H_2O_2

Gene	*WT*	ρ^0	Δ*Rip1*	Δ*Coq2*	Δ*Yap1*	Δ*Mga2*	Δ*Msn2*
SOD1	1.5644	2.4145	2.141	2.2055	1.413	1.507	1.316
HSP12	4.2932	8.124	21.921	28.114	2.577	1.511	1.252
ALD2	2.52	3.871	3.786	3.502	1.446	1.211	1.111
OLE1	1.62525	1.621	1.7085	1.705	0.892	0.97	1.213
JEN1	1.418	1.248	1.6015	1.476	0.214	3.96	1.184
CTT1	1.76225	4.1655	9.861	2.8485	0.211	1.749	1.226
SUT1	1.477	1.034	0.757	0.815	0.873	1.196	1.478
TRX2	7.4455	12.011	11.2315	11.671	1.641	2.513	1.86

Yeast were grown for 3 h in media containing glucose as a carbon source, after which they were exposed to 400 μM H_2O_2 for 40 min. Total RNA was analyzed via slot blot and conventional Northern analysis, and quantified via densitometry. Data from at least two independent experiments were normalized to Act1 and presented as an average fold-increase from controls.

To address whether the ETC-dependent hypoxic gene induction seen in glycerol media-grown yeast strains is mediated by oxidative stress, we first measured the hypoxia-induced induction of JEN1, HSP12, ALD2, and SOD1 in the presence or absence of a combination of the antioxidants *N*-acetyl cysteine (2 mM) and 2-mercaptopropionyl glycine (200 μM). All four of these genes show significant inhibition of hypoxia-induced transcription in the presence of antioxidants (Fig. 5), showing that their hypoxic induction in respiring yeast requires increased ROS production.

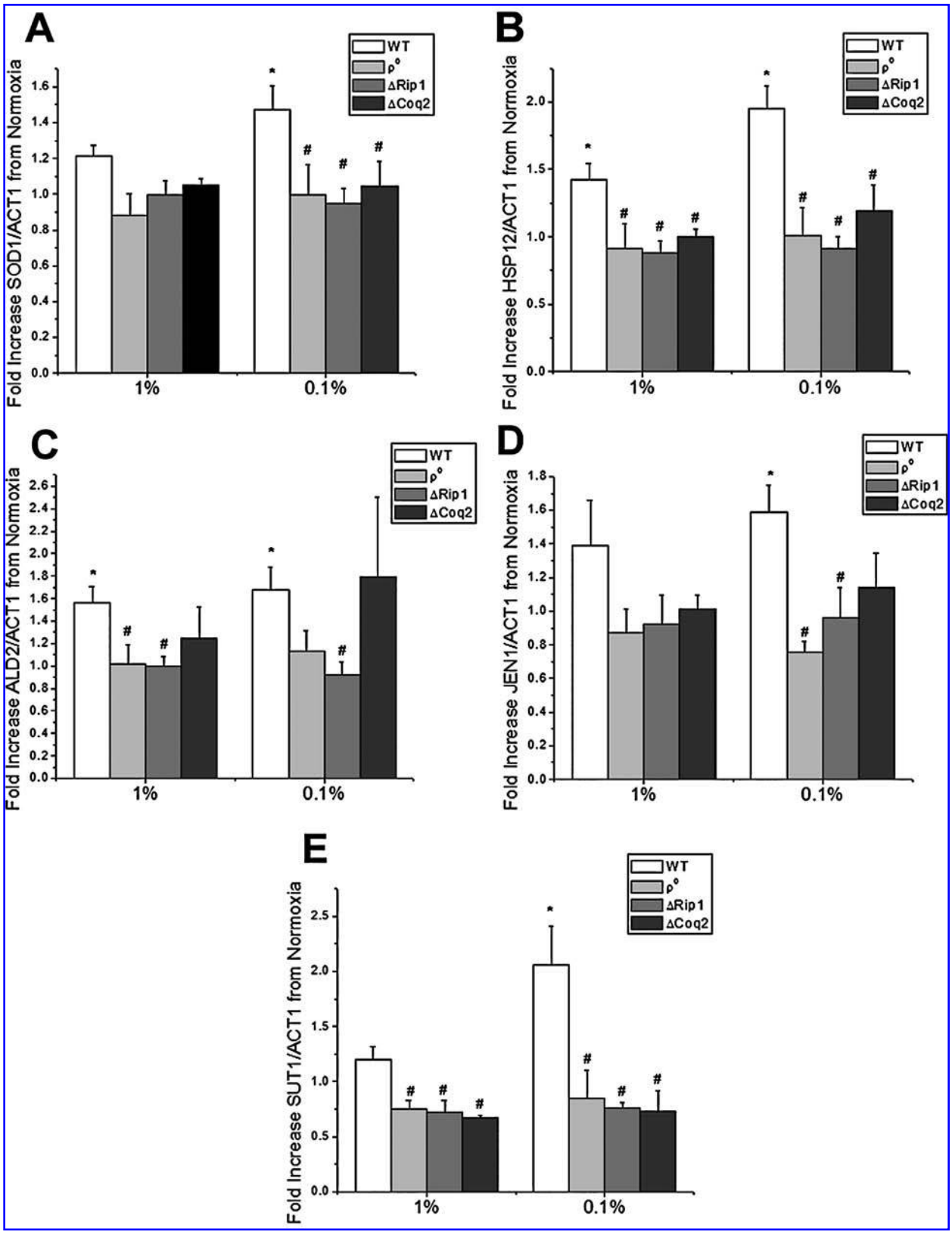
We then used yeast strains deficient in transcription factors known to be responsive to oxidative stress (14, 23). Yap1p and Msn2p have been implicated in oxidative stress-induced transcription of a number of genes, including SOD1, ALD2, CTT1, TRX2, and HSP12 (34, 39, 52, 60, 61). We also tested strains deficient in Mga2p, which is a known transcription factor involved in OLE1 regulation (30). It is possible that, while OLE1 is not dependent on a functional ETC in its overall induction, Mga2p may be sensitive to oxidative stress under conditions that do not favor OLE1 hypoxia-induced transcription, such as growth in glycerol media. ΔYap1, ΔMga2, and ΔMsn2 strains exhibited normal growth on glycerol media, oxidation of DCF in response to hypoxia when grown in glycerol media (data not shown), and normal induction of gene transcription in response to glucose-to-glycerol media growth switch (Table 1). JEN1, CTT1, SUT1, and TRX2 all had decreased induction in response to exogenous H_2O_2 in the ΔYap1 strain (Table 2). Interestingly, all three transcription factors tested were found to be important in hypoxia-induced transcription (Fig. 6). ΔYap1 and ΔMsn2 strains showed deficient hypoxic induction of ALD2 (Fig. 6C), JEN1 (Fig. 6D), SUT1 (Fig. 6E), and CTT1 (data not shown). ΔMga2 strains showed decreased transcription of all genes tested, indicating a role of Mga2p in the transcriptional activation of hypoxia-induced genes in respiratory growth media.

DISCUSSION

This study shows that yeast, when grown in conditions favoring mitochondrial respiration, are sensitive to various levels of hypoxia (1% or 0.1% O_2). Furthermore, yeast mitochondria increase their generation of ROS in response to hypoxia, as detected by DCFH oxidation. These ROS originate from the mitochondrial electron transport chain, for yeast strains deficient in respiration, specifically at Complex III, are unable to generate ROS in response to hypoxia. We also find that hypoxia-induced transcription of these genes is inhibited by antioxidants. Furthermore, we find that hypoxia-induced expression of specific genes is also decreased in yeast deficient in the transcription factors Yap1p, Msn2p, and Mga2p.

The main catalytic subunit of Complex III is the Rieske iron—sulfur protein (Rip1p). The mechanism responsible for hypoxia-induced production of ROS from Complex III is not well understood, but it is conceivable that hypoxia causes an increase in the lifetime of the semiquinone radical at Complex III (22). This could increase the production of superoxide despite the lesser availability of O_2 as a substrate for that reaction. In the present study, we examined responses in Rip1-deficient cells because an absence of the Rieske protein would prevent oxidation of ubiquinol (48), and abrogate the generation of the semiquinone, thus abolishing the ability to generate hypoxia-induced increases in ROS (21). For the same reason, deletion of Coq2p, which is a crucial enzyme in ubiquinol biosynthesis, would prevent ubiquinol formation and semi-

FIG. 3. Hypoxia-induced gene transcription occurs in glycerol-fed WT, but not ETC-deficient, yeast. Wild-type and ETC-deficient yeast strains were grown overnight in glucose media, washed in PBS, and resuspended for 3 h in glycerol media prior to hypoxic exposure for 6 h. Northern blot analysis was performed and analyzed as described in Materials and Methods. Data are presented as fold-increase from normoxia for (**A**) SOD1, (**B**) HSP12, (**C**) ALD2, (**D**) JEN1, and (**E**) SUT1, and represent an average of at least 5 experiments. * indicates a significant change from normoxia ($p < 0.05$); # indicates a significant difference from WT values ($p < 0.05$).


A
Fold Increase SOD1/ACT1 from Normoxia
WT
ρ°
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1%
0.1%
B
Fold Increase HSP12/ACT1 from Normoxia
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D
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E
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FIG. 3.

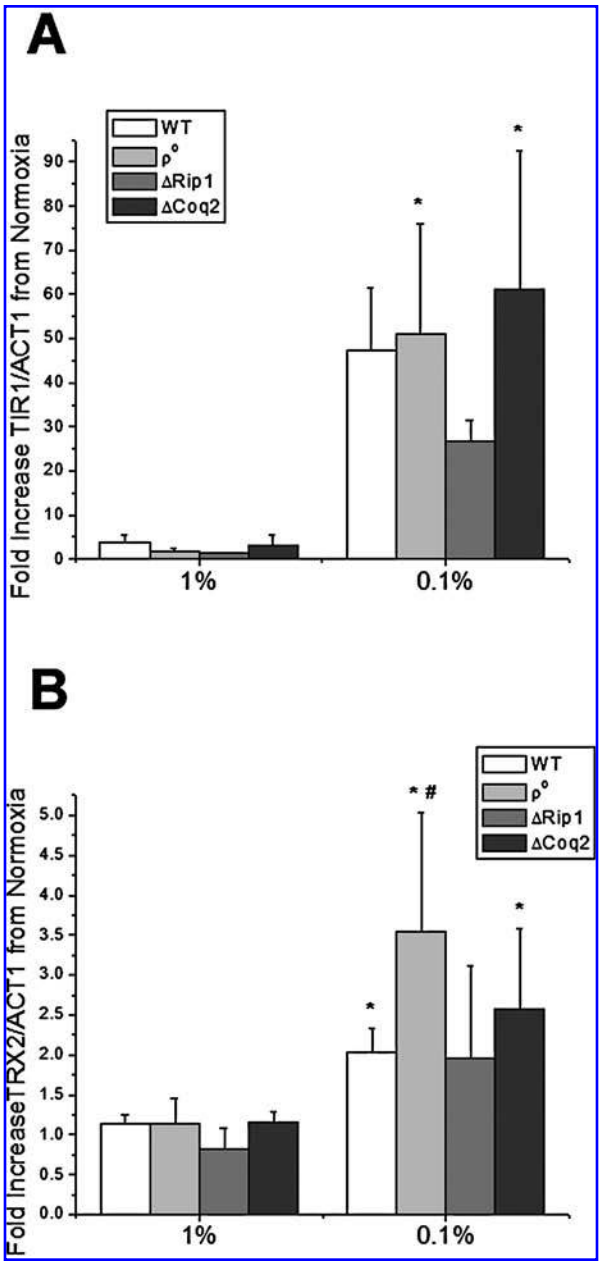


FIG. 4. TIR1 and TRX2 transcription is intact in ETC-deficient yeast in response to severe hypoxia. Wild-type and ETC-deficient yeast strains were grown for 3 h in glucose media prior to hypoxic exposure. Northern blot analysis was performed and analyzed as described in experimental procedures. Both TIR1 (**A**) and TRX2 (**B**) have increased transcription in response to 0.1% O_2 in all strains shown. $N = 3$. * indicates a significant change from normoxia ($p < 0.05$); # indicates a significant difference from WT values ($p < 0.05$).

quinone-derived ROS generation at Complex III. The ρ^0 cells have a generalized loss of electron transport chain activity due to their lack of mitochondrial DNA, and previous studies demonstrate that these cells exhibit lesser levels of protein carbonylation during transition to anoxia compared to wild-type cells (16). These strains provided distinct deletions in the ETC, and each of these strains demonstrated decreased hypoxia-induced ROS production, which supports the conclusion that hypoxia-induced mitochondrial ROS are derived from the electron transport chain, specifically from Complex III.

Wild-type yeast, when grown in conditions that support mitochondrial respiration, increase ROS production in response to hypoxia, and activate transcription of multiple genes including SOD1, HSP12, JEN1, and SUT1. SOD1 is an important antioxidant enzyme that protects yeast from superoxide and exogenous oxidants such as hydroxyurea (7). HSP12 is an important heat-shock protein that is implicated in preservation of cell wall integrity and flexibility in response to physical stress (32), and has been shown to be regulated by Msn2p (17). SOD1 and HSP12 transcription has been reported to be activated by exogenous H_2O_2 (Table 2) and other environmental stresses (10, 56). JEN1 is a lactate permease important in lactate transport, and has been noted to be upregulated in anaerobic environments (41). ALD2 (aldehyde dehydrogenase) expression has not only been shown to be activated by alcohols and aldehydes, but has also been shown to be dependent upon Msn2p for its upregulation (4;5). SUT1 is a transcription factor involved in the upregulation of genes involved in sterol biosynthesis and exogenous sterol uptake (3). SUT1 has been shown to be upregulated by hypoxia, which corresponds with the observation that yeast must import sterols in an anaerobic environment to survive (57). Given the known regulation and function of these genes, acti-

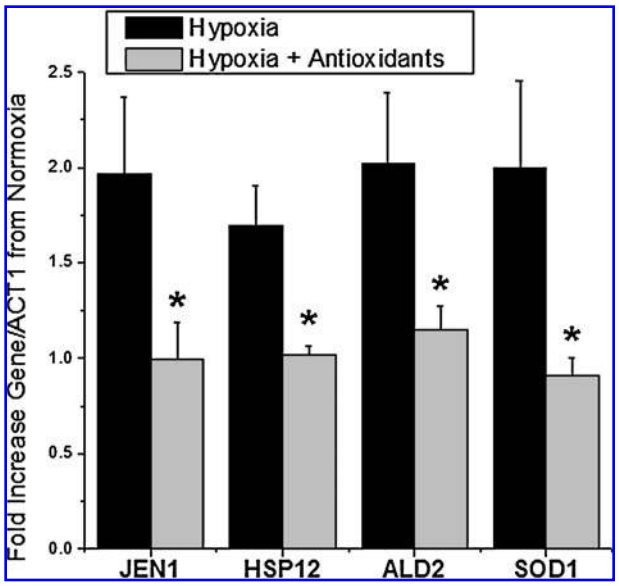
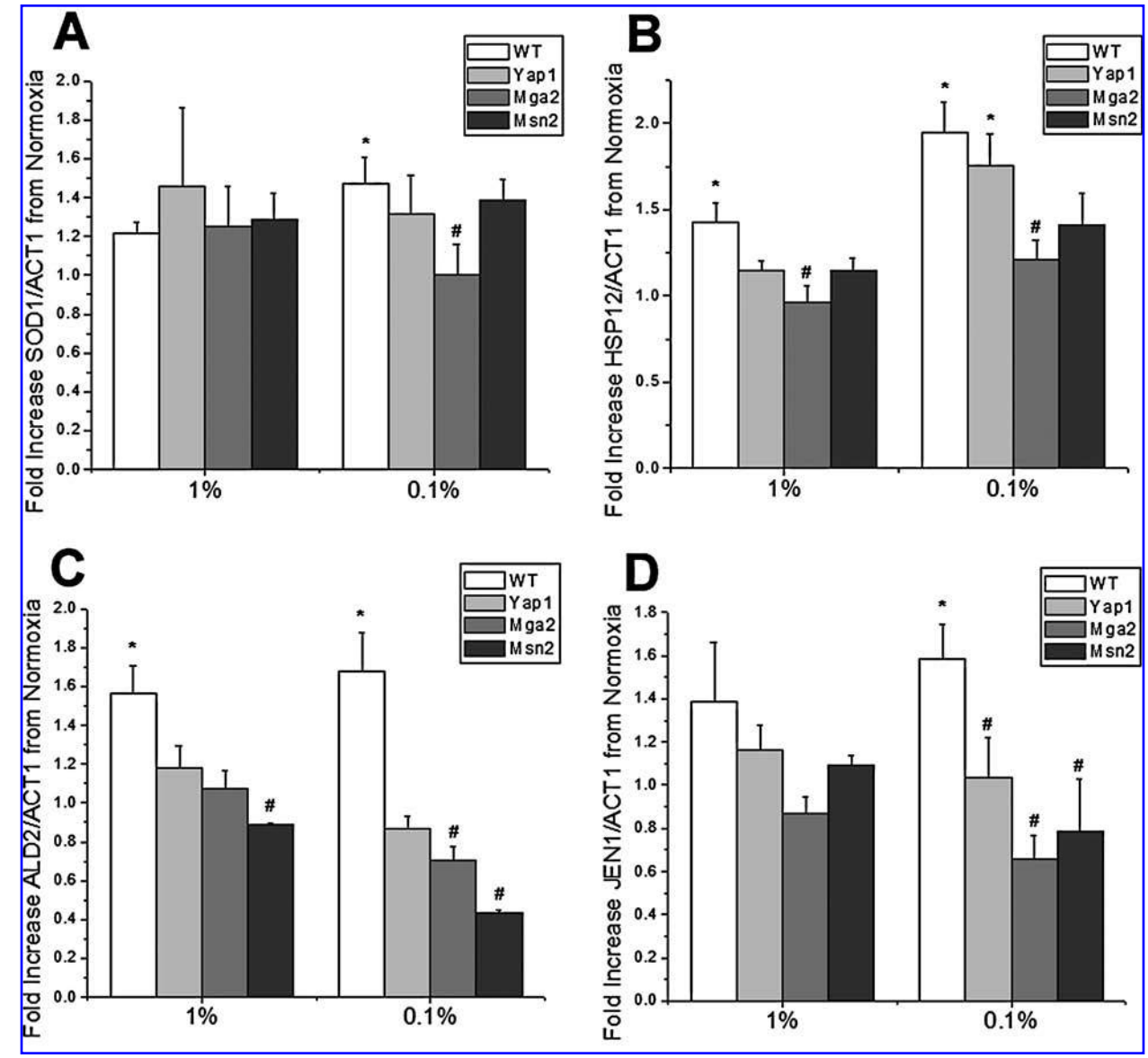


FIG. 5. Antioxidants inhibit hypoxia-induced transcription of JEN1, HSP12, ALD2, and SOD1. Wild-type yeast were grown overnight in glucose media, washed in PBS, diluted, and grown for an additional 3 h in glycerol media as described in Materials and Methods. Yeast were then administered a combination of 2 m*M* *N*-acetyl cysteine and 200 μM 2-MPG upon initiation of hypoxia (0.1% O_2) for 6 h. Northern blot analysis was performed and analyzed as described in Materials and Methods. Data are presented as fold-increase from normoxia, and represent an average of 5 experiments. * indicates a significant decrease relative to hypoxia for each gene studied ($p < 0.05$).



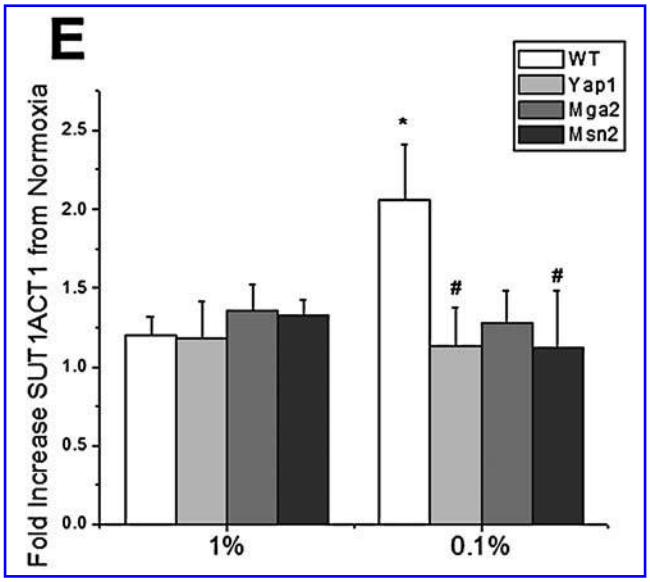


FIG. 6. Hypoxia-induced gene transcription in yeast lacking redox-sensitive transcription factors. Wild-type, ΔYap1, ΔMga2, and ΔMsn2 yeast strains were grown for 3 h in glycerol media prior to hypoxic exposure for 6 h. Northern blot analysis was performed and analyzed as described in experimental procedures. Data are presented as fold-increase from normoxia for (**A**) SOD1, (**B**) HSP12, (**C**) ALD2, (**D**) JEN1, and (**E**) SUT1. * indicates a significant change from normoxia ($p < 0.05$); # indicates a significant difference from WT values ($p < 0.05$).

vation of SOD1, HSP12, JEN1, ALD2, and SUT1 in response to hypoxia may therefore be due to an oxidant signal. This conclusion is supported by our observation that hypoxia-induced transcription of SOD1, HSP12, ALD2, and JEN1 are inhibited by antioxidants. Yeast deficient in the oxidant-sensitive transcription factor Yap1p, as well as stress-induced transcription factors Mga2p and Msn2p, show deficient hypoxic activation of a number of genes studied. The hypoxia-induced ROS signal likely originates from the mitochondria, since ETC-deficient strains that are unable to generate ROS in response to hypoxia have significantly decreased or absent hypoxic gene transcription of the same genes discussed above.

While previous reports have shown OLE1 gene transcription to be dependent upon a functional mitochondrial ETC (37), we have found that OLE1 transcription is unresponsive to mild hypoxia (1% O_2) yet it is responsive to severe hypoxia (0.1% O_2) independent of a functional ETC. Further studies regarding the dependence of OLE1 transcription on a functional mitochondrial ETC are needed to resolve this apparent discrepancy.

An intriguing result from this study is the dependence of hypoxic gene activation on the transcription factor Mga2p. This factor has been shown to be involved in hypoxic (0.1% O_2 or anoxia) activation of OLE1 (49, 67). To our knowledge, ours is the first study showing that ΔMga2 yeast strains are unable to activate other hypoxic genes, specifically under growth conditions where mitochondrial respiration occurs. While further studies are clearly necessary, these findings indicate that Mga2p, like Yap1p and Msn2p, may be sensitive to oxidative stress.

While a hypoxia-sensitive transcription factor analogous to mammalian HIF-1 has not been found in yeast, we provide evidence of a mechanism of hypoxia and anoxia signaling in yeast that has previously been shown in mammalian cells (8). Multiple reports have linked hypoxia-induced mitochondrial ROS production to HIF-1 activation and transcriptional activation of genes such as VEGF and GLUT-1 (8, 21, 45, 46). One recent and elegant report linked anoxia to gene expression via transient ROS production in yeast (16). The present study extends that work by showing that mitochondrial ROS may act as a putative second messenger that activates redox-sensitive transcription factors in the yeast response to hypoxia. As a single cell organism, hypoxia may not present a physiological stress to yeast grown in growth media containing glucose, where the primary source of energy is generated from fermentation. There are, however, growth conditions where glucose is limited or absent, such as the diauxic shift, where yeast consume all available glucose and switch to a respiratory metabolism (35, 54). Under these conditions, where yeast must use O_2 as a terminal electron acceptor to generate ATP, hypoxia presents a physiologically relevant stress. This stress may have two aspects, consisting of the potential bioenergetic consequences of O_2 deprivation, as well as an oxidative stress generated from the ETC (42). ROS generation from the electron transport chain is a normal byproduct of respiration, and a switch from glucose to glycerol media results in an upregulation of antioxidant enzymes (Table 1). Yeast that are deficient in antioxidant enzymes such as superoxide dismutase are unable to survive diauxic shift and are unable to grow on nonfermentable carbon sources because of their inability to metabolize ROS generated from normal respiration (13, 53). Under low oxygen conditions, a functional ETC increases production of ROS, necessitating an increase in antioxidant enzymes such as Sod1p and stress-response proteins such as Hsp12p and Sut1p.

For hypoxia-induced ROS generation to have an effect on gene transcription, yeast must utilize transcription factors that are either directly or indirectly regulated by oxidative stress. We found that the transcription factors Yap1p and Msn2p are required for hypoxia-induced gene transcription for a number of genes examined in this study. Furthermore, we found Mga2p, a factor known to regulate hypoxia-induced transcription of OLE1, to be required for the activation of a number of hypoxia-induced genes. While there is evidence for a direct effect of oxidative stress on Yap1p (11, 14, 15) and Msn2p (23), Mga2p has not been previously described to be redox sensitive. This report demonstrates an overlapping function of these three transcription factors during hypoxia under respiratory growth conditions. Overall, these results provide novel insight into the role of mitochondria as O_2 sensors, by showing that the generation of hypoxia-induced ROS from the ETC may be evolutionarily conserved. This response was not evident in yeast grown in glucose media. However, when grown in conditions favoring respiration over fermentation, their mitochondrial ETC appears to be sensitive to hypoxic conditions, increasing ROS production and initiating gene transcription through redox-sensitive transcription factors. Further studies are needed to better understand the mechanism by which changes in O_2 levels affect the production of ROS from mitochondria.

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ABBREVIATIONS

DCFH, dihydrodichlorofluorescin; ETC, electron transport chain; H_2O_2, hydrogen peroxide; 2-MPG, 2-mercaptopropionyl glycine; RISP, Rieske iron-sulfur protein; ROS, reactive oxygen species

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