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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.

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 *S*- or *N*-methylated xenobiotics in the liver (IARC, 2006; NTP, 2010). A genetically dangerous site of endogenous FA formation is the release of this reactive chemical in the vicinity of DNA during a continuously occurring oxidative demethylation of histones (Walport et al., 2012). Normal plasma levels of FA in humans are in the range of 30–100 μM (IARC, 2006; NTP, 2010). FA was first classified as a human carcinogen based on the increased risks for nasopharyngeal cancers in occupationally exposed populations (IARC, 2006). Recent epidemiological studies have also found a statistically significant association between occupational inhalation exposures to FA and risks of leukemia (Hauptmann et al., 2009; Schwilk et al., 2010). Unlike nasal cancers, the biological plausibility of leukemia causation is controversial, as there were no detectable FA-DNA adducts in the bone marrow of animals exposed to FA via inhalation (Lu et al., 2010).

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 (Quievryn 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; Taioli et al., 1996; Loeber 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* egg extracts 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% CO_2 humidified atmosphere in 10% serum-supplemented media (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% O_2 and 5% CO_2. Cells were treated with FA in complete growth media for 3 h.

Western blotting. Attached and floating cells were collected and combined for the preparation of protein extracts. Soluble cellular proteins were obtained as described previously (Reynolds and Zhitkovich, 2007). For detection of histones, cellular proteins were solubilized by boiling cells for 10 min in a 2% SDS buffer (2% SDS, 50 mM Tris–HCl pH 6.8, 10% glycerol, 20 mM N-ethylmaleimide) supplemented with protease and phosphatase inhibitors (Thermo Scientific). Solutions were cooled to room temperature and centrifuged at 10,000 ×*g* for 10 min to remove occasional debris. Proteins were separated by SDS-PAGE and electrotransferred to ImmunoBlot PVDF membranes. The following primary antibodies were used: anti-histone H3 phosphorylated at Ser10 (9701), anti-CHK1 phosphorylated at Ser317 (2344) and anti-p53 phosphorylated at Ser15 (9284) from Cell Signaling; anti-γ-tubulin (T6557) was from Sigma. Primary antibodies were typically used at 1:1000 dilutions except for anti-histone H3 antibodies that were diluted 1:5000. Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse IgG (12-349, Millipore; 1:5000 dilution) and goat anti-rabbit IgG (7074, Cell Signaling; 1:2000 dilution). Band intensities were quantified by ImageJ and normalized for loading.

Microscopy. Cells were seeded on human fibronectin-coated coverslips and allowed to attach overnight before treatments with 0–150 μM FA for 3 h in the complete medium. S-phase cells were labeled by incubation with 10 μM 5-ethynyl-2′-deoxyuridine (EdU) for 1 h prior to the addition of FA. After aspiration of media and a rinse with PBS, cells were fixed with ice-cold methanol for 10 min at 4 °C. Next, cells were permeabilized with PBS-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