Proteasome activity is important for replication recovery, CHK1 phosphorylation and prevention of G2 arrest after low-dose formaldehyde

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Abstract
Formaldehyde (FA) is a human carcinogen with numerous sources of environmental and occupational exposures. This reactive aldehyde is also produced endogenously during metabolism of drugs and other processes. DNA–protein crosslinks (DPCs) are considered to be the main genotoxic lesions for FA. Accumulating evidence suggests that DPC repair in high eukaryotes involves proteolysis of crosslinked proteins. Here, we examined a role of the main cellular proteolytic machinery proteasomes in toxic responses of human lung cells to low FA doses. We found that transient inhibition of proteasome activity increased cytotoxicity and diminished clonogenic viability of FA-treated cells. Proteasome inactivation exacerbated suppressive effects of FA on DNA replication and increased the levels of the genotoxic stress marker γ-H2AX in normal human cells. A transient loss of proteasome activity in FA-exposed cells also caused delayed perturbations of cell cycle, which included G2 arrest and a depletion of S-phase populations at FA doses that had no effects in control cells. Proteasome activity diminished p53-Ser15 phosphorylation but was important for FA-induced CHK1 phosphorylation, which is a biochemical marker of DPC proteolysis in replicating cells. Unlike FA, proteasome inhibition had no effect on cell survival and CHK1 phosphorylation by the non-DPC replication stressor hydroxyurea. Overall, we obtained evidence for the importance of proteasomes in protection of human cells against biologically relevant doses of FA. Biochemically, our findings indicate the involvement of proteasomes in proteolytic repair of DPC, which removes replication blockage by these highly bulky lesions.

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Introduction
Formaldehyde (FA) is a widely used industrial chemical and a ubiquitous atmospheric pollutant. Combustion processes are usually the largest sources of ambient FA but offgassing of plastics, paints and other synthetic materials also generates significant amounts of this toxicant. FA is produced in human body endogenously either as a product of normal metabolism or demethylation of toxicant. FA is produced in human body endogenously either as a product of normal metabolism or demethylation of toxicant. FA readily reacts with DNA bases producing N-hydroxymethyl adducts with dA and dG, however, these small modifications are hydrolytically unstable (IARC, 2006). The most abundant DNA lesions formed by FA in cells are DNA–protein crosslinks (DPC), which have a much greater chemical stability than small DNA adducts (Quiévryn and Zhitkovich, 2000). The dose-dependence of DPC formation and nasal cancers in FA-exposed animals showed a close correlation, leading to the use of DPC in modeling of cancer risks associated with human exposures (Subramaniam et al., 2008). Although it is frequently assumed that DPC are major contributors to FA toxicity, their role in specific toxic responses has not yet been assessed experimentally. The importance of specific lesions for agents producing multiple DNA damage forms can be most directly evaluated through the manipulations of

Abbreviations: DPC, DNA–protein crosslinks; EdU, 5-ethynyl-2′-deoxyuridine; FACS, fluorescence-activated cell sorting; FA, formaldehyde; HU, hydroxyurea; NER, nucleotide excision repair; ss, single-stranded.

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repair processes. In this approach, an increase in toxic responses to the chemical in cells with a lesion-specific repair defect provides evidence for the biological significance of the particular DNA modification.

Characterization of repair mechanisms for DPC has been slower than that for other DNA lesions despite that DPCs are formed by common cancer drugs (Santi et al., 1984; Taïloi et al., 1996; Loeb et al., 2009) and several human carcinogens (Costa et al., 1997; Voitkun and Zhitkovich, 1999; Macfie et al., 2010). Suppression of DPC removal in FA-treated cells by inhibition of proteasome activity and a normal kinetics of DPC losses in nucleotide excision repair (NER)-deficient human lines has led to a model of DPC repair through the initial proteolysis of crosslinked protein (Quievryn and Zhitkovich, 2000). Cellular repair of DPC formed by chromium(VI) was also sensitive to proteasome inhibition and independent of NER (Zecevic et al., 2010). DPCs were also found to be resistant to excision by mammalian NER in vitro (Reardon and Sancar, 2006; Nakano et al., 2009). A very recent study with DPC-containing substrates incubated with Xenopus extract clearly demonstrated a replication-dependent mechanism of DPC repair via ubiquitin-dependent proteolysis (Duxin et al., 2014). These findings are consistent with the virtual absence of active repair of FA-induced DPC in nondividing peripheral blood human lymphocytes (Quievryn and Zhitkovich, 2000). Thus, inhibition of DPC proteolysis in replicating cells can help assess a toxicological importance of these lesions.

In this work, we examined replication recovery, cell cycle changes, genotoxic signaling and survival of human cells treated with low-dose FA under the conditions of proteasome inhibition with the goal of assessing contributions of DPC to specific toxic effects and determining the importance of proteasomes in protection against FA injury.

Materials and methods

Chemicals. MG132 and bortezomib were obtained from SelleckChem and MG115 was from Santa Cruz. A stock solution of formaldehyde (F8775) and all buffers and salts were from Sigma.

Cells and treatments. Cells were purchased from the American Type Culture Collection. H460 and A549 human lung epithelial cells were cultured under 95% air/5% CO2 humidified atmosphere in 10% serum-supplemented medium (RPMI-1640 for H460 and F-12K for A549). IMR90 human normal lung fibroblasts were propagated in DMEM medium containing 10% serum. Primary human fibroblasts were grown in 5% O2 and 5% CO2. Cells were treated with FA in complete growth media containing 10% serum. Primary human IMR90 human normal lung fibroblasts were obtained as described previously (Reynolds and Zhitkovich, 2000). Cytotoxicity.

For detection of histones, cellular proteins were solubilized by trypsinization and fixed overnight before treatments with 0.5% Triton X-100 for 15 min at room temperature. Coverslips were blocked with 2% fetal bovine serum for 1 h followed by EdU staining using Click-it Edu-Alexa Fluor 488 Imaging kit (Invitrogen). Mouse monoclonal anti-phospho-histone H2AX antibodies (05-636, Millipore) were used at 1:250 dilution. The secondary antibodies were from Life Technologies (A11029 Alexa Fluor 488 goat anti-mouse, 1:500 dilution). All dilutions of antibodies were made in a PBS solution containing 1% BSA and 0.5% Tween-20. Cells were incubated with primary antibodies for 2 h at 37 °C, washed three times with PBS and then incubated with secondary antibodies for 1 h at room temperature. Coverslips were then mounted on glass slides using a fluorescence mounting media with DAPI (H-1200, Vectashield). Cells were viewed on the Nikon E-800 Eclipse fluorescent microscope.

Fluorescence-activated cell sorting (FACS). In experiments analyzing DNA synthesis, IMR90 cells were treated with 0–100 µM FA for 2 h with the addition of 10 µM EdU for the last hour. For the determination of the delayed cell cycle changes, IMR90 cells were treated with FA for 3 h, incubated with 2 µM MG132 for 6 h and taken for FACS analyses 18 h later. Cells were collected by trypsinization and fixed overnight in 80% ethanol at 4 °C. After washing with PBS, cells were permeabilized with 0.5% Triton X-100 in PBS for 30 min at room temperature and washed with PBS. Cell pellets were resuspended in a Click-it reaction mixture (Click-it Edu-Alexa Fluor 488 Flow Cytometry Assay kit from Invitrogen) and incubated for 30 min at room temperature in the dark. Cells were washed once with PBS, resuspended in 500 µl PBS containing 4 µg/ml propidium iodide, and incubated for 30 min at room temperature without light. Cells were washed once again with 2 ml PBS and resuspended in 0.5 ml PBS for flow cytometry (FACScalibur, BD Biosciences). The CellQuest Pro software package was used for data analysis.

Cytotoxicity. Measurements of the total metabolic activity of cell populations using the CellTiter-Glo luminescent cell viability assay (Promega) were used for the assessment of cytotoxicity. Cells were seeded into 96-well optical cell culture plates (2000 cells/well), grown overnight and then treated with FA for 3 h. Proteasome inhibitors were added for 6 h after FA removal. The cytotoxicity assay was performed at 72 h post-FA.

Clonogenic survival. H460 cells were seeded onto 60-mm dishes (400 cells/dish) and grown overnight. Next day, cells were treated with FA for 3 h followed by the addition of proteasome inhibitors for 6 h. Cells were fixed with methanol and Giemsa-stained after 7–8 days of growth. Groups with 30 or more cells were counted as colonies.

Statistics. Two-tailed, unpaired t-test was used for the evaluation of differences between the groups. The p-values for multiple testing were adjusted using the Bonferroni correction. Data in figures are presented as means ± SD. When not visible, error bars were smaller than symbols.

Results

Experimental models

FA is a common product of the combustion of organic matter, which results in the exposure of lung cells to relatively large doses of this carcinogen among tobacco smokers (Hecht, 2003). Therefore, we chose human lung cells as our biological models. A549 and H460 are human lung epithelial cell lines that we have previously examined for
repair of FA-induced DPC and cytotoxic mechanisms (Quievryn and Zhitkovich, 2000; Wong et al., 2012). Both cell lines contain wild-type transcriptional factor p53, which plays a major role in DNA damage-induced cell fate decisions, including those triggered by FA (Wong et al., 2012). Since some regulatory networks are altered in all transformed cell lines, signaling responses to FA were investigated in IMR90 normal human lung fibroblasts. We focused our studies on low doses of FA, which ranged from those inducing no or minimal effects in exposed cells to doses producing not more than 50% decline in the colony-forming ability. In addition to their human relevance, low doses help minimize formation and confounding effects of secondary lesions that occur in cells treated with highly toxic concentrations of FA. For example, high FA doses have been found to suppress p53 activation, resulting in a bell-shaped dose response (Wong et al., 2012). The majority of our experiments were conducted with 50–150 μM FA, which covered the physiological range of 30–100 μM FA in human serum of unexposed individuals (IARC, 2006; NTP, 2010) and included a moderately higher dosage of FA occurring locally during inhalation exposures. To mimic in vivo conditions and avoid acute cell injury, our treatments with FA were done in the presence of serum. We primarily employed a widely used proteasome inhibitor MG132 to test a role of proteasomes in cellular responses to FA. Confirmatory experiments were performed with two more inhibitors, MG115 and bortezomib (PS-341). MG132, MG115 and bortezomib are all reversible inhibitors, which affords a better control of the duration of their action and avoids prolonged inactivation of proteasomes and the resulting cytotoxicity.

Enhancement of FA toxicity by proteasome inhibition

To assess a role of proteasomes in cytotoxicity of FA, we first measured the overall metabolic activity of cell populations. Cells were treated with FA for 3 h, then incubated with proteasome inhibitors for 6 h and allowed to recover for 3 days before viability measurements. We found that the addition of the proteasome inhibitor bortezomib significantly increased cytotoxicity of FA treatments in A549 cells (Fig. 1A), a cell line in which we have previously found a loss of DPC repair by proteasome inactivation (Quievryn and Zhitkovich, 2000). The assessment of MG132 impact on cytotoxicity and clonogenic viability of H460 cells detected strong protective effects of proteasome activity against FA toxicity (Fig. 1B, C). For 50 μM FA, which alone did not produce a significant decrease in the number of colonies, the addition of MG132 caused 50–60% loss in the clonogenic survival of cells.

To further explore a role of proteasome activity in FA resistance, we examined cell cycle changes in IMR90 normal human lung fibroblasts. A microscopy-based scoring of EdU-incorporated nuclei revealed significant decreases in the number of replicating IMR90 cells that were treated with FA and MG132 but not with FA alone (Fig. 2A). Cell cycle analyses by FACS confirmed a depletion of IMR90 cells in S-phase by a combined FA + MG132 treatment, which was accompanied by a parallel increase in the frequency of G2/M cells (Fig. 2B). For 150 μM FA, proteasome inhibition caused approximately a 2-fold decrease in the number of S-phase cells and a 4-fold increase in G2/M cells whereas cells treated with FA alone did not show any significant changes. FACS analyses of EdU/DNA-stained cells are unable to differentiate between G2 and mitotic cells, which both have the 4n DNA content and are negative for EdU incorporation. To determine the phase in which IMR90 accumulate after FA + MG132 exposures, we measured the amounts of a mitotic marker, histone H3 phosphorylated at Ser10. Because of a weak attachment of mitotic cells to the dishes, we collected both attached and floating IMR90 cells for the analysis of phospho-histone H3 by western blotting. We found lower levels of this mitotic marker in FA + MG132 samples relative to FA-alone groups (Fig. 2C), indicating a diminished entry of G2 cells into mitosis. Based on these and FACS results, we concluded that proteasome inhibition in FA-treated normal human cells caused a delayed G2 phase arrest.

![Enhancement of FA toxicity by proteasome inhibition](Image)

**Fig. 1.** Proteasome inhibitors enhance FA toxicity in human lung cells. Cells were treated with FA for 3 h and then incubated for 6 h with or without proteasome inhibitors. Cell viability was measured at 72 h post-FA exposure. Data are from 3 to 4 experiments. Statistical comparisons were made relative to the corresponding MG132-unexposed groups (**p < 0.01, ***p < 0.001) and relative to the no-FA controls (**p < 0.01, ***p < 0.001). (A) Effect of 300 nM bortezomib on viability of A549 cells. (B) Effect of 2 μM MG132 on viability of H460 cells. (C) Clonogenic survival of H460 cells incubated with 0, 2 or 4 μM MG132.

Proteasome activity and replication stress by FA

G2 cell cycle arrest usually reflects the formation of genomic damage in the preceding S-phase, which activates checkpoint response upon the entry of cells into G2 phase. Therefore, we next investigated DNA synthesis by FACS measurements of incorporation of the base analogue EdU. Consistent with the strong replication-blocking properties of DPC (Duxin et al., 2014), we found that even a low dose of 50 μM FA significantly decreased DNA synthesis, which was further lowered by 100 μM FA (Fig. 3A). Replication inhibition by FA appears to be uniform throughout the S-phase, as evidenced by the lack of changes in the shape of distribution of cells with EdU incorporation (right peaks remaining symmetrical). The addition of MG132 further slowed the rate of DNA replication in FA-treated but not control cells, as evidenced by decreased EdU signals in S-phase populations. To explore how proteasome inhibition is causing inhibition of replication, we analyzed Ser139-phosphorylation of histone H2AX (known as γ-H2AX), which occurs in cells experiencing replication stress (Toledo et al., 2011). Because γ-H2AX is also produced at the sites of DNA breaks in cells outside of S-phase (Lukas et al., 2011), we first investigated the cell cycle distribution of γ-H2AX by costaining with the S-phase marker EdU. We found that all of γ-H2AX-positive IMR90 cells in FA/MG132-treated samples were in S-phase (Fig. 3B), demonstrating the suitability of γ-H2AX for detection of replication stress by FA. The addition of MG132 with FA strongly increased the number of cells with γ-H2AX in comparison to FA-alone samples (Fig. 3C). Furthermore, MG132-treated cells also had larger amounts of γ-H2AX, as evidenced by the increased staining in individual nuclei (Fig. 3D, E). Co-treatments with FA and two concentrations of another proteasome inhibitor bortezomib also significantly elevated the frequency of IMR90 cells with γ-H2AX (Fig. 3F). To further
assess the importance of functional proteasomes for DNA replication in FA-treated cells, we examined the effects of a specific inhibitor of the chymotrypsin-like activity of proteasomes, MG115. Similarly to MG132, which inhibits both chymotrypsin-like and post-glutamyl peptide hydrolase activities, a specific inactivation of the main proteasomal activity by MG115 also decreased the rate of DNA synthesis in FA-treated IMR90 cells (Fig. 3G). Overall, our results on the diminished DNA synthesis and the increased formation of γ-H2AX indicate that proteasome inhibition exacerbated replication stress by FA.

Effect of proteasome inhibition on CHK1 phosphorylation

To better understand how proteasome inhibition enhances replication stress by FA, we examined phosphorylation of checkpoint kinase CHK1. Phosphorylation of this kinase by ATR is usually dependent on the formation of single-stranded (ss)-DNA regions (Zou and Elledge, 2003), which was confirmed in FA-treated cells (Wong et al., 2012). The appearance of ssDNA and CHK1 phosphorylation during replication of DPC-containing templates required proteolysis of crosslinked protein, which allowed resumption of DNA unwinding but resulted in stalling of DNA polymerase at the sites of residual DNA–peptide crosslinks. Ongoing duplex unwinding despite blocked DNA synthesis in the leading strand generated ssDNA, which triggered CHK1 phosphorylation (Duxin et al., 2014). Thus, CHK1 phosphorylation can serve as a biochemical marker of DPC proteolysis in replicating cells. Western blots of two independent sets of IMR90 lysates showed a complete suppression of CHK1 phosphorylation by FA in the presence of 1–6 μM MG132 (Fig. 4A, B). A clear inhibition of CHK1 phosphorylation by MG132 at low–moderate doses of FA was also observed in H460 cells (Fig. 4C). The suppressive effect of MG132 was lost for the toxic 300 μM FA, which may reflect the incompleteness of proteasome inhibition or a contribution of non-DPC lesions to ATR activation. FA has also been found to induce ATR-mediated phosphorylation of the transcriptional factor p53, which occurred in the ssDNA-independent manner (Wong et al., 2012). Consistent with the postulated helicase blockage-triggered mechanism of p53 activation (Wong et al., 2012), we found that proteasome inhibition produced a dose-dependent increase in p53 phosphorylation by FA (Fig. 4A, B).

Proteasome activity and responses to hydroxyurea (HU)

Since proteasome inhibition exacerbated replication stress by FA, we wanted to test whether it would also occur for DPC-nonproducing replication stressors. HU is a classic replication stressor that causes inhibition of dNTP synthesis, which results in the stalling of DNA polymerases and accumulation of ssDNA due to ongoing replisome-associated helicase activity. Using the same treatment protocol and the range of toxicity as those for FA (Fig. 1C), we found that MG132 did not decrease survival of HU-treated H460 cells in clonogenic experiments (Fig. 5A). Proteasome inhibition had also no effect on CHK1 phosphorylation by HU in H460 and IMR90 cells (Fig. 5B, C), indicating a lack of MG132 interference with ATR activation by ssDNA that is produced independently of DPC proteolysis.

Discussion

Previous studies of cellular processes promoting recovery from FA-induced injury have been largely focused on canonical DNA repair and DNA damage tolerance mechanisms (Ridpath et al., 2007; de Graaf et al., 2009; Nakano et al., 2009). NER, which is the principal repair mechanism for large DNA adducts (Reardon and Sancar, 2005), did not have a detectable effect on the removal of FA-induced and other DPC in human cells (Quevryn and Zhitkovich, 2000; Zecевич et al., 2010) and it was unable to excise model DPC in vitro (Reardon and...
Sancar, 2006; Nakano et al., 2009). Mammalian NER in vitro was able to act on small DPC but lost its activity for crosslinks with protein size of approximately 8 kDa (Nakano et al., 2009), which is below molecular weight for histones that are the main crosslinking proteins by FA in cells (Solomon and Varshavsky, 1985). Based on the suppression of DPC removal from FA-treated human cells by inhibition of proteasome activity, it was proposed that DPC repair involves proteolysis of crosslinked proteins and not DNA excision (Quievryn and Zhitkovich, 2000). Cellular repair of other types of chemically induced DPC was also sensitive to inhibition of proteasomes (Baker et al., 2007; Zecevic et al., 2010). However, the proteolysis-based model of DPC repair has been rejected by other investigators who argued that mammalian cells are unable to remove DPC and only possess tolerance mechanisms for these lesions (Ide et al., 2011). A very recent study of DPC substrates in DNA repair- and replication-proficient Xenopus egg extracts provided a clear evidence for an ubiquitin-dependent proteolysis of crosslinked protein, which was required for the restoration of replication (Duxin et al., 2014). The activation of DPC repair was strictly dependent on replication and was triggered by stalling of replicative complexes. For DPC in the leading strand, digestion of crosslinked proteins to peptides allowed resumption of DNA unwinding and DNA synthesis in the lagging strand; however, DNA–peptide crosslinks blocked DNA polymerase in the leading strand. The formation of ssDNA in the leading strand, resulting from uncoupling of DNA unwinding and DNA synthesis, led to CHK1 phosphorylation by ATR (Duxin et al., 2014).

DPC are considered to be the main genotoxic lesions for FA and are used in human risk assessment modeling of FA exposures (Subramaniam et al., 2008). Thus, it is important to understand how human cells can cope with FA-induced DPC. In this work, we investigated the significance of proteasome activity in recovery of human cells from FA-induced injury. Considering a multitude of cellular functions that are controlled by proteasomes, their inhibition can directly or indirectly affect more than one DNA damage tolerance mechanism. We minimized general effects of proteasome inhibition by employing short-term treatments and low concentrations of reversible inhibitors. We found that proteasome activity played a protective role in survival, replication recovery and cell cycle restoration in human cells after low-dose FA treatments. These results are consistent with the involvement of proteasomes in the removal of cellular DPC, which are potent replication–blocking lesions (Duxin et al., 2014). Proteasome inhibition in FA-treated cells resulted in delayed G2 arrest, which would be expected for cells exiting S-phase with checkpoint-activating unrelicated DNA regions. Despite the involvement of a different protease, a diminished proteolysis of DPC in yeast was also found to lead to G2 arrest (Stingele et al., 2014). CHK1 phosphorylation, which serves as a biochemical marker of crosslinked protein cleavage during replication of DPC-containing DNA (Duxin et al., 2014), was suppressed by proteasome inhibition in FA-treated cells. Inhibition of proteasome activity did not have any effect on CHK1 phosphorylation by hydroxyurea in our experiments and in other studies with different replication stressors (Sakasai and Tibbetts, 2008). Similarly, increased γ-H2AX formation in the presence of proteasome inhibitors was specific to FA-treated cells, as this marker of genotoxic stress was formed normally in response to non-DPC DNA damage (Murakawa et al., 2007).

Our experimental findings provide a further support for the proteolysis-based DPC repair mechanism (Quievryn and Zhitkovich, 2000; Zecevic et al., 2010; Wong et al., 2012; Duxin et al., 2014) (Fig. 6). In this model, a movement of replisome helicase along the leading strand is blocked by DPC causing replicative stress and phosphorylation of p53 and H2AX. The recruitment of 20S proteasome causes cleavage of crosslinked proteins, which allows a passage of helicase through the residual DNA–peptide crosslinks that then block a more tightly DNA-associated polymerase. Stalling of DNA polymerase under...
conditions of the ongoing DNA unwinding results in the production of ssDNA gaps in the leading strand, which induces activating phosphorylation of CHK1 by ATR (Zou and Elledge, 2003; Duxin et al., 2014). CHK1 activity plays a well-established pro-survival role in cells experiencing replication stress (Stracker et al., 2009). A lack of specific cellular readouts does not allow us to draw conclusions regarding the role of proteasomes in repair of DPC in the lagging DNA strand. Because the presence of a DPC in the lagging strand blocks DNA synthesis of a single Okazaki fragment (~150 nucleotides long), it has only a very minor effect on the overall replication in comparison to the complete stalling of the replication fork by a DPC in the leading strand (Duxin et al., 2014). Overall, our data demonstrate the importance of proteasome activity in recovery from the primary FA-induced injury, which blocked DNA replication. Under normal conditions, a proliferative pool of cells in tissues in vivo is limited to stem cells and various progenitors, suggesting that these populations are likely to be particularly dependent on the efficient removal of replication-blocking DPC by proteasomes. It is possible that in addition to repair of DPC, proteasomes also eliminate other cellular proteins damaged by FA, which would be beneficial for both dividing and fully differentiating cells.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

**Transparency document**

The Transparency document associated with this article can be found, in the online version.

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