RESEARCH ARTICLE

CKA2 functions in H2O2-induced apoptosis and high-temperature stress tolerance by regulating NO accumulation in yeast

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One sentence summary: CKA2-mediated regulation of NO accumulation involves H2O2-induced apoptosis and high-temperature stress tolerance in yeast.

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ABSTRACT

Nitric oxide (NO) plays key roles in yeast responses to various environmental factors, such as H2O2 and high temperature. However, the gene encoding NO synthase (NOS) in yeast has not yet been identified, and the mechanism underlying the regulation of NOS-like activity is poorly understood. Here, we report on the involvement of CKA2 in H2O2-induced yeast apoptosis and yeast high-temperature stress tolerance. Our results showed that although Δcka2 mutant had reduced NO accumulation with decreased apoptosis after H2O2 exposure, treatment with a NO donor, sodium nitroprusside, resulted in similar survival rate of Δcka2 mutant compared to that of wild-type yeast when subjected to H2O2 stress. This finding occurred because H2O2-enhanced NOS-like activity in wild-type yeast was significantly repressed in Δcka2. Our additional experiments indicated that both high-temperature-enhanced NO accumulation and NOS-like activity were also suppressed in Δcka2, leading to the hypersensitivity of the mutant to high temperature in terms of changes in survival rate. Thus, our results showed that CKA2 functioned in H2O2-induced apoptosis and high-temperature stress tolerance by regulating NOS-like-dependent NO accumulation in yeast.

Keywords: yeast; nitric oxide; H2O2; high-temperature stress; NOS-like; CKA2

INTRODUCTION

Nitric oxide (NO) is a widespread signalling molecule involved in various aspects of cellular functions in yeast, including H2O2-induced apoptosis, heat shock and high hydrostatic pressure (Domitrovic et al. 2003; Almeida et al. 2007; Nishimura, Kawahara and Takagi 2013). In mammals, NO is synthesized by NO synthase (NOS) with L-arginine (L-Arg) as a substrate (Gupta et al. 2011). However, NO synthesis in yeast is much more complex compared with that in mammals. In yeast, in addition to cytochrome c oxidase, which can produce NO with nitrite as a substrate, NOS-like activity for NO synthesis with L-Arg as a substrate has been verified (Castello et al. 2006; Gupta et al. 2011; Nishimura, Kawahara and Takagi 2013). In a previous report, the treatment of yeast cells with menadione led to NO production dependent on intracellular L-Arg content (Osorio et al. 2007), suggesting the existence of NOS-like activity, but no gene or protein homologous to yeast NOS among mammal NOS has yet been identified.

The precise regulation of cell growth, proliferation and apoptosis is essential for the development and maintenance of normal cells (Ahmed, Gerber and Cochet 2002). Experiments have indicated that yeast apoptosis can be induced by many different
stimuli, such as H$_2$O$_2$, acetic acid, high salt and UV irradiation (Carmona-Gutierrez et al. 2010). NO also modulates the apoptosis signalling cascade by regulating the expression of apoptosis-related genes, mitochondrial dysfunction and the activation of caspase activity (Brune, Messmer and Sandau 1995; Kroncke, Fehsel and Kolb-Bachofen 1995). For H$_2$O$_2$-induced yeast apoptosis, NO accumulation is required, and this increase in NO production depends on NOS-like activity because the application of an NOS-specific inhibitor, N’-nitro-L-Arg-methyl ester (L-NAME), obviously inhibits H$_2$O$_2$-induced apoptosis, thus increasing the survival rate in response to H$_2$O$_2$ (Almeida et al. 2007). However, how H$_2$O$_2$ modulates NO accumulation via NOS-like activity remains unknown.

High-temperature stress tolerance is another NO-mediated biological process. Nishimura Kawahara and Takagi (2013) reported that the application of either L-NAME or the NO scavenger c-PTIO remarkably decreased the tolerance of wild-type (WT) yeast cells to high-temperature stress. Moreover, elevating NO accumulation by overexpressing TAH18 conferred higher tolerance, whereas knockdown of this gene expression resulted in greater sensitivity when the yeast cells were subjected to high-temperature stress (Nishimura, Kawahara and Takagi 2013).

Here, we report that Cka2, an α′ catalytic subunit of casein kinase 2 (CK2), functioned as a new player in H$_2$O$_2$-induced NO accumulation and apoptosis by regulating NOS-like activity. Furthermore, NO-mediated tolerance to high-temperature stress was compromised in Δcka2 mutant with reduced NOS-like activity, indicating the role of CKA2 in high-temperature stress tolerance in yeast.

**MATERIALS AND METHODS**

**Strains and treatments**

The yeast Saccharomyces cerevisiae WT strain BY4741 (MATα; his3Δ1; leu2Δ0; metαΔ0; ura3Δ0), the deletion strain YOR061W Δcka2 (YOR061W::kanMX4) and a collection of yeast deletion mutants were purchased from EUROSCARF (Frankfurt, Germany).

For H$_2$O$_2$ treatment assay, 4 mM H$_2$O$_2$ was used to treat the yeast cells for 30 min as previously reported (Almeida et al. 2007). For the screening of the collection of yeast deletion mutants, deep 96-well plates containing YPD medium with glass beads were used to culture WT yeast and mutants with agitator (150 rpm) at 26°C for 2 days. The yeast cells (10$^6$ cells mL$^{-1}$) were treated with H$_2$O$_2$ at a final concentration of 4 mM for 30 min. After treatment, serial dilutions of the cells were spread on YPD agar plates, and the viability was calculated by counting colony-forming units after 2 days of incubation at 26°C. For high-temperature stress tolerance testing, experiments were performed as previously reported (Nishimura, Kawahara and Takagi 2013).

**NO detection**

Free intracellular NO levels were detected with the NO-specific fluorescence dye DAF-FM DA (diaminofluorescein-FM diacetate, Sigma). Treated or untreated yeast cells were incubated in 50 mM potassium phosphate buffer (pH 7.4) with 5 μM DAF-FM DA in the dark for 30 min. Then, the yeast cells were rinsed and suspended in potassium phosphate buffer. For visualization, images of yeast NO fluorescence were obtained under a fluorescence microscope (BX60, Olympus) equipped with a CCD camera, and images of cell morphology were obtained by different interference contrast.

**Flow cytometry**

The WT and deletion strain yeast cells were treated with 4 mM H$_2$O$_2$ for 30 min and then were washed with 50 mM potassium phosphate buffer (pH 7.4) to remove the H$_2$O$_2$. Treated or untreated yeast cells were incubated in potassium phosphate buffer (pH 7.4) with 5 μM DAF-FM DA in the dark for 30 min. Flow cytometry assays were performed as previously reported (Almeida et al. 2007).

**Measurement of NOS-like activity**

To assay NOS-like activity, S. cerevisiae cells were cultured to the stationary growth phase in YPD medium at 26°C; then, the cells collected and resuspended in ice-cold extraction buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 1 mM leupeptin, 1 mM pepstatin and 1 mM phenylmethlysulfonyl fluoride) and were vortexed with glass beads. The mixture was centrifuged at 12 000 rpm for 15 min at 4°C. The supernatant was used for NOS-like activity determination with a NOS assay kit (Beyotime, Haimen, China), as previously described (Xiong et al. 2009; Shi et al. 2012; Xue et al. 2012). Briefly, 0.1 mL of supernatant was added to a 0.1 mL assay mixture (containing NADPH, L-Arg, NOS assay buffer and DAF-FM DA) and reacted at 37°C in the dark for 1 h. The production of NO was measured using a fluorimeter with 488 nm excitation and 510 nm emission filters.

**Determination of L-Arg content in yeast**

To extract L-Arg in yeast according to a previously reported method by Nishimura et al. (2010), the treated or untreated yeast cells were harvested, washed twice with 50 mM potassium phosphate buffer (pH 7.4) and resuspended in 200 μL of distilled water. The suspension was transferred to boiling water, and intracellular amino acids were extracted by boiling for 15 min. After centrifugation for 15 min at 14000 g, the supernatant was used to measure Arg content as previously reported (Shi et al. 2013). Briefly, in an ice water bath, 100 μL of the supernatant was mixed with 40 μL of 0.03% thymol (W/V, in 4% NaOH) for 20 min; then, 20 μL of 0.7% sodium hypobromite (W/V, in 5% NaOH) was added to the mixture and vortexed, immediately followed by the addition of 20 μL of 40% urea for 2 min. Finally, the mixtures were used to measure the absorbance at 470 nm.

**Assessment of superoxide anion accumulation in yeast cells**

Superoxide anion accumulation in yeast cells was detected by dihydroethidium (DHE) staining, as previously reported (Almeida et al. 2007). Briefly, treated or untreated yeast cells were harvested by centrifugation, were resuspended in 50 mM potassium phosphate buffer (pH 7.4) with 2.5 μg mL$^{-1}$ DHE and then were incubated in the dark for 10 min. For the visualization and analysis of the fluorescence, images of yeast ROS fluorescence were obtained under the fluorescence microscope (BX60, Olympus).

**RESULTS**

**CKA2 is involved in H$_2$O$_2$-induced NO accumulation and apoptosis**

Previous reports have shown that H$_2$O$_2$ induced yeast apoptosis to decrease the survival rate by accumulating NO (Almeida et al. 2007). Therefore, identifying yeast mutants with lower NO
levels for higher survival rates in response to H$_2$O$_2$ might shed light on how H$_2$O$_2$ modulates changes in NO levels in yeast. To achieve this goal, we screened the collection of yeast deletion mutants for possible mutants with higher survival rates and lower NO levels than WT cells by treating the collection with 4 mM H$_2$O$_2$ for 30 min and testing the survival rates. Our results showed that several yeast mutants, including Δcka2 (YOR061W) (see Table S1, Supporting Information), exhibited increased survival rates compared with WT yeast without H$_2$O$_2$ treatment (Fig. 1A). CKA2 encodes the α′-catalytic subunit of casein kinase 2 (CK2), which plays roles in cell growth and proliferation (Schmidt et al. 2011). Whether and how Cka2 functions in H$_2$O$_2$-induced NO production have not been previously reported. Thus, we further assayed possible changes in NO levels in mutant Δcka2 by flow cytometry analysis. These assays revealed that a high percentage of H$_2$O$_2$-treated WT yeast cells contained high NO levels, but far fewer H$_2$O$_2$-treated Δcka2 cells showed high NO accumulation (Fig. 1B). This result was confirmed using the NO-specific fluorescent dye DAF-FM DA. Although NO production was remarkably increased in H$_2$O$_2$-treated WT yeast cells, H$_2$O$_2$-induced NO accumulation was largely compromised in Δcka2, compared with WT yeast (Fig. 1C and 1D). Treatment with sodium nitroprusside (SNP), a widely used NO donor, resulted in the similar survival rate of Δcka2 mutants to that of the WT yeast (Fig. 1A). Taken together, our results demonstrated that CKA2 was involved in H$_2$O$_2$-induced yeast apoptosis by changing NO accumulation.

Δcka2 mutant exhibits reduced NOS-like activity upon H$_2$O$_2$ exposure

It was reported that H$_2$O$_2$ treatment could increase L-Arg content for NO accumulation via NOS-like activity in yeast (Almeida et al. 2007). Thus, we first examined L-Arg content in Δcka2 mutants upon H$_2$O$_2$ exposure. Similar to the previous report (Almeida et al. 2007), H$_2$O$_2$ induced L-Arg accumulation in WT yeast. L-Arg content was also increased in H$_2$O$_2$-treated Δcka2 (Fig. 2A). In addition, both H$_2$O$_2$-treated WT yeast and Δcka2 mutants had similar L-Arg contents (Fig. 2A). Taken together with our above finding that H$_2$O$_2$-induced NO accumulation was suppressed in Δcka2, these data suggested that reduced NO accumulation in Δcka2 mutant was not due to the insufficiency of L-Arg.

An alternative explanation for the low NO level in H$_2$O$_2$-treated Δcka2 was that H$_2$O$_2$-treated WT yeast and Δcka2 mutants could have different NOS-like activities. Indeed, although Δcka2 mutant exhibited slightly reduced NOS-like activity compared with WT yeast without H$_2$O$_2$ treatment, H$_2$O$_2$-enhanced NOS-like activities in WT yeast were significantly repressed in...
Δcka2 (Fig. 2B), suggesting that yeast CKA2 functioned in H₂O₂-induced NO accumulation by regulating NOS-like activity.

Δcka2 is hypersensitive to high temperature with reduced NO production via NOS-like effects

Yeast also responds to high-temperature stress by increasing NO accumulation (Nishimura, Kawahara and Takagi 2013). CKA2 might also be involved in the yeast response to high-temperature stress because of its capacity to modulating NO accumulation, as shown above. To test this theory, we first examined the tolerance of Δcka2 mutant to high-temperature stress as previously reported (Nishimura, Kawahara and Takagi 2013). Both WT yeast and Δcka2 were exposed to 39°C for 4 h, and then both survival rates and NO levels were determined. Δcka2 was more sensitive to high-temperature stress than the WT in terms of changes in survival rates (Fig. 3A). Consistently, lower NO levels in Δcka2 mutant were found than in the WT upon high-temperature stress (Fig. 3B and C). This reduced survival rate of Δcka2 was due to the suppression of high-temperature-induced NO accumulation in the mutants because SNP application could increase the survival rate of high-temperature-treated Δcka2 (Fig. 3A). These results indicated that CKA2 played a vital role in high-temperature stress tolerance in yeast cells by changing NO accumulations.

This suppression of high-temperature-induced NO accumulation in Δcka2 mutant could be the result of WT yeast and Δcka2 mutants having different NOS-like activities when challenged with high temperature. Thus, we measured NOS-like activity and tested the survival rates of WT and Δcka2 yeast cells. Our results revealed that NOS-like activity was induced by high-temperature stress in WT yeast, but the induction of NOS-like activity was compromised in Δcka2 (Fig. 4), indicating the role of CKA2 in high-temperature-induced NOS-like activity. Furthermore, whereas treatment with L-NAME, a NOS-specific inhibitor, resulted in enhanced sensitivity of WT yeast to high-temperature stress in terms of decreased survival rates, L-NAME-treated Δcka2 did not show a significant reduction in survival rate in response to high-temperature stress (Fig. 3A). Thus, our results suggested that Cka2 modulated the NOS-like activity of yeast in response to high-temperature stress.

DISCUSSION

It has been documented that NO is involved in the response of yeast to high-temperature stress and H₂O₂-induced yeast apoptosis (Dimitrovic et al. 2003; Almeida et al. 2007; Nishimura, Kawahara and Takagi 2013). Here, we further showed that CKA2 played roles in both of these processes. CKA2 is a Ser/Thr protein kinase essential for mammals, plants and yeast. In yeast, this kinase comprises two catalytic α (A1) or α′ (A2) subunits and two regulatory β subunits that can form a holoenzyme with an α2β2, αα′β2 or α2β′2 tetrameric structure (Padmanabha et al. 1996; Litchfield et al. 2002; Mulekar and Huq 2014). Yeast cells carrying disruptions in the both CKA1 and CKA2 genes were not viable (Padmanabha et al. 1996; Litchfield et al. 2002), suggesting the crucial role of CKA in yeast. Our study here identified Cka2 as a new player in the response of yeast to H₂O₂ exposure and high-temperature stress.

We note that mammal CK2 can repress the transcription of endothelial NOS (eNOS) by phosphorylating transcription factor Sp1, which binds to eNOS promoter, whereas protein phosphatase 2A activates eNOS transcription by dephosphorylating Sp1 (Cieslik et al. 1999). In contrast, in Arabidopsis, salicylic acid-induced NOS-like-dependent NO accumulation is decreased by CK2 inhibitor (Zottini et al. 2007). In this study, our results showed that H₂O₂- and high-temperature-induced NO accumulation was compromised in Δcka2 in yeast because CK2 was involved in the regulation of NOS-like activity, but how CK2 modulates NO production by affecting NOS directly or indirectly remains elusive.

Although our data indicated that CK2 was involved in H₂O₂-induced apoptosis and high-temperature stress tolerance by changes in the NO accumulation of yeast, we also noted that NO reduced the survival rate of yeast cells during oxidative stress but increased the survival rate of yeast cells subjected to high-temperature stress (Figs 1A and 3A), consistent with previous reports (Almeida et al. 2007; Nishimura, Kawahara and Takagi 2013). Almeida et al. (2007) indicated that H₂O₂ induced yeast apoptosis by accumulating NO, whereas NO increased the survival rate of high-temperature-treated yeast cells, as shown by Nishimura, Kawahara and Takagi (2013). Similar phenomena have also been observed in plants. NO was involved in hypersensitivity-induced cell death in plants to enhance plant immune responses (Yun et al. 2011). However, NO protected root cells from salinity-mediated damage by upregulating plasma membrane H+-ATPase activity to increase the K-to-Na ratio (Zhao et al. 2004). These interesting phenomena suggest that NO could play different roles in cells responding to different stresses. However, the mechanisms underlying these phenomena remain elusive. In addition, NO is also involved in the physiological scenario of yeast apoptosis over its chronological life.
Figure 3. Survival rate and NO accumulation in WT yeast and Δcka2 after exposure to high-temperature stress. (A) The survival rate of WT yeast and Δcka2 after exposure to 39 °C for 4 h with or without 1 mM SNP or 1 mM L-NAME. (B) NO production is shown as fluorescence from DAF-FM DA staining in WT yeast and Δcka2 after exposure to 39 °C for 4 h (right panels). Cell morphology was observed by different interference contrast (left panels). (C) The relative intensities of NO fluorescence were obtained by dividing whole-field fluorescence measured with total cell numbers in 10 whole fields obtained by the microscope with three replicates. The relative NO intensity of NO fluorescence of the untreated WT was expressed as the standard. All of the experiments were repeated at least three times. Data shown are the means ± SEs. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t test.

Figure 4. NOS-like activity of WT yeast and Δcka2 upon high-temperature stress. NOS-like activity in WT yeast and Δcka2 exposed to 39 °C for 4 h. The relative NOS-like activity was expressed using the NOS-like activity of the untreated WT yeast as the standard. All of the experiments were repeated at least three times. Data shown are the means ± SEs. **P < 0.001 by Student’s t test.

It has been documented that NO is needed for the accumulation of superoxide anion during CLS (Almeida et al. 2007), and superoxide anion plays a major role in age-associated yeast cell death (Fabrizio et al. 2004). Further, NO-induced production of superoxide anion and yeast apoptosis can be inhibited by the NO scavenger OxyHb (Almeida et al. 2007), indicating that NO-induced superoxide anion accumulation functions in yeast CLS. Although CKA2 was shown in our study to be a key player in H₂O₂-induced yeast apoptosis by regulating NO accumulation, whether NO also affects superoxide anion accumulation in H₂O₂-stressed yeast cells has not been span (CLS) (Almeida et al. 2007). Almeida et al. (2007) reported that chronologically aged cells accumulated more NO than early stationary-phase yeast cells, and reduced NO content due to oxyhaemoglobin (OxyHb) resulted in faster cell growth and a delay in cell death during yeast CLS. Our data indicated the involvement of CKA2 in stress-induced NO accumulation for changes in yeast viability. Thus, whether CKA2 is also involved in the physiological scenario of yeast apoptosis should be worthy of testing experimentally.
reported. Thus, we assayed by DHE staining possible changes in superoxide anion accumulation in both WT yeast and Δcka2 mutants when they were subjected to H$_2$O$_2$ stress. Our data showed that superoxide anion was dramatically induced in WT yeast by H$_2$O$_2$ treatment, but this H$_2$O$_2$-induced accumulation of superoxide was significantly repressed in both Δcka2 and L-NAME-treated WT yeast (Fig. S1, Supporting Information), revealing that Cka2-mediated NOS-like activity was needed for H$_2$O$_2$-induced accumulation of superoxide anion. Combined with our above findings that Cka2 functioned in H$_2$O$_2$-induced yeast apoptosis, our results suggested that the production of superoxide anion is involved in CKA2-mediated H$_2$O$_2$-induced apoptosis.

In summary, CKA2 was involved in both H$_2$O$_2$-induced yeast apoptosis and yeast high-temperature stress tolerance by regulating NO accumulation via changes in NOS-like activity.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSYR online.

**Conflict of interest.** None declared.

**REFERENCES**


