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Nitric oxide levels and *CYP55* expression in *Chlamydomonas reinhardtii* under normoxia and hypoxia

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Summary

Nitric oxide (NO) is a vitally important molecule in many organisms, including *Chlamydomonas reinhardtii*. Given that NO can be toxic, its generation and degradation have to be tightly regulated. The intracellular NO can be the substrate for NO reductases producing nitrous oxide (N_2O), and the cytochrome P450, CYP55, catalyzes this NO conversion in the darkness. Here we show that nitrite-dependent NO accumulation in dark cells is directly dependent on O_2 levels: under hypoxia, very little NO is detected, whereas under normoxia, substantial amount of NO is measured. Following dark incubation, NO level is negatively correlated with the *CYP55* expression. Moreover, we found that the transcription of *CYP55* is under the complex influence of NO- and O_2-dependent pathways. Finally, removal of extracellular Ca^{2+} by the application of EGTA leads to an increase in *CYP55* transcript levels both in hypoxic and aerobic dark cells, demonstrating the importance of membrane Ca^{2+} influx in the regulation of this gene. Taken together, our results suggest that *CYP55* expression might be the regulatory point involved in controlling of NO accumulation in the dark-incubated *Chlamydomonas*.

Key words: *Chlamydomonas*, cytochrome P450, hypoxia, nitric oxide, normoxia

Introduction

Nitric oxide (NO) is now regarded as a vitally important molecule in many organisms, including unicellular green algae (Leon and Costa-Broseta, 2020). In *Chlamydomonas reinhardtii* (further referred to as *Chlamydomonas*), NO is involved in the regulation of various cell functions, such as nitrate assimilation (Sanz-Luque et al., 2013), macronutrient stress responses (Wei et al., 2014; Minaeva et al., 2017; De Mia et al., 2019; Filina et al., 2019), salt stress acclimation (Chen et al., 2016) and impact on putrescine and proline synthesis (Zalutskaya et al., 2020). Moreover, NO has also been implicated in responses to hypoxia (Hemschemeier et al., 2013).

The accumulated evidence suggests that *Chlamydomonas* produce NO from nitrite using a dual enzymatic system of nitrate reductase (NR) and NO-forming nitrite reductase (Chamizo-

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Ampudia et al., 2016) or the mitochondrial electron transport chain (Plouviez et al., 2017; Ostroukhova and Ermilova, 2019). Although another (Arg-dependent) mechanism of NO generation has been defined, this process yet has to be characterized at the molecular level (De Mia et al., 2019).

Importantly, NO can be toxic, and therefore, its generation and degradation have to be tightly regulated. In *Chlamydomonas*, truncated hemoglobins might be NO scavengers that modulate NO and thereby control NO-based signaling cascades (Sanz-Luque et al., 2015; Minaeva et al., 2017; Filina et al., 2019; Grinko et al., 2021). Moreover, the intracellular NO can also be the substrate for NO reductases producing nitrous oxide (N_2O) in mitochondria and chloroplasts (Plouviez et al., 2017, 2019; Burlacot et al., 2020).

Interestingly, NO reduction to N_2O has been described in both prokaryotic (Kuypers et al., 2018) and non-photosynthetic eukaryotic organisms (Shoun et al., 2012). In eukaryotes (fungi), the cytochrome P450, CYP55, which is involved in the pathway $NO \rightarrow N_2O$, appears to be acquired by a horizontal gene transfer from bacteria (Shoun et al., 2012). The *Chlamydomonas* genome also contains a gene encoding for CYP55.

Given that in *Chlamydomonas* CYP55 is responsible for N_2O production in the darkness (Plouviez et al., 2017; Burlacot et al., 2020), it is likely that the cytochrome P450 plays an important role in the regulation of cellular NO levels. Nevertheless, the ratio of NO in the two types of dark cells, aerobic and hypoxic, is not known to date. In this study, we investigated nitrite-dependent NO accumulation in the dark-incubated NR-deficient cells upon normoxia and hypoxia. Our analysis demonstrates that there is a negative correlation between the NO amount and the levels of *CYP55* transcripts. The expression level of *CYP55* increased in the dark cells and was negatively associated with both O_2 levels and membrane-regulated entry of extracellular Ca^{2+} ions. These data suggest that *CYP55* expression might be the regulatory point involved in the control of NO levels in dark cells.

Material and methods

Growth conditions and cell treatments

The strain CC4533 (cw15 mt-) was obtained from *Chlamydomonas* Resource Center (USA). Cells were grown in tris-acetate-phosphate (TAP) medium (http://www.chlamy.org/TAP.html) under continuous illumination by white light (fluence rate of 45 μmol/m^2 s^1) at 22 °C with a constant orbital agitation at 90 rpm. Depending on the nitrogen source, four variants of the TAP medium were used: with 7.5 mM NH_4Cl, 4 mM KNO_3, 10 mM or 20 mM KNO_2.

To induce anoxic conditions, the cells grown in TAP-NH_4 to log phase (2–2.5 10^6 cells/ml) in 1 l flasks were collected by centrifugation (4000× g, 5 min), washed with TAP medium containing 20 mM KNO_2, and then 50 ml aliquots of cells were transferred into sealed 50 ml square glass bottles. The flasks were subsequently incubated in the dark, allowing a gradual removal of dissolved oxygen by respiratory activity. Dark-aerobic conditions were established by incubating 50 ml of cell suspension in 250 ml glass beakers loosely covered with aluminum foil. Dark-aerobic and dark-anoxic experiments were performed in parallel from a single batch culture in each biological experiment. Dissolved oxygen was measured using a Clark-type electrode to ensure that anoxic conditions were achieved.

2-(N,N diethylamino)-diazenolate 2-oxide sodium salt (DEA-NONOate, Sigma-Aldrich, USA), S-nitroso-N-acetylpenicillamine (SNAP, Sigma-Aldrich, USA) and ethyleneglycol- bis (β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EG TA, Sigma-Aldrich, USA) were used at final concentrations of 100 μM, 2 mM, and 2 mM, respectively.

At each harvesting time, the number of cells was measured employing a counting chamber and the viable cells were estimated microscopically with use of 0.05% (v/v) Evans blue (DIA-M, Russia) as described by Baker and Mock (1994).

Chlorophyll extraction was carried out using 80% acetone. The resulting supernatant, which contains total chlorophyll, was isolated, and absorption was measured by a spectrophotometer (SmartSpec Plus, BioRad) at 652 nm. The total chlorophyll content (μg ml^{-1}) was quantified according to Harris (1989).

Quantitative real-time PCR

The total RNA was isolated with Trizol according to the manufacturer's instructions (Invitrogen, USA). DNA contamination was avoided by treatment of the RNA samples with RNase-Free DNase I (Fermentas, Lithuania). Reverse transcription was performed with Revert Aid H Minus First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Thermo Scientific,

USA). Gene expression analysis was carried out by real-time quantitative RT-PCR (RT-qPCR) on the Light Cycler Instrument (CFX96 Real--Time PCR Detection System, Bio-Rad) using SYBR Green I following a previously reported protocol (Zalutskaya et al., 2016). The primer pairs used for RT-qPCR were as follows: 5'-AG GCCAAGGCAGACAAGCTG-3' and 5'-GC CTCGGGAATGCCGATGAA-3' for *CYP55* (Cre01.g007950), and 5'- CTTCTCGCCCATG ACCAC-3' and 5'-CCCACCAGGTTGTTCT TCAG-3' for *RACK1* (receptor of activated protein kinase C; Cre06.g278222, formerly termed *CBLP*). The relative gene expression ratios were normalized with *RACK1* using the ΔC_T and $\Delta\Delta C_T$ methods (Livak and Schmittgen, 2001). Values were obtained from at least three biological replicates; each replicate was analyzed three times. Student's t-tests were used for statistical comparisons. P-values of <0.05 were considered as significant.

Measurement of NO

Chlamydomonas cells (45 μg chlorophyll ml^{-1}) were incubated in TAP medium containing 20 mM KNO_2 in the presence of 1 μM (4-amino-5-methylamino-2'7'-difluorofluorescein diacetate) dye (DAF-FM DA, Sigma-Aldrich). After 15 min of treatment, the cells were washed, resuspended in the same medium, incubated for additional 30 min to allow complete de-esterification of the intracellular diacetates, and then intracellular generation of NO was evaluated using a microplate reader CLARIOstar (BMG). Excitation and emission wavelengths were set at 483±14 and 530±30 nm, respectively. Cell autofluorescence was subtracted from the total fluorescence obtained. Fluorescence levels were expressed as arbitrary units (per μg chlorophyll 10^6 cells). Three technical replicates per condition were included on each plate, and each experiment was performed three times independently.

Confocal microscopy

For the NO detection by confocal microscopy, cells were treated as described above. Images were acquired with a Leica TCS-SP5 confocal microscope (Leica-Microsystems, Germany) equipped with a HC PL APO 63× oil immersion objective. Excitation was performed with a 488-nm argon laser. The signals arising from the DAF-FM DA were collected on the channel between 500 and 544 nm. Chlorophyll autofluorescence was monitored across a window of 600±680 nm. The experiment was performed in triplicate.

Results

NO production in the dark-incubated cells under normoxia and hypoxia

It has been shown previously that NO is important for hypoxic growth of *Chlamydomonas* in the dark (Hemschemeier et al., 2013). Moreover, NO was generated in the dark aerobic wild-type cells (Burlacot et al., 2019). To investigate the potential components involved in the control of NO levels in the dark-incubated cells, we used the strain CC4533. Because this strain was unable to grow on nitrate and lacked NR activity, this enzyme cannot be responsible for NO generation in these cells. However, CC4533 exhibits the growth on nitrite as nitrogen source (Fig. 1, A).

To compare the nitrite-dependent NO production in aerobic and hypoxic cells, we carried out two types of experiments. In the first type of analysis, cells grown in the light were transferred to open flasks with nitrite-containing medium in the dark. In these experiments, no significant drop in the dissolved oxygen levels was monitored, showing only slight decrease (about 5 %) after 3 h of incubation in the dark (Fig. 1, B). During the second type of experiments, illuminated aerobic cultures were transferred to sealed flasks in the dark to create hypoxic conditions. In these conditions, about 80% drop in oxygen levels was monitored after 1 h of incubation (Fig. 1, B). To detect NO production, an NO-sensitive fluorescent dye, DAF-FM diacetate, was added to the nitrite-containing medium before cells were transferred to a dark condition. Notably, NO amounts were 5-6 fold higher in cells under normoxia than under hypoxia (Fig. 1, B).

We confirmed these results using confocal microscopy. After incubation with the NO-specific dye DAF-FM DA, the dark hypoxia-treated cells demonstrated either no signal or a very weak one in the cytosol (Fig. 1, C). When *Chlamydomonas* was incubated in the dark aerobic conditions, the cells displayed green NO fluorescence. Thus, NO is generated under normoxia and hypoxia at very different levels.

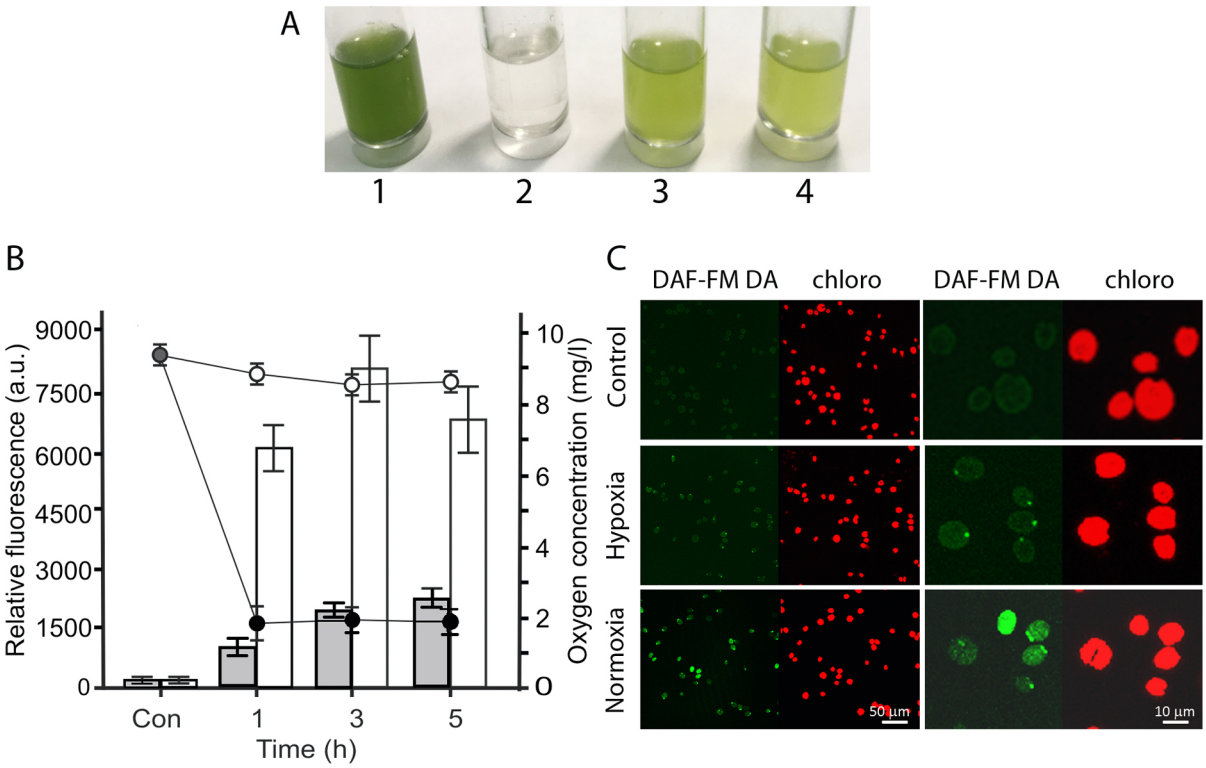

Fig. 1. Effects of nitrite on NO production in the dark-incubated *Chlamydomonas* cells under normoxia and hypoxia. A – The cell growth was analyzed in 7.5 mM ammonium (1)-, 4 mM nitrate (2)-, 10 mM nitrite (3)- or 20 mM (4) nitrite-containing media; B – NO production in cells. Vegetative cells were grown in TAP medium (Con) and transferred to a 20 mM nitrite-containing medium in the dark under hypoxia or normoxia. Fluorescence intensity (indicated by grey and white squares under hypoxia and normoxia, respectively) due to intracellular NO was determined using DAF-FM DA and was expressed as arbitrary units per µg chlorophyll 10 cells. Cell autofluorescence was subtracted from the total fluorescence obtained. Data are the means ± SE from three independent experiments. Levels of dissolved oxygen (indicated by and white circles under hypoxia and normoxia, respectively) are given as mg per l of medium; C – NO visualization by confocal microscopy. Images of cell populations grown in TAP (Con) or incubated in 20 mM nitrite-containing medium in the dark for 3 h under hypoxia or normoxia. The left-hand panels show DAF-FM fluorescence (green color) while the right-hand panels show chlorophyll autofluorescence (red color). Scale bars: 50 µm or 10 µm.

Effects of oxygen levels on *CYP55* expression in the dark-incubated cells

Previous studies have indicated that the dark reduction of NO to N_2O is mediated by cytochrome P450 (CYP55) in *Chlamydomonas* (Plouviez et al., 2017; Burlacot et al., 2019). We therefore wondered whether the *CYP55* mRNA levels correlated with NO levels in the dark-incubated cells. We found that *CYP55* mRNA transcript abundance increased 10-fold after 1 h and declined after 3 h exposure to the dark under normoxia (Fig. 2). When *Chlamydomonas* cells were exposed to hypoxia, we observed a more dramatic induction in the *CYP55* transcript levels (29-fold increase after 1 h with the highest induction of 62-fold after 3 h). Altogether, our results indicate a negative correlation between the *CYP55* mRNA levels and NO content in *Chlamydomonas* cells subjected to darkness under aerobic or hypoxic conditions indicating that the transcription step is potentially the key regulatory point of the dark reduction of NO.

NO regulates *CYP55* expression

In order to check whether the observed *CYP55* transcript levels were related to NO, we first measured the *CYP55* mRNA abundances in response to DEANONOate or SNAP as NO donors. Unexpectedly, after the treatment with 100 µM DEA

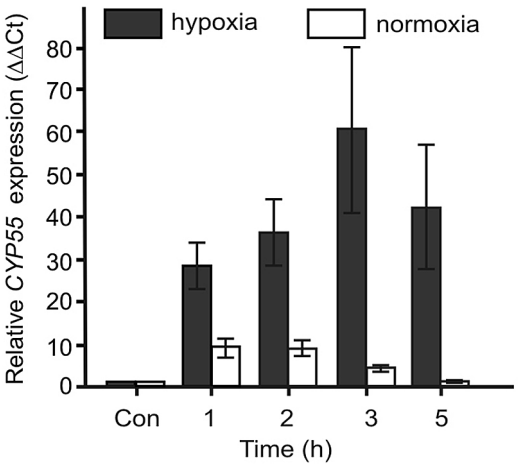

Fig. 2. Effects of nitrite on *CYP55* expression in the dark-incubated *Chlamydomonas* cells under normoxia and hypoxia. Cells were treated as described in Fig. 1, B. Levels of gene transcripts are calculated as times of relative abundance with respect to the housekeeping control gene (*RACK1*) that has a value of 1. Data are the means ± SE from three biological and three technical replicates obtained by RT-qPCR.

NONOate for 0.5 h or 1 h, transcripts of the *CYP55* gene were strongly induced (Fig. 3, A). Furthermore, the addition of SNAP also strikingly enhanced the *CYP55* expression (Fig. 3, A). However, in the dark-hypoxic cells, the high *CYP55* mRNA transcript abundance was maintained under low levels of NO (Fig. 2, A). A possible explanation of this difference is that the effects of DEA NONOate and SNAP were found in the TAP grown aerobic cells in the light. We hypothesized that NO may interfere with the hypoxia-specific components involved in the regulation of this gene in the darkness. In order to test this possibility, we analyzed the effects of DEA NONOate that was added to dark cells in the TAP-NO_2 (Fig. 3, B). The reduction of *CYP55* expression levels in dark-hypoxic cells in the presence of exogenous NO indicates that NO-dependent signal network appears to be not the only pathway influencing the transcript levels of *CYP55* mRNA under hypoxia. In contrast, the addition of DEA NONOate resulted in the increase of the dark-induced expression of *CYP55* in aerobic cells (Fig. 3, B). Therefore, our results highlight that NO may negatively control the dark-induced *CYP55* gene expression at low O_2 levels by operating in a reverse regulatory loop. This mechanism may help to fine-tune NO levels depending on the cells' physiological status.

CYP55 gene responds to Ca^{2+}

Since Ca^{2+} appears to play a role in adaptation to hypoxic conditions (Düner et al., 2018), the question arises whether this second messenger exerts any effect on the hypoxia-induced *CYP55* expression. As shown in Fig. 4, the addition of the calcium chelator EGTA to washed cells prior to hypoxia treatment did affect the *CYP55* transcription: the presence of 2 mM EGTA led to enhanced amplitude of hypoxia-induced *CYP55* gene expression. Moreover, this induction was diminished when an excess extracellular Ca^{2+} (2.1 versus 0.34 mM) was provided, despite the presence of EGTA.

Then we also tested the cells that were incubated in darkness, but in the presence of O_2. Notably, EGTA had an even stronger effect on the *CYP55* transcription in dark aerobic cells (Fig. 4). The results suggest that a membrane-regulated entry of extracellular Ca^{2+} ions is essential to regulate the dark-induced *CYP55* expression and this effect is dependent on O_2 levels.

Discussion

In this study, we demonstrate that dark-incubated *Chlamydomonas* cells are able to the nitrite-dependent NO production, which is not mediated by nitrate reductase. Moreover, we unveil that NO accumulation in dark cells is directly dependent on O_2 levels: under hypoxia, very little NO is detected, whereas under normoxia, substantial amount of NO is measured (Fig. 1). This effect differs from higher plants that produce high levels of NO under hypoxic conditions (Gupta et al., 2005; Wany et al., 2017).

In *Chlamydomonas*, NO homeostasis is based on the balance between reduction of nitrite to NO and re-oxidation of NO to nitrate (Bellido-Pedraza et al., 2020). It is believed that truncated hemoglobins might be NO scavengers that protect *Chlamydomonas* cells from an accumulation of the radical molecule (Sanz-Luque et al., 2015; Chamizo-Ampudia et al., 2016; Minaeva et al., 2017; Filina et al., 2019; Grinko et al., 2021). Recently it was shown that *Chlamydomonas* can reduce NO to N_2O in the dark (Plouviez et al., 2017, 2019) and this dark reaction is mediated by CYP55 (Burlacot et al., 2020). Interestingly, we found that expression of *CYP55* is under the complex influence of NO- and O_2-dependent pathways (Figs 2, 3). The NO level in dark cells is negatively correlated with the *CYP55* expression (Figs 1, 2). An important ques-

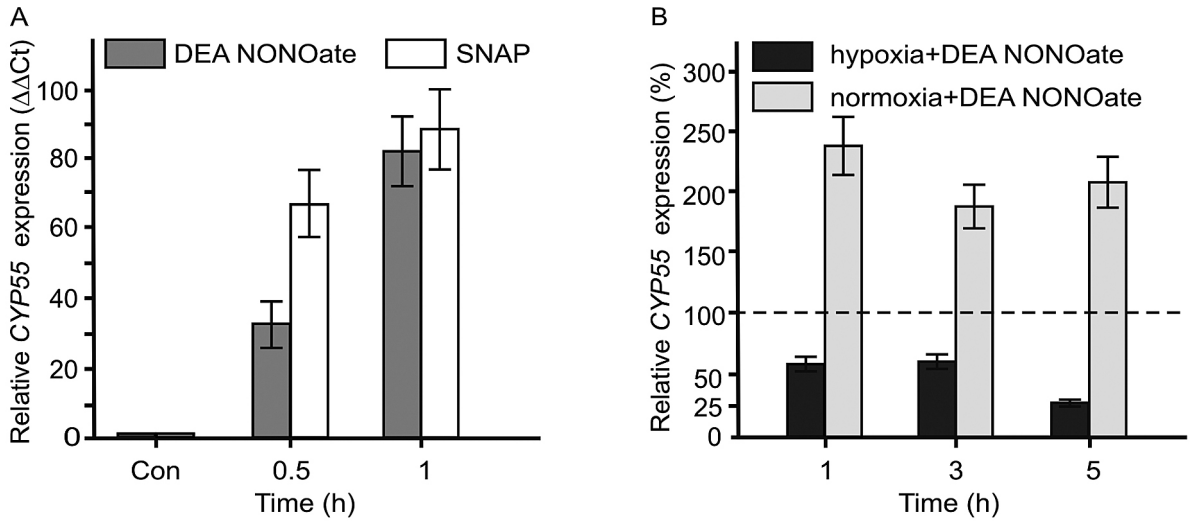

Fig. 3. NO-dependent expression of *CYP55*. A – Effects of DEA-NONOate and SNAP on the expression of *CYP55* gene. DEA NONOate (100 μM) or SNAP (2 mM) were added to TAP grown cells (Con). B – Effects of DEA-NONOate on the expression of *CYP55* gene under hypoxia and normoxia. Cells grown in TAP were transferred to 20 mM nitrite-containing medium and incubated for 1 h, 3 h or 5 h in the dark. DEA NONOate (100 μM) was added 40 min before the indicated times. Expression levels of *CYP55* are given as a percentage with respect to the variant without DEA NONOate at each time point, which has a value of 100%. Relative gene expression was determined as described in Fig. 2.

tion is why O_2-dependent signals are required to control the expression of *CYP55*. One possibility is that lower NO levels have emerged to be required for the acclimation to dark hypoxia than to dark normoxia. Therefore, important regulatory layer in dark *Chlamydomonas* could be that the CYP55 protein amounts must be balanced in order to ensure the NO signaling mechanism to function properly.

In higher plants, NO cross talks with Ca^{2+} (Besson-Bard et al., 2008; Verma et al., 2019; Lau et al., 2021). The regulation of *CYP55* transcription by Ca^{2+} in the cells incubated under hypoxia overlaps with that under normoxia. As we found a strict negative correlation between NO levels and the accumulation of *CYP55* transcripts (Fig. 2), a retrograde signal appears to exist that is integrated at the level of CYP55. Here, one scenario would be that NO triggers Ca^{2+} influx through membrane Ca^{2+} channels, which, via a signaling cascade, finally results in a negative control of *CYP55* expression. Given that we observed a more dramatic induction in the *CYP55* transcript levels by EGTA in dark aerobic cells (Fig. 4), additional components might be used to regulate the transcription of this gene by Ca^{2+} under normoxia. Moreover, we still cannot rule out the role of NO production mechanisms. Besides, the major players involved in NO generation in dark cells at different O_2 levels remain unknown.

This study provides a basis for further research on elucidating the components involved in the relationship between NO- and Ca^{2+}-dependent signaling, and on the question at which level the mechanisms of NO production are coordinated with the mechanisms of NO conversion in dark cells.

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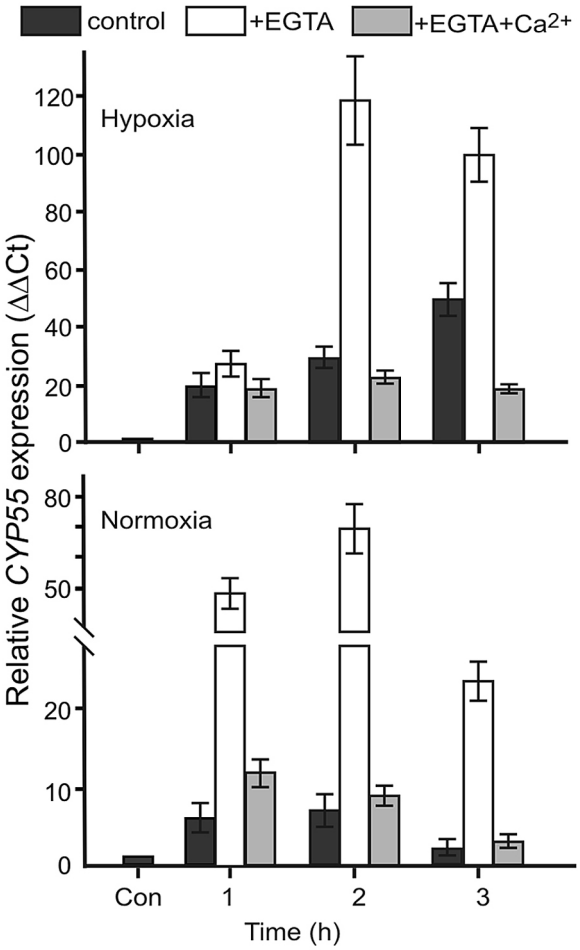

Fig. 4. Effects of EGTA on *CYP55* transcript accumulation induced by dark under hypoxia or normoxia. Cells were exposed to dark hypoxia or normoxia in the absence (Control) and in the presence of 2 mM EGTA with or without 2.1 mM $CaCl_2$. Relative gene expression was determined as described in Fig. 2. Data are the means ± SE from three biological and three technical replicates obtained by RT-qPCR.

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