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Cold atmospheric pressure plasma causes protein denaturation and endoplasmic reticulum stress in *Saccharomyces cerevisiae*

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Abstract

Cold atmospheric pressure plasma (CAP) does not cause thermal damage or generate toxic residues; hence, it is projected as an alternative agent for sterilization in food and pharmaceutical industries. The fungicidal effects of CAP have not yet been investigated as extensively as its bactericidal effects. We herein examined the effects of CAP on yeast proteins using a new CAP system with an improved processing capacity. We demonstrated that protein ubiquitination and the formation of protein aggregates were induced in the cytoplasm of yeast cells by the CAP treatment. GFP-tagged Tsa1 and Ssa1, an H_2O_2-responsive molecular chaperone and constitutively expressed Hsp70, respectively, formed cytoplasmic foci in CAP-treated cells. Furthermore, Tsa1 was essential for the formation of Ssa1-GFP foci. These results indicate that the denaturation of yeast proteins was caused by CAP, at least partially, in a H_2O_2-dependent manner. Furthermore, misfolded protein levels in the endoplasmic reticulum (ER) and the oligomerization of Ire1, a key sensor of ER stress, were enhanced by the treatment with CAP, indicating that CAP causes ER stress in yeast cells as a specific phenomenon to eukaryotic cells. The pretreatment of yeast cells at 37 °C significantly alleviated cell death caused by CAP. Our results strongly suggest that the induction of protein denaturation is a primary mechanism of the fungicidal effects of CAP.

Keywords Cold atmospheric pressure plasma · Fungicidal efficacy · Sterilization · Endoplasmic reticulum stress · Protein denaturation · *Saccharomyces cerevisiae*

Introduction

Cold atmospheric pressure plasma (CAP) mainly consists of ions and electrons and emits reactive species and UV photons (Gaunt et al. 2006). CAP has been used in various medical applications including wound healing and non-inflammatory anti-cancer therapy (Kong et al. 2009; Weltmann and von Woedtke 2016; Tanaka and Hori 2017). Especially, argon (Ar)-based CAP is widely used for coagulation therapy (Manner 2008) and known to enhance wettability and osseointegration on dental implant metals (Duske et al. 2012; Giro et al. 2013; Canullo et al. 2016). Additionally, exposure to CAP is projected as a new sterilization method in the fields of food processing, sanitation, and medicine (Heinlin et al. 2010; Hoffmann et al. 2013; Shaw et al. 2015; Mir et al. 2016). The CAP system as a sterilization technique is advantageous because of the low-associated costs and minimal generation of residual toxicity and thermal damage (Hoffmann et al. 2013; Lackmann and Bandow 2014). Previous studies reported the sterilization effects of CAP on bacterial cells such as *Escherichia coli*, *Salmonella enterica*, and *Staphylococcus aureus* (Klämfl et al. 2012; Maisch et al. 2012a, b; Fernández et al. 2013; Homma et al. 2013; Niemira et al. 2014; Sun et al. 2014; Ziuzina et al. 2014; Maeda et al. 2015). Bacterial cells are mainly inactivated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) induced by CAP exposure (Gaunt et al. 2006; van Gils et al. 2013; Lackmann and Bandow 2014).

Inactivation of spoilage yeasts including *Saccharomyces cerevisiae* is an important issue to be solved in food industries (Loureiro and Malfeito-Ferreira 2003; Krisch et al. 2016), and

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CAP exposure is expected as an effective way to prevent the spoilage. However, the fungicidal effects of CAP have not yet been investigated as extensively as its bactericidal effects. Previous studies reported that yeast cells were also inactivated by a CAP treatment via the accumulation of ROS and RNS (Feng et al. 2010; Koban et al. 2010; Maisch et al. 2012a, b; Ryu et al. 2013). However, the fungicidal mechanisms of CAP and the cellular responses of eukaryotic microorganisms to CAP stress have not yet been elucidated in detail.

We recently demonstrated via a fluorescence microscopic analysis that CAP promoted the formation of Hsp104 aggregates (Itooka et al. 2016). Hsp104 is a stress-responsive chaperone that plays a role in the segregation of denatured proteins (Glover and Lindquist 1998; Bösl et al. 2006). Since Hsp104 forms complexes with denatured proteins, it is possible to visualize protein aggregates using Hsp104-GFP (Liu et al. 2010; Zhou et al. 2011; Escusa-Toret et al. 2013; Wallace et al. 2015). Therefore, our finding of the formation of Hsp104-GFP aggregates following a CAP treatment strongly suggests that CAP promotes the denaturation of yeast proteins. Additionally, we found that CAP induced changes in the intracellular localization of Ire1, a key sensor of ER stress and trigger of the unfolded protein response (UPR) (Kimata et al. 2003, 2004, 2007; Mori 2009). Yeast UPR is induced via the activation of the Ire1-Hac1 pathway (Mori et al. 1996; Mori 2009; Brodsky and Skach 2011). The accumulation of misfolded proteins in the endoplasmic reticulum (ER) leads to the activation of Ire1 via the self-association and oligomerization of Ire1 in yeast cells (Kimata et al. 2007). Activated Ire1 subsequently splices *HAC1* mRNA, leading to the synthesis of the Hac1 protein, which functions as a transcription factor and induces the transcriptional activation of UPR-related genes (Chapman and Walter 1997; Kawahara et al. 1997). Although the non-activated form of Ire1-GFP diffuses throughout the ER, highly self-oligomerized Ire1-GFP exhibits a punctate intracellular localization (Kimata et al. 2007; Promlek et al. 2011; Mathuranyanon et al. 2015; Kawazoe et al. 2017). Since Ire1-GFP in CAP-treated cells shows a punctate localization (Itooka et al. 2016), CAP has been proposed to cause ER stress in yeast cells.

Although the findings of our previous fluorescence microscopy study suggested that CAP causes protein denaturation and ER stress in yeast (Itooka et al. 2016), it was almost impossible to perform more detailed analyses because of the limited processing capacity of the CAP system used in that study. In order to conduct biochemical and molecular biological analyses, we developed a new CAP system using a glass funnel with improved processing capacity. We herein demonstrated that CAP elevated the levels of insoluble aggregated proteins and ubiquitinated proteins and also impaired protein folding in the ER. Additionally, we found that the pretreatment of yeast cells at 37 °C alleviated damage and cell death caused by CAP exposure. Our results strongly indicate that the fungicidal effects of CAP are partially derived from protein damage.

Materials and methods

Strains and medium

S. cerevisiae YPH250 (*MATα trp1-1 his3-200 lys2-801 leu2-1 ade2-101 ura3-52*) and its isogenic *tsa1*Δ null mutant were used in the present study. The *tsa1*Δ null mutant was constructed by introducing the DNA fragment encoding *tsa1*Δ*::kanMX*, which was amplified from the genomic DNA of the *tsa1*Δ*::kanMX* strain in the Yeast Knockout Collection (Open Biosystems Inc., Huntsville, AL). In order to monitor the localization of Ire1-GFP, KMY1015 (*MATα leu2-3112 ura3-52 his3-*Δ*200 trp1-*Δ*901 lys2-801 ire1*Δ*::TRP1*) expressing Ire1-GFP was used (Mathuranyanon et al. 2015). The methods of cell cultivation and medium were described in our previous study (Itooka et al. 2016). Exponentially growing cells were harvested at an optical density at 600 nm (OD_{600}) of 0.5 and treated with CAP.

The CAP system and CAP treatment

CAP was produced in dielectric barrier discharges on a glass funnel. Copper tape (5.0 mm in width) was attached to the inner and outer surfaces of the funnel (Fig. 1). A high voltage of 20.0 kV was applied at 11 kHz between the outer and inner tapes. The inner tape was grounded for an electrical circuit. Ar gas was introduced into the funnel at a flow rate of 0.8 standard liters per minute (SLM), and the distance between the bottom of the glass funnel and ground was adjusted to 3.0 mm. Copper tape was cooled by a blower (YMS-A107, Yamazen, Osaka, Japan) to prevent overheating. In the CAP treatment of yeast cells, 25 ml of the liquid culture (OD_{600} = 0.5) was centrifuged, and precipitated cells were resuspended in 2 ml of fresh SD medium. The cell solution was added to a petri dish (diameter, 53 mm; Corning Falcon® 351007) and exposed to CAP. The CAP treatment was carried out in a room at 25 °C, and the sample temperature was measured using a radiation thermometer (AD-5611A, A&D Company, Limited, Tokyo, Japan).

Plasmid construction

YIp-*SSA1*-GFP A part of the open reading frame (ORF) of *SSA1* was amplified by a polymerase chain reaction (PCR) using the primers 5′-GGACCCAGTTGAAAAGGTCT AGAGAGATGCTAAATTGGACAAATCTC-3′ and 5′-CGTTATTATTCAATTGCCGCACCAATCTCGAG AATCACTTCTTCAAC-3′. The amplicon was cloned into

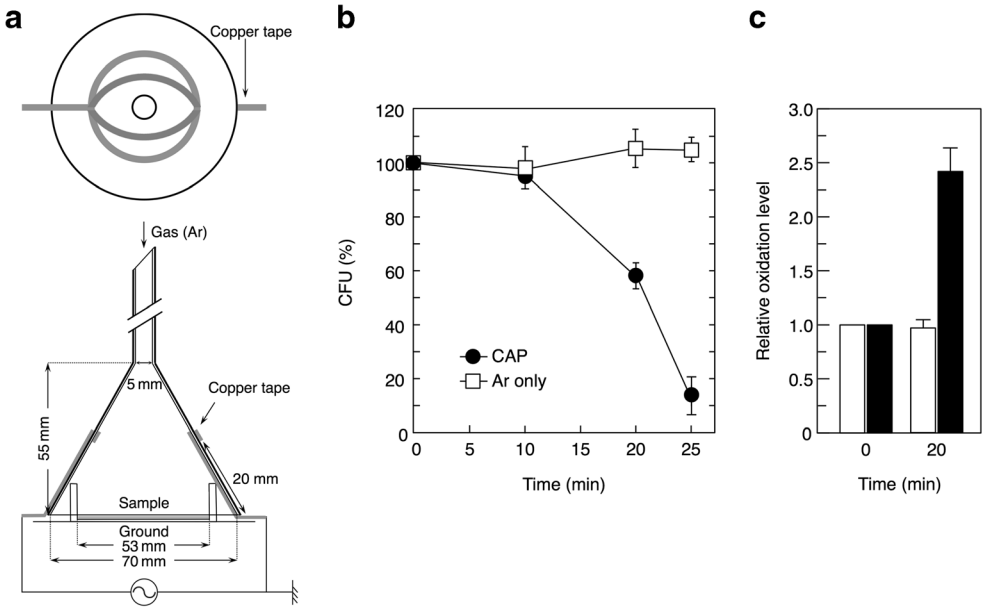

Fig. 1 Schematic of the experimental setup for CAP exposure. **a** CAP was produced in dielectric barrier discharges on a glass funnel. Copper tape (5.0 mm in width) was attached to the inner and outer surfaces of the funnel. A high voltage of 20.0 kV at 11 kHz between the inside and outside copper tape caused a barrier discharge using a dielectric of the glass. Ar gas was fed to the funnel at a flow rate of 0.8 SLM. Samples in a petri dish were exposed to CAP produced inside the funnel. **b**, **c** Yeast cells in SD medium were treated with CAP (closed circles and black bars) or Ar gas alone (open squares and white bars). **b** The relative survival rate was calculated as colony-forming units (CFU). **c** Intracellular oxidation levels were assayed using an oxidant-sensitive probe H_2DCFDA. Data are shown as the mean ± SE ($n = 3$)

the *Xba*I/*Xho*I sites of YIp-*SSA4*-GFP (Itooka et al. 2016) after digestion with *Xba*I/*Xho*I to construct YIp-*SSA1*-GFP. Regarding the integration of the *SSA1-GFP* gene at the chromosomal *SSA1* locus, YIp-*SSA1*-GFP was linearized by *Sal*I and introduced into cells.

YIp-*TSA1*-GFP A part of the ORF of *TSA1* was amplified by PCR using the primers 5′-CAACCGAGCTCATTGCTTTC TCAGAAGCTGCTAAG-3′ and 5′-GCAAGCTCGAGATT TGTTGGCAGCTTCGAAGTATT-3′. The amplicon was digested with *Sac*I/*Xho*I and cloned into the *Sac*I/*Xho*I sites of YIp-*SSA4*-GFP to construct YIp-*TSA1*-GFP. YIp-*TSA1*-GFP was linearized by *Bst*XI and introduced into yeast cells.

The construction of the YIp-*HSP104*-GFP plasmid was previously described (Itooka et al. 2016).

Western blotting analysis

A BiP aggregation analysis was performed as described by Promlek et al. (2011). Briefly, total cell lysates treated with CAP were fractionated by centrifugation at 19,300×*g* for 10 min. Pellet samples were applied to a 8.0% polyacrylamide gel for an SDS-PAGE analysis. BiP protein levels were monitored using an anti-BiP antibody and quantified using ImageJ software (http://imagej.nih.gov/ij/).

Protein ubiquitination was analyzed as described by Collins et al. (2010). Extracted proteins were resolved on an 8.0% polyacrylamide gel for the SDS-PAGE analysis. Ubiquitinated proteins were detected using an anti-ubiquitin antibody (P4D1, Santa Cruz Biotechnology, Dallas, TX). Ponceau S staining was used for the verification and normalization of protein loading abundance.

Measurement of insoluble aggregated proteins

Insoluble aggregated proteins were analyzed as described by Koplin et al. (2010) with modifications. In order to prepare cell lysates, cells were frozen rapidly in liquid nitrogen and resuspended in lysis buffer (50 mM potassium phosphate buffer, pH 7.0, 1.0 mM EDTA, and 5% glycerol). After an incubation with Zymolyase 20 T (2.5 mg/ml) and protease inhibitors at 25 °C for 20 min, cells were disrupted by vortexing with glass beads. In order to remove intact cells, samples were centrifuged at 200×*g* for 20 min. The total protein concentration of each sample was measured using the Protein Assay CBB Solution kit (Nacalai Tesque, Kyoto, Japan) and normalized. Insoluble aggregated proteins were obtained by centrifugation at 16,000×*g* for 20 min. These proteins were washed twice with lysis buffer containing 2% Nonidet® P-40 (NP-40), and centrifuged at 16,000×*g* at 4 °C for 20 min to remove membrane proteins. The final sediment proteins were solubilized in 50 μl of urea buffer (50 mM Tris-HCl, pH 7.5, 6.0 M urea, and 5% SDS), separated by a 10% polyacrylamide gel, and visualized by silver staining using Sil-Best Stain One (Nacalai Tesque, Kyoto, Japan). The quantities of insoluble aggregated proteins were measured by the

BCA assay using the Protein Assay Bicinchoninate kit (Nacalai Tesque, Kyoto, Japan).

Microscopic analysis and survival assay

A fluorescence microscope system (IX83, Olympus, Tokyo, Japan) was used for the microscopic analysis. Cells treated with CAP were immediately observed without fixation. The relative survival rate was calculated as colony-forming units (CFU), as previously described (Itooka et al. 2016). Levels of intracellular oxidation were measured using an oxidant-sensitive probe 2′,7′-dichlorofluorescein diacetate (H_2DCFDA; Molecular Probes, OR, USA) as described previously (Allen et al. 2010; Itooka et al. 2016).

Results

Fungicidal activity of the new CAP system

In our previous study, we used a typical laboratory-scale CAP system that processes only 100 μl of culture medium each time (Itooka et al. 2016). In order to increase this capacity, we developed a new CAP system using a glass funnel that functions as a non-conductor as well as a cover. This allows the efficient exposure of CAP to the target via the retention of Ar plasma inside the funnel (Fig. 1a). We initially examined the effects of the new CAP system on the survival of yeast cells (Fig. 1b). The CAP exposure efficiently killed yeast cells, while exposure of Ar gas alone did not kill yeast cells. Furthermore, the CAP treatment for 20 min elevated the level of intracellular oxidation (Fig. 1c). On the other hand, exposure of Ar gas alone did not elevate the intracellular oxidation level, as reported previously (Itooka et al. 2016). The sample temperature hardly changed before and after CAP exposure for 25 min (from 23.6 ± 0.3 to 23.5 ± 0.4 °C). These results were consistent with previous findings (Koban et al. 2010; Maisch et al. 2012a, b; Itooka et al. 2016), and clearly indicate an improvement of the processing capacity of our CAP systems; the new CAP system processed 2 ml of culture medium each time and efficiently killed yeast cells without thermal stress.

CAP increased insoluble aggregated protein levels in yeast cells

Since we succeeded to improve the processing capacity of our CAP system (from 100 μl to 2 ml), it became possible to conduct biochemical and molecular biological analyses to find out more about the effects of CAP on yeast cells. Previous studies reported that CAP causes the inactivation and denaturation of bacterial proteins (Yasuda et al. 2008; Lackmann et al. 2013; Lackmann and Bandow 2014). We also demonstrated that Hsp104-GFP, a marker of insoluble protein aggregation (Liu et al. 2010; Zhou et al. 2011; Escusa-Toret et al. 2013; Wallace et al. 2015), formed foci in CAP-treated yeast cells (Itooka et al. 2016). This finding suggested that CAP causes the accumulation of denatured proteins in yeast cells. In order to verify this possibility, we used the new system to examine whether CAP promotes the formation of insoluble protein aggregates. Consistent with our previous study, the new CAP system also induced the formation of Hsp104-GFP foci (Fig. 2a). We reconfirmed that the formation of Hsp104-GFP foci was not induced by exposure of Ar gas alone (data not shown), as reported in our previous report (Itooka et al. 2016). We assayed the levels of insoluble aggregated proteins in CAP-treated cells. As shown in Fig. 2b, c, the CAP treatment for more than 20 min significantly elevated the levels of insoluble aggregated proteins to a similar extent as those in cells exposed to heat shock at 42 °C. These results clearly indicate that CAP induces the denaturation of yeast proteins.

CAP promoted protein ubiquitination

In the ubiquitin-proteasome system, ubiquitin functions as a signal for protein degradation. Denatured proteins conjugated with ubiquitin are degraded via the proteasome (Finley et al. 2012; Amm et al. 2014). Since CAP induced the denaturation of yeast proteins (Fig. 2), we performed western blotting using an anti-ubiquitin antibody to examine whether protein ubiquitination is enhanced in cells treated with CAP. The CAP treatment, as well as heat shock at 42 °C, significantly increased the levels of ubiquitinated proteins (Fig. 3). This result supports the finding that CAP induces protein denaturation in yeast cells.

H_2O_2-dependent protein aggregation was induced in CAP-treated cells

Tsa1, a major thioredoxin peroxidase in yeast (Garrido and Grant 2002), is a molecular chaperone and protects against the accumulation of H_2O_2-induced denatured protein aggregates (Jang et al. 2004; Rand and Grant 2006; Hanzén et al. 2016). Since sulfenylated Tsa1 recruits Hsp70 to denatured proteins in a H_2O_2-dependent manner and forms aggregates, Tsa1-GFP and Ssa1-GFP form foci in the cytoplasm under H_2O_2 stress, but not under heat shock conditions (Hanzén et al. 2016; Hill et al. 2017). We verified that the formation of the Tsa1-GFP focus was induced by H_2O_2, but not by heat shock, and the formation of Tsa1-GFP focus was also induced by the treatment with CAP (Fig. 4a). Additionally, the formation of the Ssa1-GFP focus was not observed in *tsa1*Δ cells treated with CAP (Fig. 4b). These results suggest that the formation of protein aggregates in CAP-treated cells was induced, at least partially, in a H_2O_2-dependent manner.

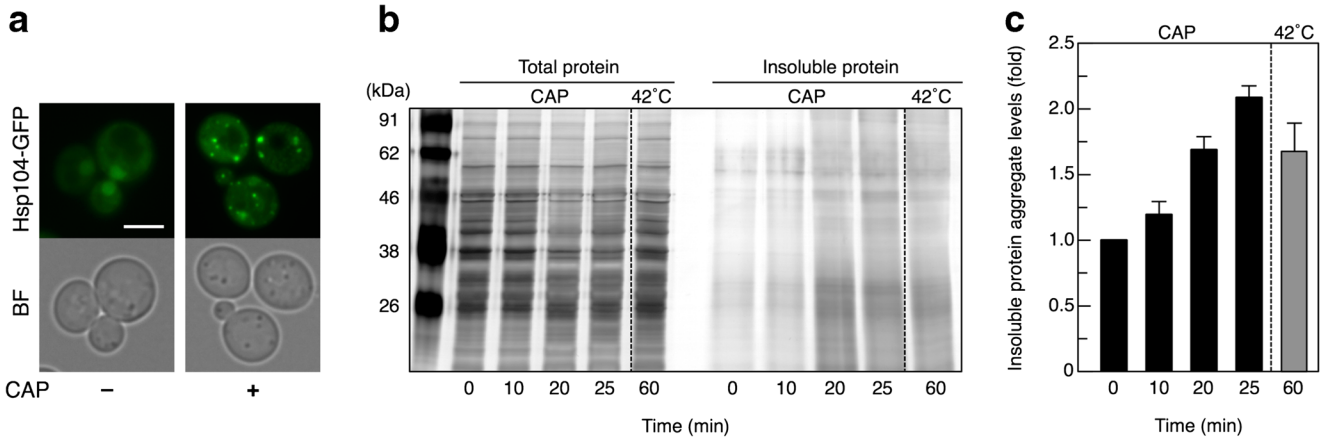

Fig. 2 CAP enhanced insoluble aggregated protein levels in yeast cells. **a** Cells carrying a GFP-tagged chromosomal copy of *HSP104* were treated with CAP for 20 min. GFP signals were immediately observed after the treatment without cell fixation. BF bright field. The white bar indicates 3 μm. **b**, **c** Cell lysates were prepared after the treatment with CAP or heat shocked at 42 °C for the indicated time. Insoluble aggregated proteins were isolated by centrifugation and washed with NP-40 containing buffer, and then solubilized in buffer containing urea and SDS. **b** Samples were separated using 10% polyacrylamide gel electrophoresis and visualized by silver staining. **c** The quantities of insoluble aggregated proteins were measured by the BCA assay. The levels of insoluble aggregated proteins in non-stressed cells were considered to be onefold. Data are represented as the mean ± SE ($n = 3$)

The CAP treatment promoted the accumulation of unfolded proteins in the ER

We previously reported that CAP but not Ar gas alone induced changes in the localization of Ire1, a key sensor of ER stress (Kimata et al. 2003, 2004, 2007; Mori 2009), which suggests that CAP causes the oligomerization of Ire1 and ER stress in yeast cells (Itooka et al. 2016). In the present study, we investigated whether CAP enhances the levels of unfolded proteins in the ER. Previous studies reported that BiP, a major ER chaperone (Rose et al. 1989; Mori et al. 1996), binds unfolded proteins to form aggregates under ER stress conditions (Kimata et al. 2003). Therefore, it is possible to detect ER stress by measuring the levels of sedimentable BiP (Promlek et al. 2011; Kawazoe et al. 2017). A treatment with dithiothreitol (DTT), an ER stress inducer that disrupts disulfide bond formation (Kimata et al. 2003, 2007), elevated the levels of sedimentable BiP (Fig. 5a). The treatment with CAP also increased the levels of sedimentable BiP over time in the pellet fraction. The levels of sedimentable BiP in cells treated with CAP for 25 min were similar to those in cells treated with DTT. These results clearly indicate that the CAP treatment promotes the formation of unfolded protein aggregates in the yeast ER.

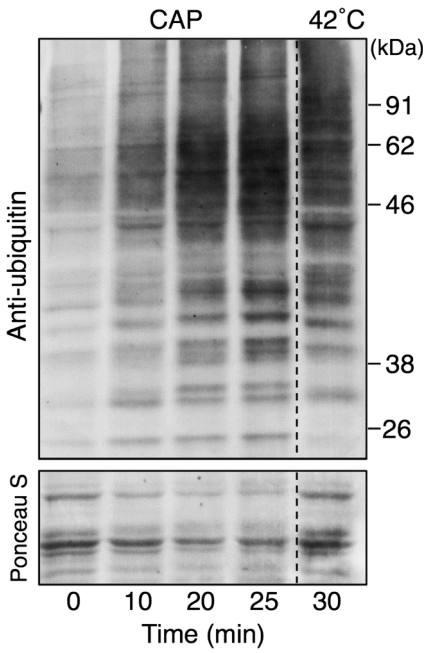

Fig. 3 CAP promoted protein ubiquitination. Cells were treated with CAP for the indicated time or heat shocked at 42 °C for 30 min and then lysed using glass beads in EZ buffer. Extracted proteins were resolved on an 8% polyacrylamide gel, and ubiquitinated proteins were detected by western blotting using an anti-ubiquitin antibody. The protein loading quantity was verified by Ponceau S staining

Since BiP levels in the pellet fraction were increased by CAP, the oligomerization of Ire1 was re-examined using the new CAP system. We observed that Ire1-GFP formed punctate foci following the CAP treatment (Fig. 5b), confirming that CAP induced the oligomerization of Ire1 and ER stress in yeast cells.

A pretreatment with mild heat shock improved yeast resistance to CAP

Since we found that, as described above, CAP as well as heat shock induced protein denaturation, we next examined whether the pretreatment of cells with mild heat shock improves cell survival under CAP stress. Cells pretreated with sublethal mild heat shock at 37 °C exhibit increased resistance to subsequent severe stress because the pretreatment causes adaptive

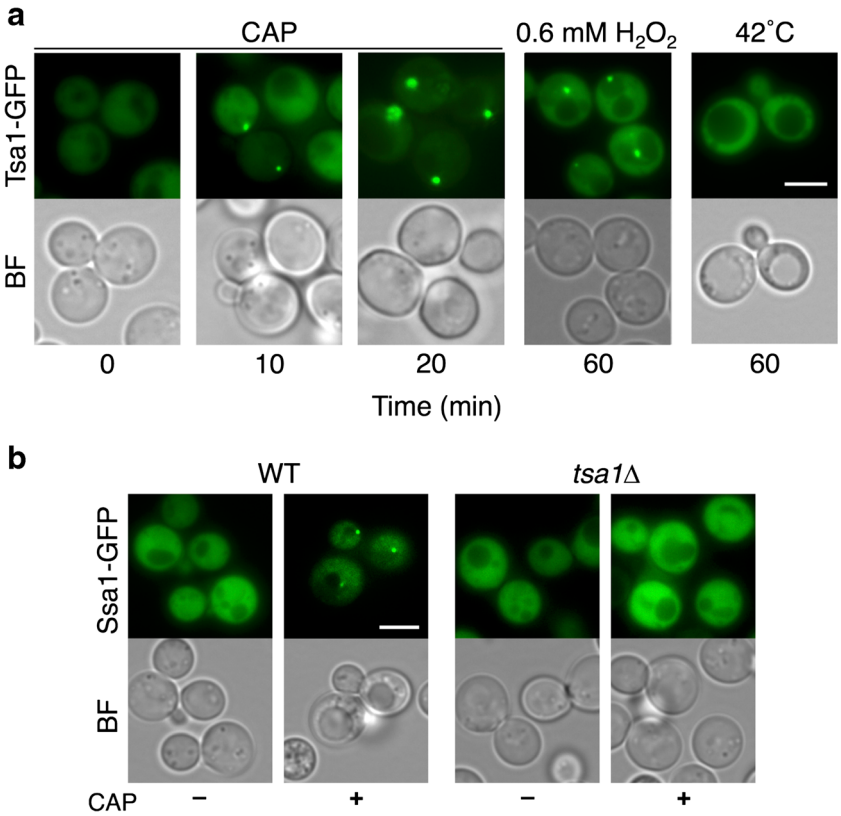

Fig. 4 CAP caused the formation of the Tsa1-GFP focus in the cytoplasm. **a** Yeast cells carrying the GFP-tagged chromosomal copy of *TSA1* were treated with CAP or the indicated stress conditions. Tsa1-GFP was immediately observed after the CAP treatment without fixation. **b** Wild-type and *tsa1*Δ cells carrying a *GFP*-tagged chromosomal copy of *SSA1* were treated with CAP for 20 min. BF bright field. The white bar indicates 3 μm

responses including the induced expression of molecular chaperones and intracellular accumulation of trehalose via the activation of the stress-responsive transcription factors Hsf1 (heat shock factor 1) and Msn2/Msn4 (general stress

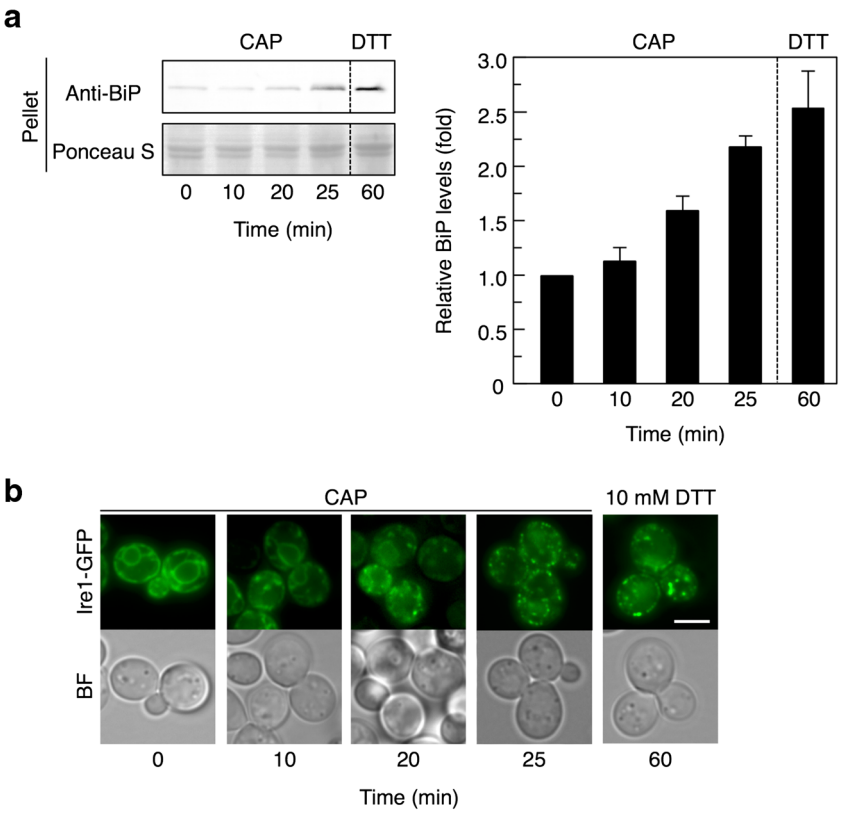

Fig. 5 CAP increased BiP aggregate levels in the ER. **a** After the treatment with CAP or 10 mM DTT for the indicated time, total cell lysates were prepared using glass beads in Triton X-100-containing buffer. Pellet fractions were prepared by the centrifugation of total cell lysates and subjected to a western blotting analysis with an anti-BiP antibody. The protein levels of sedimentable BiP were quantified using ImageJ, and the intensity of the BiP band in cells treated without stress was considered to be onefold. Data are shown as the mean ± SE ($n = 3$). **b** Cells were treated with CAP for the indicated time or with 10 mM DTT for 60 min. Ire1-GFP was immediately observed after the CAP treatment

responsive transcription factors) (Sanchez and Lindquist 1990; Izawa et al. 2004; Morano et al. 2012; Verghese et al. 2012; Gibney et al. 2013). In order to examine the effects of the pretreatment on yeast resistance to CAP, cells in the exponential phase were pretreated at 37 °C for 1 h and then exposed to CAP. We found that the survival rate under CAP stress was significantly increased by the pretreatment at 37 °C (Fig. 6a), indicating that the pretreatment with mild heat shock improved resistance to the subsequent CAP treatment.

The pretreatment also affected the formation of Hsp104-GFP foci upon CAP exposure. The formation of Hsp104-GFP foci was significantly less in cells treated with CAP after the treatment at 37 °C for 1 h than in those directly challenged with the CAP treatment (Fig. 6b). Additionally, the oligomerization of Ire1-GFP was hardly induced by CAP in pretreated cells (Fig. 6b). These results suggest that the pretreatment at 37 °C alleviated the accumulation of denatured proteins in CAP-treated yeast cells.

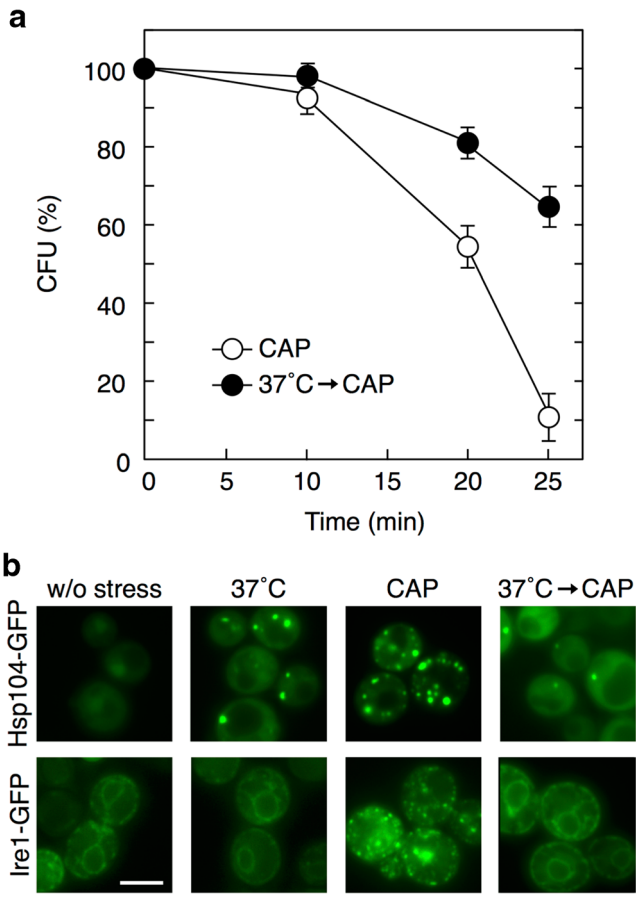

Fig. 6 A pretreatment with mild heat shock alleviated cell death caused by CAP. Cells were pretreated with or without mild heat shock at 37 °C for 1 h prior to the CAP treatment. **a** The relative survival rate was calculated as colony-forming units (CFU). Data are shown as the mean ± SE ($n = 3$). **b** Cells were treated with CAP for 20 min after or without the pretreatment at 37 °C for 1 h. The intracellular localization of Hsp104-GFP and Ire1-GFP was immediately observed after the stress treatment without fixation. The white bar indicates 3 μm

Discussion

In the present study, we improved the processing capacity of our CAP system by preventing the rapid diffusion of Ar plasma using a glass funnel as a cover. We increased the amount of culture medium that may be processed by CAP each time, with efficient fungicidal effects. Therefore, this system became available to perform molecular biological analyses in order to confirm that CAP causes protein denaturation in yeast cells. CAP led to increased levels of insoluble protein aggregates and ubiquitinated proteins, clearly indicating that CAP causes severe damage to yeast proteins. Protein denaturation appears to have strongly contributed to the inactivation of yeast cells by CAP. Previous studies reported that CAP causes protein denaturation in bacterial cells (Yasuda et al. 2008; Lackmann et al. 2013; Lackmann and Bandow 2014). Thus, the induction of protein denaturation may be a common cytotoxic activity utilized by CAP against bacteria and eukaryotic microorganisms.

However, ER stress is a phenomenon specific to eukaryotic cells. CAP enhanced the levels of unfolded proteins in the ER as well as Ire1 oligomerization, indicating that CAP induces ER stress. The induction of ER stress by CAP indicates that CAP damages not only preexisting proteins but also newly synthesized secretory and transmembrane proteins in yeast cells. The accumulation of these inadequacies may lead to the quick inactivation of yeast cells by CAP.

This idea may be supported by our novel result showing that the pretreatment at 37 °C alleviated cell death caused by CAP (Fig. 6). A treatment with mild heat shock induces the expression of molecular chaperones via the activation of Hsf1 and Msn2/Msn4, and they counteract protein denaturation and prevent intracellular disorder (Vabulas et al. 2010; Kim et al. 2013; Mackenzie et al. 2016). Therefore, pretreated cells appear to have an enhanced ability to cope with protein damage caused by CAP and improved tolerance to CAP stress.

CAP emits ROS and causes the endogenous production of ROS in yeast cells (Ryu et al. 2013). Indeed, we observed elevated levels of intracellular oxidation in cells treated with CAP (Fig. 1c) (Itooka et al. 2016). ROS are known to induce protein denaturation and aggregation in yeast cells (Costa and Moradas-Ferreira 2001; Hanzén et al. 2016; Weids et al. 2016). Additionally, the formation of the Tsa1-GFP focus in the cytoplasm, which is induced in a H_2O_2-dependent manner and forms aggregates with denatured proteins (Hanzén et al. 2016; Hill et al. 2017), was also induced in CAP-treated yeast cells. Collectively, these results indicate that ROS, including H_2O_2, are the main cause of protein denaturation in the cytoplasm of CAP-treated cells. Another possible cause of protein denaturation is UV photons derived from CAP (Park et al. 2003; Heise et al. 2004; Gaunt et al. 2006). It is widely known that UV radiation causes modifications to proteins in cells via ROS production (Ichihashi et al. 2003) and a direct

photochemical reaction (Pattison and Davies 2006; Pattison et al. 2012). Lackmann et al. (2013) demonstrated the synergistic effects of UV photons and particles, such as ROS, on enzyme activities and protein structures; thus, ROS and UV photons may synergistically contribute to protein denaturation in yeast cells treated with CAP.

Although CAP is projected as an alternative method of sterilization in various fields, its fungicidal effects have not fully been examined. Only a small amount of samples may be processed each time in the typical laboratory-scale CAP system; therefore, we improved the processing capacity of CAP in order to acquire sufficient amounts of CAP-treated cells for biochemical and molecular biological analyses. In the present study, we demonstrated that CAP caused the denaturation of yeast proteins, at least partially, in a H_2O_2-dependent manner. We also showed that CAP-induced ER stress in yeast was specific to eukaryotic cells. Furthermore, a pretreatment at 37 °C significantly alleviated cell death caused by CAP. Our results strongly suggest that the induction of protein denaturation is a common cytotoxic function of CAP against prokaryotic and eukaryotic microorganisms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Allen SA, Clark W, McCaffery JM, Cai Z, Lanctot A, Slininger PJ, Liu ZL, Gorsich SW (2010) Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol Biofuels 3(1):2. https://doi.org/10.1186/1754-6834-3-2

Amm I, Sommer T, Wolf DH (2014) Protein quality control and elimination of protein waste: the role of the ubiquitin-proteasome system. Biochim Biophys Acta 1843(1):182–196. https://doi.org/10.1016/j.bbamcr.2013.06.031

Bösl B, Grimminger V, Walter S (2006) The molecular chaperone Hsp104—a molecular machine for protein disaggregation. J Struct Biol 156(1):139–148. https://doi.org/10.1016/j.jsb.2006.02.004

Brodsky JL, Skach WR (2011) Protein folding and quality control in the endoplasmic reticulum: recent lessons from yeast and mammalian cell systems. Curr Opin Cell Biol 23(4):464–475. https://doi.org/10.1016/j.ceb.2011.05.004

Canullo L, Genova T, Tallarico M, Gautier G, Mussano F, Botticelli D (2016) Plasma of argon affects the earliest biological response of different implant surfaces: an in vitro comparative study. J Dent Res 95(5):566–573. https://doi.org/10.1177/0022034516629119

Chapman RE, Walter P (1997) Translational attenuation mediated by an mRNA intron. Curr Biol 7(11):850–859. https://doi.org/10.1016/S0960-9822(06)00373-3

Collins GA, Gomez TA, Deshaies RJ, Tansey WP (2010) Combined chemical and genetic approach to inhibit proteolysis by the proteasome. Yeast 27(11):965–974. https://doi.org/10.1002/yea.1805

Costa V, Moradas-Ferreira P (2001) Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: insights into ageing, apoptosis and diseases. Mol Asp Med 22(4-5):217–246. https://doi.org/10.1016/S0098-2997(01)00012-7

Duske K, Koban I, Kindel E, Schröder K, Nebe B, Holtfreter B, Jablonowski L, Weltmann KD, Kocher T (2012) Atmospheric plasma enhances wettability and cell spreading on dental implant metals. J Clin Periodontol 39(4):400–407. https://doi.org/10.1111/j.1600-051X.2012.01853.x

Escusa-Toret S, Vonk WIM, Frydman J (2013) Spatial sequestration of misfolded proteins by a dynamic chaperone pathway enhances cellular fitness during stress. Nat Cell Biol 15(10):1231–1344. https://doi.org/10.1038/ncb2838

Feng H, Wang R, Sun P, Wu H, Liu Q, Fang J, Zhu W, Li F, Zhang J (2010) A study of eukaryotic response mechanisms to atmospheric pressure cold plasma by using *Saccharomyces cerevisiae* single gene mutants. Appl Phys Lett 97(13):131501–131503. https://doi.org/10.1063/1.3491180

Fernández A, Noriega E, Thompson A (2013) Inactivation of *Salmonella enterica* serovar Typhimurium on fresh produce by cold atmospheric gas plasma technology. Food Microbiol 33:24–29

Finley D, Ulrich HD, Sommer T, Kaiser P (2012) The ubiquitin-proteasome system of *Saccharomyces cerevisiae*. Genetics 192(2):319–360. https://doi.org/10.1534/genetics.112.140467

Garrido EO, Grant CM (2002) Role of thioredoxins in the response of *Saccharomyces cerevisiae* to oxidative stress induced by hydroperoxides. Mol Microbiol 43(4):993–1003. https://doi.org/10.1046/j.1365-2958.2002.02795.x

Gaunt L, Beggs C, Georghiou G (2006) Bactericidal action of the reactive species produced by gas-discharge nonthermal plasma at atmospheric pressure: a review. IEEE Trans Plasma Sci 34(4):1257–1269. https://doi.org/10.1109/TPS.2006.878381

Gibney PA, Lu C, Caudy AA, Hess DC, Botstein D (2013) Yeast metabolic and signaling genes are required for heat-shock survival and have little overlap with the heat-induced genes. Proc Natl Acad Sci U S A 110(46):E4393–E4402. https://doi.org/10.1073/pnas.1318100110

Giro G, Tovar N, Witek L, Marin C, Silva NRF, Bonfante EA, Coelho PG (2013) Osseointegration assessment of chairside argon-based nonthermal plasma-treated Ca-P coated dental implants. J Biomed Mater Res A 101(1):98–103. https://doi.org/10.1002/jbm.a.34304

Glover JR, Lindquist S (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell 94(1):73–82. https://doi.org/10.1016/S0092-8674(00)81223-4

Hanzén S, Vielfort K, Yang J, Roger F, Andersson V, Zamarbide-Forés S, Andersson R, Malm L, Palais G, Biteau B, Liu B, Toledano MB, Molin M, Nyström T (2016) Lifespan control by redox-dependent recruitment of chaperones to misfolded proteins. Cell 166(1):140–151. https://doi.org/10.1016/j.cell.2016.05.006

Heinlin J, Morfill G, Landthaler M, Stolz W, Isbary G, Zimmermann JL, Shimizu T, Karrer S (2010) Plasma medicine: possible applications in dermatology. J Dtsch Dermatol Ges 8(12):968–976. https://doi.org/10.1111/j.1610-0387.2010.07495.x

Heise M, Neff W, Franken O, Muranyi P, Wunderlich J (2004) Sterilization of polymer foils with dielectric barrier discharges at atmospheric pressure. Plasmas Polym 9(1):23–33. https://doi.org/10.1023/B:PAPO.0000039814.70172.c0

Hill SM, Hanzén S, Nyström T (2017) Restricted access: spatial sequestration of damaged proteins during stress and aging. EMBO Rep 18: 377–391

Hoffmann C, Berganza C, Zhang J (2013) Cold atmospheric plasma, methods of production and application in dentistry and oncology. Med Gas Res 3(1):21. https://doi.org/10.1186/2045-9912-3-21

Homma T, Furuta M, Takemura Y (2013) Inactivation of *Escherichia coli* using the atmospheric pressure plasma jet of Ar gas. Jpn J Appl Phys 52(3R):036201. https://doi.org/10.7567/JJAP.52.036201

Ichihashi M, Ueda M, Budiyanto A, Bito T, Oka M, Fukunaga M, Tsuru K, Horikawa T (2003) UV-induced skin damage. Toxicology 189(1-2):21–39. https://doi.org/10.1016/S0300-483X(03)00150-1

Itooka K, Takahashi K, Izawa S (2016) Fluorescence microscopic analysis of antifungal effects of cold atmospheric pressure plasma in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 100:9295–9304

Izawa S, Takemura R, Inoue Y (2004) Gle2p is essential to induce adaptation of the export of bulk poly(A) mRNA to heat shock in *Saccharomyces cerevisiae*. J Biol Chem 279:35469–35478

Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, Lee JR, Lee SS, Moon JC, Yun JW, Choi YO, Kim WY, Kang JS, Cheong GW, Yun DJ, Rhee SG, Cho MJ, Lee SY (2004) Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. Cell 117(5): 625–635. https://doi.org/10.1016/j.cell.2004.05.002

Kawahara T, Yanagi H, Yura T, Mori K (1997) Endoplasmic reticulum stress-induced mRNA splicing permits synthesis of transcription factor Hac1p/Ern4p that activates the unfolded protein response. Mol Biol Cell 8(10):1845–1862. https://doi.org/10.1091/mbc.8.10.1845

Kawazoe N, Kimata Y, Izawa S (2017) Acetic acid causes endoplasmic reticulum stress and induces the unfolded protein response in *Saccharomyces cerevisiae*. Front Microbiol 8:1192. https://doi.org/10.3389/fmicb.2017.01192

Kim YE, Hipp MS, Bracher A, Hayer-Hartl M, Hartl FU (2013) Molecular chaperone functions in protein folding and proteostasis. Annu Rev Biochem 82(1):323–355. https://doi.org/10.1146/annurev-biochem-060208-092442

Kimata Y, Kimata I, Shimizu Y, Abe H, Farcasanu IC, Takeuchi M, Rose MD, Kohno K (2003) Genetic evidence for a role of BiP/Kar2 that regulates Ire1 in response to accumulation of unfolded proteins. Mol Biol Cell 14(6):2559–2569. https://doi.org/10.1091/mbc.E02-11-0708

Kimata Y, Oikawa D, Shimizu Y, Ishiwata-Kimata Y, Kohno K (2004) A role for BiP as an adjustor for the endoplasmic reticulum stress-sensing protein Ire1. J Cell Biol 167:445–456

Kimata Y, Ishiwata-Kimata Y, Ito T, Hirata A, Suzuki T, Oikawa D, Takeuchi M, Kohno K (2007) Two regulatory steps of ER-stress sensor Ire1 involving its cluster formation and interaction with unfolded proteins. J Cell Biol 179(1):75–86. https://doi.org/10.1083/jcb.200704166

Klämfl TG, Isbary G, Shimizu T, Li YF, Zimmermann JL, Stolz W, Schlegel J, Morfill GE, Schmidt HU (2012) Cold atmospheric air plasma sterilization against spores and other microorganisms of clinical interest. Appl Environ Microbiol 78(15):5077–5082. https://doi.org/10.1128/AEM.00583-12

Koban I, Matthes R, Hübner NO, Welk A, Meisel P, Holtfreter B, Sietmann R, Kindel E, Weltmann K, Kramer A, Kocher T (2010) Treatment of *Candida albicans* biofilms with low-temperature plasma induced by dielectric barrier discharge and atmospheric pressure plasma jet. New J Phys 12:1–15

Kong MG, Kroesen G, Morfill G, Nosenko T, Shimizu T, van Dijk J, Zimmermann JL (2009) Plasma medicine: an introductory review. New J Phys 11(11):115012. https://doi.org/10.1088/1367-2630/11/11/115012

Koplin A, Preissler S, Ilina Y, Koch M, Scior A, Erhardt M, Deuerling E (2010) A dual function for chaperones SSB-RAC and the NAC nascent polypeptide associated complex on ribosomes. J Cell Biol 189:57–68

Krisch J, Chandrasekaran M, Kadaikunnan S, Alharbi NS, Vágvölgyi C (2016) Latest about spoilage by yeasts: focus on the deterioration of beverages and other plant-derived products. J Food Prot 79(5):825–829. https://doi.org/10.4315/0362-028X.JFP-15-324

Lackmann JW, Bandow JE (2014) Inactivation of microbes and macromolecules by atmospheric-pressure plasma jets. Appl Microbiol Biotechnol 98(14):6205–6213. https://doi.org/10.1007/s00253-014-5781-9

Lackmann J, Schneider S, Edengeiser E, Jarzina F, Brinckmann S, Steinborn E, Havenith M, Benedikt J, Bandow JE (2013) Photons and particles emitted from cold atmospheric-pressure plasma inactivate bacteria and biomolecules independently and synergistically. J R Soc Interface 10(89):2013059. https://doi.org/10.1098/rsif.2013.0591

Liu B, Larsson L, Caballero A, Hao X, Oling D, Grantham J, Nyström T (2010) The polarisome is required for segregation and retrograde transport of protein aggregates. Cell 140:257–267

Loureiro V, Malfeito-Ferreira M (2003) Spoilage yeasts in the wine industry. Int J Food Microbiol 86(1-2):23–50. https://doi.org/10.1016/S0168-1605(03)00246-0

Mackenzie RJ, Lawless C, Holman SW, Lanthaler K, Beynon RJ, Grant CM, Hubbard SJ, Eyers CE (2016) Absolute protein quantification of the yeast chaperome under conditions of heat shock. Proteomics 16(15-16):2128–2140. https://doi.org/10.1002/pmic.201500503

Maeda K, Toyokawa Y, Shimizu N, Imanishi Y, Sakudo A (2015) Inactivation of *Salmonella* by nitrogen gas plasma generated by a static induction thyristor as a pulsed power supply. Food Control 52: 54–59

Maisch T, Shimizu T, Li YF, Heinlin J, Karrer S, Morfill G, Zimmermann JL (2012a) Decolonisation of MRSA, *S. aureus* and *E. coli* by cold atmospheric plasma using a porcine skin model *in vitro*. PLoS One 7(4):e34610. https://doi.org/10.1371/journal.pone.0034610

Maisch T, Shimizu T, Isbary G, Heinlin J, Karrer S, Klämpfl TG, Lin YF, Morfill G, Zimmermann JL (2012b) Contact-free inactivation of *Candida albicans* biofilms by cold atmospheric air plasma. Appl Environ Microbiol 78(12):4242–4247. https://doi.org/10.1128/AEM.07235-11

Manner H (2008) Argon plasma coagulation therapy. Curr Opin Gastroenterol 24(5):612–616. https://doi.org/10.1097/MOG.0b013e32830bf825

Mathuranyanon R, Tsukamoto T, Takeuchi A, Ishiwata-Kimata Y, Tuchiya Y, Kohno K, Kimata Y (2015) Tight regulation of the unfolded protein sensor Ire1 by its intramolecularly antagonizing subdomain. J Cell Sci 128:1762–1772

Mir SA, Shah MA, Mir MM (2016) Understanding the role of plasma technology in food industry. Food Bioprocess Technol 9(5):734–750. https://doi.org/10.1007/s11947-016-1699-9

Morano KA, Grant CM, Moye-Rowley WS (2012) The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. Genetics 190(4):1157–1195. https://doi.org/10.1534/genetics.111.128033

Mori K (2009) Signalling pathways in the unfolded protein response: development from yeast to mammals. J Biochem 146(6):743–750. https://doi.org/10.1093/jb/mvp166

Mori K, Kawahara T, Yoshida H, Yanagi H, Yura T (1996) Signaling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. Genes Cells 1:803–817

Niemira BA, Boyd G, Sites J (2014) Cold plasma rapid decontamination of food contact surfaces contaminated with *Salmonella* biofilms. J Food Sci 79:917–922

Park BJ, Lee DH, Park JC, Lee IS, Lee KY, Hyun SO, Chun MS, Chung KH (2003) Sterilization using a microwave-induced argon plasma

system at atmospheric pressure. Phys Plasmas 10(11):4539–4544. https://doi.org/10.1063/1.1613655

Pattison DI, Davies MJ (2006) Actions of ultraviolet light on cellular structures. EXS 96:131–157

Pattison DI, Rahmanto AS, Davies MJ (2012) Photo-oxidation of proteins. Photochem Photobiol Sci 11(1):38–53. https://doi.org/10.1039/C1PP05164D

Promlek T, Ishiwata-Kimata Y, Shido M, Sakuramoto M, Kohno K, Kimata Y (2011) Membrane aberrancy and unfolded proteins activate the endoplasmic reticulum stress sensor Ire1 in different ways. Mol Biol Cell 22(18):3520–3532. https://doi.org/10.1091/mbc.E11-04-0295

Rand JD, Grant CM (2006) The thioredoxin system protects ribosomes against stress-induced aggregation. Mol Biol Cell 17(1):387–401. https://doi.org/10.1091/mbc.E05-06-0520

Rose MD, Misra LM, Vogel JP (1989) *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. Cell 57(7):1211–1221. https://doi.org/10.1016/0092-8674(89)90058-5

Ryu Y, Kim Y, Lee J, Shim G, Uhm H, Park G, Choi EH (2013) Effects of background fluid on the efficiency of inactivating yeast with non-thermal atmospheric pressure plasma. PLoS One 8:e66231. https://doi.org/10.1371/journal.pone.0066231

Sanchez Y, Lindquist SL (1990) HSP104 required for induced thermotolerance. Science 248(4959):1112–1115. https://doi.org/10.1126/science.2188365

Shaw A, Shama G, Iza F (2015) Emerging applications of low temperature gas plasmas in the food industry. Biointerphases 10:029402. https://doi.org/10.1116/1.4914029

Sun S, Anderson NM, Keller S (2014) Atmospheric pressure plasma treatment of black peppercorns inoculated with *Salmonella* and held under controlled storage. J Food Sci 79(12):E2441–E2446. https://doi.org/10.1111/1750-3841.12696

Tanaka H, Hori M (2017) Medical applications of non-thermal atmospheric pressure plasma. J Clin Biochem Nutr 60(1):29–32. https://doi.org/10.3164/jcbn.16-67

Vabulas RM, Raycchaudhuri S, Hayer-Hartl M, Hartl FU (2010) Protein folding in the cytoplasm and the heat shock response. Cold Spring Harb Perspect Biol 2(12):a004390. https://doi.org/10.1101/cshperspect.a004390

van Gils CAJ, Hofmann S, BKHL B, Brandenburg R, Bruggeman PJ (2013) Mechanisms of bacterial inactivation in the liquid phase induced by a remote RF cold atmospheric pressure plasma jet. J Phys D Appl Phys 46:175203. https://doi.org/10.1088/0022-3727/4617/175203

Verghese J, Abrams J, Wang Y, Morano KA (2012) Biology of the heat shock response and protein chaperones: budding yeast (*Saccharomyces cerevisiae*) as a model system. Microbiol Mol Biol Rev 76(2):115–158. https://doi.org/10.1128/MMBR.05018-11

Wallace EWJ, Kear-Scott JL, Pilipenko EV, Schwartz MH, Laskowski PR, Rojek AE, Katanski CD, Riback JA, Dion MF, Franks AM, Airoldi EM, Pan T, Budnik BA, Drummond DA (2015) Reversible, specific, active aggregates of endogenous proteins assemble upon heat stress. Cell 162(6):1286–1298. https://doi.org/10.1016/j.cell.2015.08.041

Weids AJ, Ibstedt S, Tamás MJ, Grant CM (2016) Distinct stress conditions result in aggregation of proteins with similar properties. Sci Rep 18(1):24554. https://doi.org/10.1038/srep24554

Weltmann KD, von Woedtke T (2016) Plasma medicine—current state of research and medical application. Plasma Phys Control Fusion 59(1):014031. https://doi.org/10.1088/0741-3335/59/1/014031

Yasuda H, Hashimoto M, Rahman MM, Takashima K, Mizuno A (2008) States of biological components in bacteria and bacteriophages during inactivation by atmospheric dielectric barrier discharges. Plasma Process Polym 5:615–621

Zhou C, Slaughter BD, Unruh JR, Eldakak A, Rubinstein B, Li R (2011) Motility and segregation of Hsp104-associated protein aggregates in budding yeast. Cell 23:1186–1196

Ziuzina D, Patil S, Cullen PJ, Keener KM, Bourke P (2014) Atmospheric cold plasma inactivation of *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* inoculated on fresh produce. Food Microbiol 42:109–116. https://doi.org/10.1016/j.fm.2014.02.007