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Hypochlorous Acid Induces Caspase Dependent Apoptosis in *Saccharomyces cerevisiae*

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Abstract

Hypochlorous acid (HOCl) is a strong oxidant produced by activated neutrophils via the myeloperoxidase (MPO) enzyme in order to fight against infections. Because of their antimicrobial and antiviral properties, stabilized HOCl solutions were produced to be used as a disinfectant and became a recommended disinfectant against COVID-19 by the US Environmental Protection Agency. Aberrant MPO enzyme activity results in abundant HOCl production which is related to the development and/or progression of several diseases including atherosclerosis, cardiovascular and neurodegenerative diseases. Previous studies investigating the effect of HOCl on the mode of cell death in different cell types reported that HOCl induces both apoptosis and necrosis depending on its concentration. However, the data on the apoptotic pathway triggered by HOCl is controversial. In this study, we investigated the mode of cell death induced by different concentrations of HOCl in *Saccharomyces cerevisiae*. Our data revealed that HOCl leads to cell death within 1 minute at 170 μM and above. At 340 μM, HOCl causes a rapid necrosis, while 170 μM HOCl leads to apoptosis. HOCl-induced apoptosis is mostly caspase dependent and Aif1 doesn't have a significant role.

Keywords

Hypochlorous Acid, *Saccharomyces cerevisiae*, Viability, Apoptosis

1. Introduction

Reactive oxygen species (ROS) are oxygen containing small, reactive chemical molecules. They include both radicals and non-radical ROS. Radicals, including superoxide ($O^{2}\bullet-$) and hydroxyl (OH•), contain at least one unpaired electron, and therefore are highly reactive. In order to gain stability, they tend to donate the unpaired electron or pair it by rupturing from another molecule [1]. Hy-

pochlorous acid (HOCl) and hydrogen peroxide (H_2O_2) are non-radical ROS which differ from radicals by having no unpaired electrons. Non-radical ROS exhibit their toxicity by easily initiating free radical reactions [2]. ROS are double-edged swords because of their benefits and risks in living organisms. ROS produced by neutrophils and macrophages play important roles in fighting against infections [3]. Besides, ROS acts as cellular signaling molecules involved in several biological processes at physiologic concentrations [4]. However, ROS levels above physiologic concentrations cause damage to important cellular macromolecules, such as lipids, proteins and DNA, leading to cell death as well as several diseases, including atherosclerosis and Alzheimer's disease [5] [6].

HOCl is generated by neutrophils during inflammation. At the sites of inflammation, respiratory bursts of activated neutrophils produce hydrogen peroxide (H_2O_2) via nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and superoxide dismutase (SOD) enzymes. In the presence of chlorine ion, myeloperoxidase (MPO) catalyzes H_2O_2 to HOCl [7] [8]. Since HOCl is a very strong oxidant, it reacts with bacterial proteins and destroys bacteria [9]. Owing to its antimicrobial activities, stabilized HOCl solutions are synthesized to be used as disinfectants. HOCl is also used in wound care and protection by providing antisepsis without damaging the healthy tissue [10]. Virucidal activity of HOCl solutions against several types of viruses has also been reported [11] [12] [13] [14]. For HOCl being a natural product of human's own defense system and an effective antiviral agent, HOCl solutions became recommended disinfectants against COVID-19 during the pandemic by the US Environmental Protection Agency [15].

Overproduction of HOCl due to aberrant MPO enzyme activity is associated with many pathologies including rheumatoid arthritis [16], glomerular injury [17] as well as neurodegenerative [18] [19] and cardiovascular diseases [20]. Similar to other ROS, HOCl induces oxidative DNA damage. However, unlike many other ROS, HOCl can also chlorinate the DNA, forming chlorinated adenine and cytosine (5-Cl adenine and 5-Cl cytosine) [21]. Overproduction of HOCl causes accumulation of ROS-induced damage to important cellular macromolecules: excess HOCl levels may damage collagen by forming 3-chlorotyrosine [22], attack the double bonds of unsaturated fatty acids and cholesterol to form chlorohydrins, which can disrupt the cell membrane structure [23]. Depending on the severity of the HOCl-induced cellular damage, cells may undergo a programmed or rapid cell death. There are previous studies examining the effect of HOCl on the mode of cell death in primary cultured neurons as well as a number of mammalian cell lines *in vitro* to better understand its toxicity leading to development and/or progression of several diseases [9] [24]-[30]. These studies suggested that HOCl treatment results in cell death by apoptosis or necrosis depending on the concentration [9] [24] [25] [26] [27] [28]. However, the current data on the apoptotic pathways induced by HOCl are conflicting: some studies reported that HOCl induces caspase dependent apoptosis [24] [25] [27], while others suggested that caspase-independent apoptotic pathway is triggered by

HOCl [28] [30]. The only previous study investigating the cytotoxic effects of HOCl in budding yeast reported that HOCl induced apoptosis is caspase independent, but they used NaOCl as HOCl source [31]. Since the current data on the mode of cell death triggered by HOCl are controversial, in this study we aimed to investigate the effects of different concentrations of HOCl on the viability and the mode of cell death in *Saccharomyces cerevisiae* by using a genetic approach.

2. Materials and Methods

2.1. Yeast Strains and Cultivation

Wild type (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), *yca1Δ* (*MATa leu2 ura3 his3 met3 yca1::KANMX4*) and *aif1Δ* (*MATb leu2 ura3 his3 met3 aif1::KANMX4*) *S. cerevisiae* stains were used in this study. Yeast cells were cultured in yeast extract, peptone, dextrose (YPD) medium (yeast extract (1%), peptone (2%), and glucose (2%) in distillated water) by shaking at 180 rpm at 30˚C.

2.2. Exposure of *S. cerevisiae* Cells to HOCl

Wild type and mutant yeast cells were grown in YPD media to mid-log (OD600 = ~1). Cultures were washed once with phosphate buffered saline (PBS) and exposed to HOCl in PBS [32]. PBS was used as control. Serial dilutions of HOCl (2720 µM, 1360 µM, 680 µM, 340 µM, 170 µM, 85 µM and 42.5 µM) were prepared from 5440 µM HOCl stock solution in PBS. Then, cells were exposed to different concentrations of HOCl for 0, 1, 5, 10, 15 or 30 minutes (T_0, T_1, T_5, T_{10}, T_{15} and T_{30}) in PBS. 0 min (prior to exposure) samples were used as control (T_0).

2.3. Spotting and Colony Forming Unit (CFU) Assays

Spotting and CFU assays were performed as described previously [33]. Briefly for the spotting assay, 5 µL of 10-fold serial dilutions of each sample were spotted onto YPD. The plates were photographed following the incubation at 30˚C for 2 days or at 23˚C for 4 days. For the CFU assay, 100 µL of appropriate dilutions of samples were spread onto YPD plates and incubated for 2 days at 30˚C or for 4 days at 23˚C. Number of the colonies on each plate was divided by the spread volume (0.1 mL) and dilution factor in order to determine the number of CFU/mL in the original sample. Data were normalized to T_0 (100%). Experiments were performed at least three times for each treatment and the average cell viability was graphed with standard deviation (SD).

2.4. Hoechst 33342 & PI Double Staining

Cells were stained with Hoechst and PI (Propidium Iodide) (Chromatin Condensation/Dead Cell Apoptosis Kit with Hoechst 33342 and PI, Invitrogen), according to the manufacturer's instructions. Briefly, 100 µL of samples were collected from each culture, washed with cold PBS once and resuspended in 1 mL of PBS. Then, 1 µL of the Hoechst 33342 stock solution (Component A) and 1 µL

of the PI stock solution (Component B) were added to each 1 mL of cell suspension and incubated on ice for 20 minutes. Stained cells were analyzed under a fluorescent microscope (Leica DM1000 LED, Leica Microsystems, Germany) and categorized as necrotic (PI positive), apoptotic (Hoechst positive, PI negative) or live (very low level/no fluorescence). At least 200 cells were analyzed for each sample and the average of % necrotic or apoptotic cells of three independent experiments was reported with standard deviations (SD).

2.5. Statistical Analysis

All experiments were repeated at least three times. Data are presented as mean ± standard deviation (SD). Differences between groups were analyzed by two tailed student's T-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Determining the Minimal Lethal Concentration of HOCl in *S. cerevisiae*

In order to investigate the cytotoxicity of HOCl, we first determined the minimal lethal concentration of HOCl. For this purpose, we examined the viability of wild type yeast cells exposed to different concentrations of HOCl by the spotting assay: Cells were exposed to HOCl at 2720 µM, 1360 µM, 680 µM, 340 µM, 170 µM, 85 µM and 42,5 µM for 1, 5, 10, 15 and 30 minutes (**Figure 1(A)**). 2720 µM, 1360 µM, 680 µM, 340 µM and 170 µM HOCl led to cell death within 1 minute (**Figure 1(B)**). Whereas, 85 µM and 42.5 µM HOCl did not dramatically affect the viability of wild type cells compared to the untreated control (T_0).

The viability of wild type cells exposed to 340 µM, 170 µM, or 85 µM HOCl for 5 minutes were quantified by the CFU assay and compared to the untreated

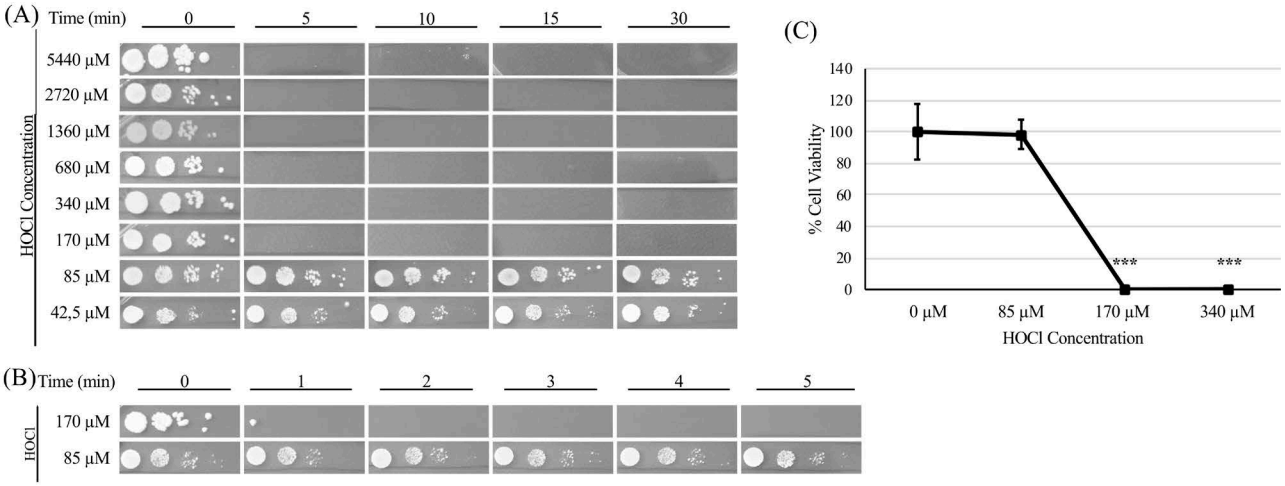

Figure 1. Effect of different HOCl concentrations on the viability of wild type (WT) cells. A. Viability of cultures exposed to 5440, 2720, 1360, 680, 340, 170, 85 and 42.5 µM HOCl for 5, 10, 15 and 30 minutes by the spotting assay. B. Viability of WT cultures exposed to 170 and 85 µM HOCl for 5 minutes by the spotting assay. C. Viability of WT cells exposed to 0, 85, 170 and 340 µM HOCl for 5 minutes by the CFU assay (***$p < 0.001$, Student's t-test).

control (Figure 1(C)). Although, the percentage of viability in cultures exposed to 340 μM (0.0028%) and 170 μM (0.0014%) HOCl for 5 minutes were significantly reduced relative to T_0 (0 min) (100%) ($p < 0.001$), exposure to 85 μM (98.38%) HOCl did not significantly affect the viability compared to the control (100%) ($p > 0.05$). Therefore, we determined the minimal lethal concentration of HOCl to be 170 μM.

3.2. HOCl Activates Different Cell Death Pathways at Different Concentrations

Wild type cells were exposed to 340 μM and 170 μM HOCl for 5 minutes and examined by Hoechst and propidium iodide (PI) staining to determine the mode of cell death induced by HOCl. Hoechst & PI staining is a valid method to evaluate chromatin condensation and cell membrane integrity to differentiate apoptotic cells from necrotic cells [34] [35]. After 5 minute-HOCl exposure, cells were stained with Hoechst & PI and categorized as apoptotic, necrotic or viable (Figure 2). We observed that exposure to 340 μM HOCl significantly increased the percentage of necrotic cells (99.83%) compared to the untreated control (0.16%) ($p < 0.001$). Whereas, percentage of apoptotic cells was very low (0.16%) and not significantly different relative to the control (0.33%) ($p > 0.05$). Notably, in the 340 μM HOCl exposed culture, the ratio of necrotic cells was significantly higher than the ratio of apoptotic cells ($p < 0.001$). Exposure to 170 μM HOCl resulted in a significant increase in the ratio of apoptotic cells (99.02%) compared to the untreated control ($p < 0.001$), while the percentage of necrotic cells was not significantly different (0.97%) relative to the untreated control (0.16 %) ($p > 0.05$). Besides, the percent apoptotic cells in 170 μM HOCl exposed culture was significantly higher compared to that of necrotic cells ($p < 0.001$). These data together indicate that stabilized HOCl activates necrosis or apoptosis at 340 μM and 170 μM, respectively in *S. cerevisiae*.

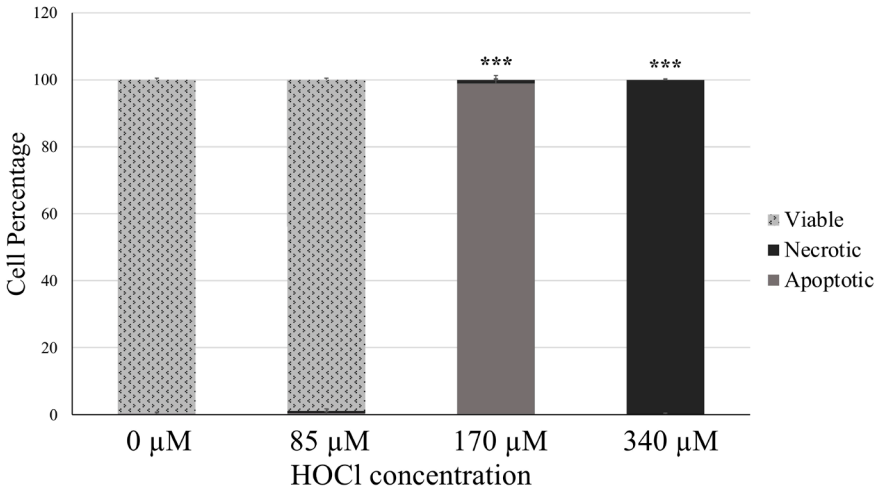

Figure 2. Ratios of apoptotic, necrotic and viable WT cells following 5 minute-exposure to 0, 85, 170 and 340 μM HOCl by Hoechst/PI staining (***$p < 0.001$, Student' t-test).

3.3. The Apoptotic Mechanism Induced by HOCl

Metacaspases are structural homologs of caspases in non-metazoan organisms. Yeast metacaspase 1 (Yca1) is the only metacaspase identified in *S. cerevisiae* [36]. Yeast Aif1, is a caspase-independent effector of apoptosis in *S. cerevisiae* [37] [38]. Similar to its mammalian counterpart, Aif1 translocates from mitochondria to the nucleus in response to various apoptosis stimulators [39]. After we observed that 170 μM HOCl resulted in cell death by apoptosis, we investigated whether the apoptotic pathway induced is either Yca1 or Aif1 dependent. For this purpose, wild type (WT), *yca1Δ* and *aif1Δ* deletion mutant strains were exposed to apoptotic concentration of HOCl (170 μM) for 5 minutes and cell viabilities were assessed by the CFU assay (**Figure 3(A)**). Our data revealed that 100.28% of *yca1Δ* cells were viable, which was significantly higher compared to WT cells (0.001%) ($p < 0.001$). Whereas, no significant difference was observed in percent viable cells between WT (0.0014%) and *aif1Δ* (0.0016%) cultures exposed to 170 μM HOCl for 5 minutes ($p > 0.05$).

Next, we evaluated the percentages of apoptotic and necrotic cells in WT, *yca1Δ* and *aif1Δ* cultures exposed to 170 μM HOCl for 5 min by Hoechst/PI staining (**Figure 3(B)**). In compliance with the viability data, the ratio of apoptotic cells in the *yca1Δ* culture (5.43%) was significantly lower compared to that of WT (99.02%) ($p < 0.001$). Whereas, the proportion of apoptotic cells in the *aif1Δ* culture exposed to 170 μM HOCl (96.46%) was not significantly different relative to WT (99.02%) ($p > 0.05$). These data together suggest that apoptosis induced by 170 μM HOCl is caspase dependent and Aif1 may have no role in HOCl-induced apoptosis in yeast.

4. Discussion

In the present study, we evaluated the toxicity of HOCl in a unicellular model

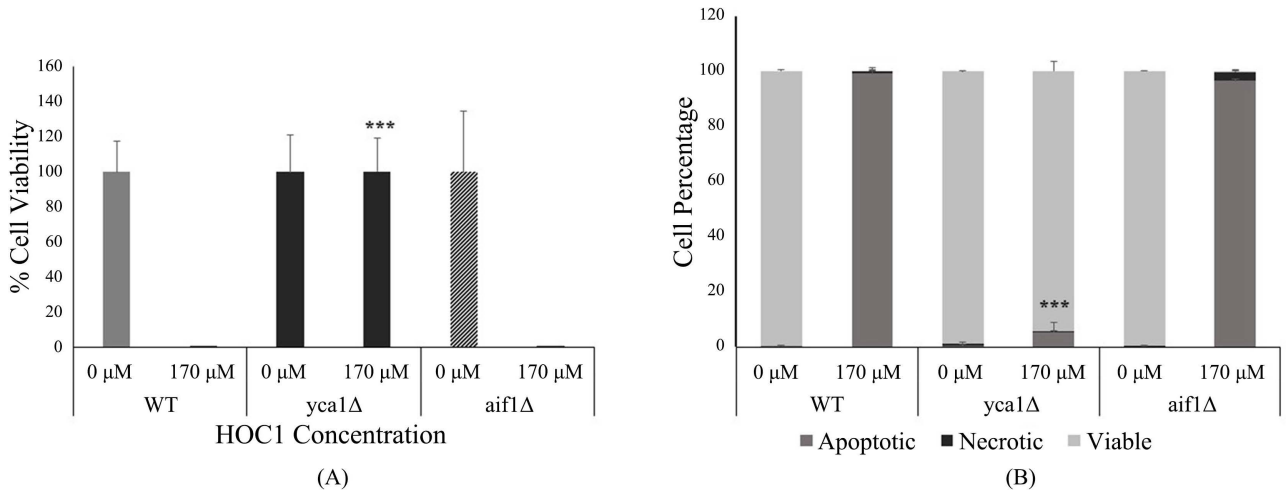

Figure 3. Determining the apoptotic pathway(s) induced by HOCl in yeast. A. Relative cell viability ratios of WT, *yca1Δ* and *aif1Δ* cultures exposed to 170 μM HOCl for 5 minutes by the CFU assay. (B) Ratios of apoptotic, necrotic and viable cells in WT, *yca1Δ* and *aif1Δ* cultures exposed to apoptotic concentration of HOCl for 0 and 5 minutes by Hoechst/PI staining (***$p < 0.001$; Student's t-test).

organism, *S. cerevisiae*. Since HOCl is used as an antimicrobial agent, determining the accurate killing time for HOCl is important. Our data revealed that minimal lethal concentration of HOCl is 170 μM in budding yeast. Above this concentration, HOCl treatment resulted in cell death within 1 minute. Sakarya *et al.* previously reported the accurate killing time for 5.5 ppm (~170 μM) HOCl in green fluorescent protein (GFP)-transfected *Pseudomonas aeruginosa* cells to be 12 seconds by fluorescent microscopy. They also showed that the minimal bactericidal concentration is 2.75 ppm (~85 μM) for all standard microorganisms and ranges from 5.5 ppm (~170 μM) to 2.75 ppm (~85 μM) for clinical isolates they tested [32]. Different minimal killing concentrations and accurate killing time determined in our study and Sakarya *et al.* may be due to the different species used, and/or the presence or lack of a cell wall. Different techniques used in these two studies might also have led to different results for accurate killing time.

Endogenous HOCl is produced by the immune cells of the organism in response to invading microorganisms. Excessive HOCl production in response to the pathogens as well as the significant increase in the number of HOCl producing immune cells bring the risk of damaging nearby tissues, leading to development and/or progression of several diseases. Therefore, it is important to understand the mechanism of cell death caused by HOCl. In this study we report that a high concentration of HOCl (340 μM) induces rapid necrotic cell death whereas, at a lower concentration (170 μM), cells undergo caspase-dependent apoptosis within 5 minutes. The mode of cell death induced by the endogenous HOCl production has been previously investigated in different tissues. HOCl was suggested to play an important role in the progression of atherosclerotic lesions as both HOCl-damaged proteins and MPO were increased in damaged tissue [40] [41]. Since endothelium is the first contact point of activated neutrophils and macrophages are abundant in erosive sites of coronary plaques, a number of in vitro studies have been conducted to evaluate the toxicity of HOCl in human umbilical vein endothelial (HUVEC), human saphenous vein endothelial (HSVEC) and human coronary artery endothelial (HCAEC) cells [26] [27] [28]. In these studies, HOCl was shown to trigger different apoptotic pathways. Similar to our results, studies conducted in HUVEC and HSVEC cells revealed that HOCl induces caspase-dependent apoptosis. On the other hand, HOCl has been reported to induce caspase independent apoptosis in HCAEC cells [28].

Carmona-Gutierrez *et al.* investigated the microbicidal effects and protein modifications caused by HOCl in *S. cerevisiae* and showed that HOCl induced apoptosis at 300 μM. However, they reported that HOCl induced apoptosis in a caspase-independent and Kex1-dependent manner [31]. Different findings between this study and ours may be explained by the differences in the source of the HOCl used: In our study, we used commercially available stabilized HOCl solution, which was produced by electrolyzation of NaCl containing water. On the other hand, Carmona-Gutierrez *et al.* prepared HOCl from sodium hypochlorite (NaOCl). Components of the culture media, even batch-specific differences, have been shown to affect HOCl toxicity [31]. In this study, we treated cells

with HOCl in PBS, whereas Carmona-Gutierrez *et al.* performed their HOCl treatments in culture media. Exposure times are also different between two studies: We performed HOCl treatments for 5 minutes, while Carmona-Gutierrez *et al.* performed 16-hour treatments. All these methodological differences might have led to a higher concentration at which apoptosis was observed and a different apoptotic pathway found to be triggered in Carmona-Gutierrez *et al.*'s study.

Monocyte-derived microglia in brain can also generate HOCl [42] [43]. Consistently, HOCl production has been reported in Parkinson's and Alzheimer's diseases [44]. A limited number of studies conducted in primary murine neocortical neurons and PC12 cells concluded that HOCl treated cells undergo caspase-dependent apoptosis in vitro [24] [25]. Similarly, we show that Aif1 does not have a significant role in HOCl induced apoptosis in yeast. HOCl-induced apoptosis was reported to be AIF/EndoG dependent in only one previous study conducted in human mesenchymal progenitor cells differentiated into a chondrocytic phenotype [30]. These data together indicate that, the pathway of HOCl-induced apoptosis may vary among different cell types.

5. Conclusion

In this study, we showed that HOCl results in cell death within 1 minute in budding yeast at concentrations of 170 μM and above, while it does not significantly affect the viability at concentrations of 85 μM and below. We also demonstrated that 340 μM HOCl results in necrosis, whereas 170 μM HOCl triggers apoptosis mainly in a caspase dependent manner. In summary, our data reveal that exposure to HOCl triggers either caspase dependent apoptosis or necrosis depending on its concentrations.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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