in vivo *26*: 501-518 (2012)

Amino Acid Chloramine Damage to Proliferating Cell Nuclear Antigen in Mammalian Cells

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Abstract. *Amino acid chloramines (AACLs) are reactive secondary products of activated neutrophils. To understand AACL damage in cell nuclei, we exploited proliferating cell nuclear antigen (PCNA) as a nuclear protein damage reporter, using western blotting and mass spectrometry. Chloramines of proline, arginine, and glycine caused significant damage to PCNA in cells. Chloramines of taurine and histidine caused slight damage to PCNA in cells. Other AACLs caused no PCNA damage in intact cells. Evidence supports a sulfonamide, sulfinamide, or sulfenamide crosslinking mechanism involving cysteine 148 at the PCNA subunit interface, methionine sulfoxide formation as the basis of electrophoretic mobility shifting, and tyrosine and/or methionine residues as the likely targets of AACL damage to the PCNA antibody epitope. An interstitial fluid model experiment showed that physiological amino acids can mediate HOCl damage to PCNA in the presence of proteins that would otherwise completely quench the HOCl. Conclusion: PCNA is a sensitive biomarker of AACL damage in cell nuclei. Arginine chloramine and proline chloramine, or reactive species derived from them, were shown to enter cells and damage PCNA. Amino acids were shown to have at least two different mechanisms for suppressing PCNA damage in cells by their corresponding AACLs. Cysteine 148 was shown to be essential for PCNA subunit crosslinking by AACLs, and a crosslinking mechanism was proposed.*

This work was supported by NIH/NCI CA097107 to RMS, NIH/NCI CA118208, NCI P30CA016058.

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Key Words: Inflammation, HOCl, amino acid chloramines, protein damage, PCNA.

Neutrophils infiltrating infected tissues produce reactive oxygen species (ROS), including nitric oxide, superoxide, hydrogen peroxide, and hypochlorous acid (HOCl) in a respiratory burst that accompanies pathogen ingestion (1, 2). Normal tissue damage from the release of ROS is thought to be the link between inflammation and heart disease, dementia, rheumatoid diseases, cancer, and other diseases of aging (3-6). HOCl, a major inflammatory ROS, is produced from hydrogen peroxide and chloride by neutrophil myeloperoxidase (7). The high reactivity and correspondingly short diffusion distance of HOCl in biological fluids argues strongly against the idea that it might diffuse into the cell nucleus after release from activated neutrophils in the interstitial spaces of inflamed tissues (2, 8).

The reaction of HOCl with amines produces chloramines. Because of their high concentrations in biological fluids and tissues, amino acids are major amine reactants of phagocyte-produced HOCl (9). The resulting amino acid chloramines (AACLs) are much more specific than HOCl in their reactions and very reactive, allowing them to diffuse greater distances in cells and tissues (10, 11). Computer modeling suggests that extracellular proteins in blood plasma will quench most of the HOCl produced by monocytes, leaving little to form AACLs, and that any AACLs formed will also be quenched by extracellular proteins (8). Finally, several reports indicate that only small uncharged AACLs are cell membrane-permeant (12, 13) and capable of entering cells to react with cytoplasmic biomolecules. However, there is also evidence that other AACLs can enter cells to react with glutathione (GSH) and other cytoplasmic probes and targets (14-16). AACLs are also reported to be mediators of HOCl toxicity leading to apoptotic cell death (17, 18).

The aim of our studies was to experimentally test the idea that extracellular proteins can protect cellular targets from HOCl damage in the presence of amino acids and to determine which AACLs could mediate this damage. Using proliferating cell nuclear antigen (PCNA) as a sensitive reporter of protein damage in cell nuclei, we have tested

serum components and AACLs for their ability to mediate or suppress HOCl damage in the cell nucleus.

Materials and Methods

Reagents. HOCl, all amino acids, histamine dihydrochloride, L-carnosine, 5,5-dimethyl-pyrroline *N*-oxide (DMPO), *N*-acetyl L-cysteine (NAC), GSH, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 3,3',5,5'-tetramethylbenzidine (TMB) were from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were dimethylformamide (DMF) (JT Baker, Phillipsburg, NJ, USA), analytical grade sodium iodide from Mallinckrodt Inc (Paris, KY, USA), and purified recombinant human PCNA from RayBiotech (Norcross, GA, USA). Molecular weight markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were Precision Plus Protein Dual color standards (BioRad, Hercules, CA, USA). Broad spectrum protease inhibitor cocktail (Complete Mini) was from Roche (Indianapolis, IN, USA). Purified ovalbumin grade V and hydrogen peroxide (H_2O_2) were from Sigma.

Cells and cell culture. CV-1 African green monkey kidney cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in minimal essential medium (MEM; Invitrogen, Carlsbad, CA, USA) containing 10% calf serum (Invitrogen) and 14 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; Sigma) (MEM/HEPES). Simian virus 40-transformed human fibroblasts GM637 and GM639 were obtained from the Human Genetic Mutant Repository (Coriell Institute, Camden, NJ, USA) and were maintained in MEM and DMEM respectively, with 10% fetal bovine serum (Invitrogen). *Schizosaccharomyces pombe* cell extract was a gift of Jian-Qiu Wu and Ran Zhao. *Drosophila melanogaster* cells were a gift of Amanda Simcox.

Preparation of AACLs and treatment of cells and cell lysates. HOCl is not stable (19), so it was assayed frequently and the concentration was adjusted immediately before preparation of AACLs (20). AACLs were measured using TMB reagent and spectrophotometry (21). AACLs were prepared by vortex mixing of HOCl with the amino acids in phosphate buffered saline (PBS). To ensure complete consumption of HOCl, an excess of the amino acid was used for all amino acids except proline. Specifically, 5 mM amino acid (final concentration) was mixed with 3 mM HOCl (final concentration) by vortexing. Proline chloramine (Pro-CL, 3 mM) was prepared by mixing equimolar concentrations of proline and HOCl. Unless otherwise indicated, concentrations of AACLs are 3 mM. Individual AACLs are indicated by the standard three letter amino acid abbreviation with '-CL' as in Pro-CL above. Lower concentrations of AACLs were prepared by rapid dilution with vortex mixing in PBS. For glycine, arginine and proline, the resulting chloramines and dichloramines were measured within one minute of mixing (22). To increase the ratio of glycine monochloramine to glycine dichloramine, glycine chloramine (Gly-CL) was prepared at pH 13 followed by adjustment of the pH to 7.4 before addition to cells (22). The levels of monochloramine and dichloramine were determined spectrophotometrically by the Vierodt's method (22). Guanidino chloramine formed on the arginine side chain was measured as described elsewhere (22). In experiments with cells or hypotonic cell lysates, the AACLs were prepared as described above and immediately added to the cells in drained plates or cell lysates. Hypotonic cell lysates were prepared by incubation of the cells in deionized water for 10 min at room temperature, removal of the water, and lysis of the cells by vigorous shearing with a rubber policeman.

Western blot analysis. Intact cells and hypotonic cell lysates were incubated with AACLs for 10 min at 37˚C. Treated intact cells in 35-mm culture plates were lysed with 90 μl of SDS lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol). For chloramine-treated cell lysates, 60 μl of SDS cell lysis buffer was added. Lysates were heated for 10 min at 100˚C and centrifuged to remove any insoluble materials. Protein samples were heated in Laemmli sample buffer containing 100 mM dithiothreitol (DTT) for 5 min, followed by 10% SDS-PAGE (40 μg protein per lane). The proteins were transferred to nitrocellulose membranes (Protran; PerkinElmer, Shelton, CT, USA) by semi-dry transfer. The membranes were then blocked with 5% non-fat dry milk solution (0.5% for methionine sulfoxide western blots) in tris-buffered saline (TBS; 0.05 M, Tris; 0.15 M, NaCl; 0.05%, Tween; pH 7.4) at room temperature for 1 h, incubated with primary antibody overnight at 4˚C. They were then rinsed with TBS followed by incubation with horseradish peroxidase-conjugated goat anti mouse secondary antibody (BioRad) for 1 h at room temperature, rinsed again with TBS, and imaged with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and X-ray film. Primary antibodies were monoclonal mouse anti-human PCNA, PC10, (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or mouse anti-SV40 large T antigen PAb101 (Lab Vision Products, Fremont, CA, USA). Western blots were probed separately for actin to confirm accurate loading (anti-actin monoclonal AC-40; Sigma). Antibody to methionine sulfoxide was from Novus Biologicals (Littleton, CO, USA). The apparent molecular weights of PCNA forms induced by AACLs were estimated by comparison to molecular weight markers.

Mass spectrometry. Experiments were performed on a Micromass Q-TOF-II (Micromass, Wythenshawe, UK) mass spectrometer equipped with electrospray source, operated in positive ion mode. Liquid chromatography-mass spectrometry (LC-MS) analysis was acquired at m/z 100-1000 with a 1 s integration time. Collision induced dissociation was used for the MS/MS fragmentation. Q1 was set to pass ions from m/z 644.152 and the collision energy was ramped until acceptable parent ion fragments were obtained.

Identification of PCNA oligomers. PCNA, a circular homotrimer, is dissociated into monomers in SDS-PAGE. Protein crosslinking agents produce denaturant-stable PCNA oligomers, including PCNA dimers, trimers, double trimers, as well as numerous weak PCNA-positive minor bands migrating at positions corresponding to molecular weights from >154 to ~60 kDa (23-25). The minor bands include crosslinked PCNA dimers. The relative intensities of PCNA oligomer bands on western blots are typically: monomer >> trimer >> double trimer >> dimer >> minor bands. Optimal detection or visualization of any specific PCNA oligomer is a function of the level of PCNA crosslinking and the fluorographic exposure time of the western blot. Their identification is based on the very high sensitivity and specificity of the PC10 antibody and the accurate measurement of their molecular weights by SDS-PAGE. When low levels of specific oligomers are present, long western blot exposures are necessary to detect them, resulting in overexposure of the PCNA monomer band.

Results

Serum components strongly affect HOCl damage to nuclear PCNA. HOCl treatment of cells in PBS causes cell death by necrosis, while HOCl treatment of cells in the presence of amino acid-containing serum-free media or complete media causes cell death by apoptosis (26). To gain insights into this significant difference, we compared PBS and serum-free media for HOCl damage to PCNA in cells. In PBS, HOCl caused a concentration-dependent loss of the western blot signal (Figure 1A). At higher HOCl concentrations, distinct high molecular weight PCNA-positive bands were detected with long fluorographic exposures of the western blot that overexposed the PCNA monomer band. These bands are identified as PCNA oligomers caused by covalent subunit crosslinking (see Materials and Methods). Pronounced loss of western blot signal necessitated a long film exposure to demonstrate the presence of PCNA oligomers, resulting in overexposure of the monomer band in the untreated lane. When the same experiment was carried out with serum-free medium, western blot signal was unaffected, and an HOCl concentration-dependent formation of the PCNA trimer was seen, along with mobility shifting of the PCNA monomer (Figure 1B). The presence of amino acids dramatically changed the effects of HOCl, consistent with the idea that they can mediate its damage but with very different reactivity.

Interstitial fluid model. HOCl is so highly reactive that it is consumed within ~0.4-0.6 μm of its point of production in biological fluids, and so it is not able to penetrate cells significantly (8). However, reaction of HOCl with biological fluids produces long lasting (~16 h at 37˚C) chloramines with high antimicrobial activity (27, 28). A computational model of HOCl reaction with blood plasma components has indicated that for plasma HOCl concentrations up to 1 mM, approximately 99% of the HOCl would be consumed by plasma proteins, antioxidants, thiocyanate, and free amino acids. About 1% of the HOCl would produce AACLs which would also be quenched by plasma proteins (8), thus protecting intracellular proteins from HOCl. Neutrophils in the interstitium of inflamed tissue can generate concentrations of 25-50 mM HOCl per hour (29). HOCl does not accumulate to those levels, but reacts instantly with proteins, amino acids and other molecules. Using the value of 74-177 fmol of HOCl produced per zymosan-activated neutrophil (30, 31) and 2.5×10^8 neutrophils per ml of interstitial fluid in inflamed tissue (28), we calculate ~20-44 mM HOCl produced per hour in the interstitium of inflamed tissue, in good agreement with others (29). AACLs produced in these reactions can diffuse over several cell diameters (8). The most relevant biological fluid for tissue inflammation is the interstitial fluid between cells of tissues, not blood plasma. Due to filtering effects, the protein concentration of interstitial fluid is only ~40-45% of that of blood plasma (32, 33). Since proteins are the major scavengers of HOCl in the plasma, decreased protein concentration could result in more extensive reaction of neutrophil-derived HOCl with free amino acids.

To determine if the interstitial fluid proteins could react with all or most of the HOCl produced by activated monocytes, we carried out an experiment with solutions approximating the protein and amino acid composition of the interstitial fluid. Amino acid concentrations in the interstitial fluid (34) are essentially the same as in blood plasma (35), and the total protein concentration in interstitial fluid is about 40% of that in blood plasma. To mimic the protein concentration of interstitial fluid (33, 36), calf serum was dialyzed to remove low molecular weight components of blood serum such as amino acids, ammonia, and thiocyanate, then diluted to reduce the protein concentration to 50% of the plasma concentration. This interstitial fluid protein mimic was brought to 25 mM HOCl by rapid addition of HOCl with vortex mixing for 2 s, and was then applied to confluent CV-1 for 10 min at 37˚C. No PCNA covalent oligomers were detected (Figure 1C) even after overexposure of the western blot (Figure 1C, lower panel). Thus, serum proteins completely quenched the HOCl and protected PCNA in the cells, from damage by HOCl. To determine if AACLs can mediate HOCl damage in the cell nucleus in the presence of serum proteins, the experiment was repeated as in Figure 1C, but with the major serum amino acids added to the levels found in blood and interstitium. To focus the experiment on the ability of proteins to compete with amino acids for HOCl, ammonia, and other low molecular weight blood components were not included. After treatment of cells with HOCl (up to 25 mM HOCl, Figure 1D, top), a PCNA oligomer migrating at the position of the PCNA trimer was easily detected. Long film exposure of a separate replicate western blot, detected the PCNA trimer in samples treated with 3 mM and 6 mM HOCl (Figure 1D, lower image) and resulted in great overexposure of the PCNA monomer in all lanes and the PCNA oligomers in the 25 mM HOCl lane. PCNA-positive bands at positions above and below the position of the PCNA trimer are covalently crosslinked PCNA oligomers that have been well-characterized (see Materials and Methods).

PCNA damage by individual AACLs is affected by cell membrane integrity. We studied each of the AACLs separately for their ability to cause PCNA damage in intact cells or cells with disrupted membranes. Freshly prepared AACLs were applied to the cells within a minute of preparation. The AACLs were stable for at least 30 min, as determined by the TMB assay. Pro-CL (91.4% proline monochloramine, 8.6% proline dichloramine) caused a dose-dependent increase in covalent oligomerization of PCNA, forming the 93 kDa PCNA crosslinked trimer in intact cells

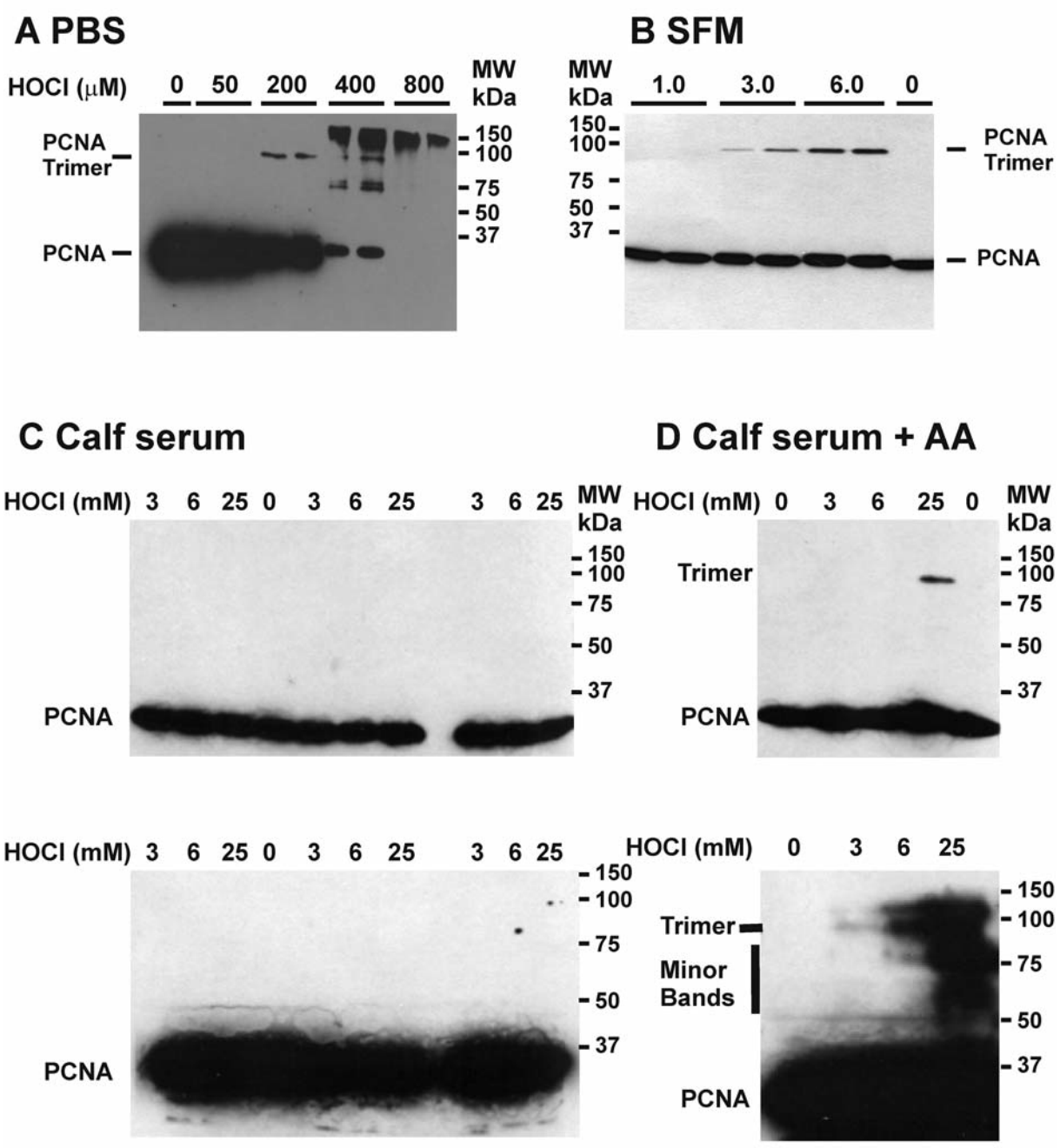


Figure 1. *Serum components greatly modify HOCl damage to cellular PCNA. Western blots were performed to detect PCNA subunit crosslinking caused by exposure of CV-1 cells to HOCl in PBS, serum-free medium, or model interstitial fluid. A: CV-1 cells were incubated with increasing concentrations of HOCl (0-0.8 mM) in PBS for 10 min at 37˚C before lysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and anti-PCNA western blotting. B: CV-1 cells were incubated with increasing concentrations of HOCl (1.0-6 mM) in serum-free MEM/HEPES medium (10 min at 37˚C), then lysed and processed for western blotting as in A. C: Effective scavenging of HOCl by serum proteins at the protein concentration of interstitial fluid. After removal of medium and PBS rinses of the cultured cells, HOCl was rapidly added with vortexing (2 s) to PBS containing dialyzed calf serum (4 g protein/100 ml) so that the final concentrations of HOCl were 3 mM, 6 mM, or 25 mM (each in duplicate). The cell layer was covered with the HOCl-treated protein solution. The plates were placed at 37˚C for 10 min, the medium was then removed and the cells were lysed, and processed by SDS-PAGE and western blotting. Anti-PCNA western blot film exposures are shown for 1 min (top), and 30 min for a replicate experiment (lower). D: Interstitial fluid mimic experiment with amino acids at physiological concentrations. The experiment was done as described above in C, using PBS and dialyzed calf serum but with the 20 common amino acids added to the levels found in human plasma (35), since these levels do not differ significantly from those of the interstitium or lymph (34). The anti-PCNA western blots are shown for a 1 min film exposure (top), and a 30 min film exposure of a replicate experiment (lower).*

(Figure 2A). Arginine chloramine (Arg-CL, 76.5% arginine alpha-monochloramine, 23.5% arginine alpha-dichloramine, 0% guanidino-chloramine) and Gly-CL (82.6% glycine monochloramine, 17.4% glycine dichloramine) caused covalent oligomerization of PCNA in cells comparable to that caused by Pro-CL (Figure 2B). Long fluorographic exposures (~30 min) were able to detect covalently crosslinked PCNA in cells treated with 0.4 mM Gly-CL or Arg-CL (Figure 3A).

In intact cells, taurine chloramine (Tau-CL) caused very weak PCNA crosslinking that was detectable only by long film exposure of the 93-kDa region of the western blot (Figure 2B, lower). To rule out the possibility that the weak PCNA crosslinking in Tau-CL-treated cells might be due to traces of Tau-CL remaining on the cell surface after rinsing, we carried out a chloramine scavenging experiment in which the rinses following Tau-CL treatment included NAC to scavenge any Tau-CL that might be loosely associated with the outer cell membrane and which might be released after the hypotonic lysis. Incubation with NAC did not affect PCNA crosslinking in cells by Tau-CL (Figure 3B). Histidine chloramine (His-CL) also caused weak PCNA crosslinking in cells, with the PCNA trimer band being visible only with long film exposure (Figure 4A).

PCNA crosslinking by AACLs was associated with a decrease in the electrophoretic mobility of the PCNA monomer as seen in Figure 2B for Gly-CL and Arg-CL. Oxidation of methionine to methionine sulfoxide has been shown to cause electrophoretic mobility shifts for other proteins (37-39), and chloramines oxidize methionine to methionine sulfoxide, but not to methionine sulfone (22). Treatment of proteins with acidified H_2O_2 selectively oxidizes methionine residues to methionine sulfoxide (40), and this treatment reproducibly caused the electrophoretic mobility shift for purified recombinant human PCNA (Figure 4D). Western blotting with anti-methionine sulfoxide antibody (41) confirmed that acidified H_2O_2 and Gly-CL, both produce methionine sulfoxide on ovalbumin (Figure 4C).

In intact cells, the chloramines of the other common amino acids (Ala, Asn, Asp, Glu, Gln, Ile, Leu, Lys, Phe, Ser, Thr, Trp, Tyr, Val) caused no detectable crosslinking or electrophoretic mobility shifting of PCNA. The lack of PCNA damage in cells treated with serine chloramine (Ser-CL) or lysine chloramine (Lys-CL) is shown in Figure 2B. Reaction of cysteine and methionine with HOCl (molar ratio HOCl: amino acid=0.6) did not produce chloramines, as determined by the TMB assay.

Molar ratio of amino acid monochloramine to dichloramine. Gly-CL prepared at pH 7.4 for our experiments with PCNA crosslinking in CV-1 cells was 82.6% glycine monochloramine and 17.4% glycine dichloramine (dichloramine: monochloramine ratio = 0.21). To test the possible role of dichloramines in PCNA crosslinking, Gly-CL was synthesized at pH 13, followed by adjustment of the pH to 7.4 before addition to cells (22), resulting in a dichloramine: monochloramine ratio of 0.13 (88.4% glycine monochloramine, 11.6% glycine dichloramine). No difference in PCNA crosslinking was detected as a result of this 33% decrease in the percentage of the dichloramine, suggesting that the dichloramine was not contributing significantly to PCNA crosslinking by Gly-CL (data not shown).

Disruption of cell membranes markedly changes AACL damage to PCNA. To explore the role of membrane permeability and/or transport, we studied damage to PCNA in hypotonic cell lysates in which cell membranes were mechanically disrupted. All of the intact cell contents were still present, without added reagents, and the only difference from intact cells was a slight dilution from hypotonic swelling and loss of membrane integrity. Although Lys-CL and Ser-CL did not cause PCNA crosslinking in intact cells, they caused marked PCNA crosslinking when added to disrupted cells (Figure 2C). Significant PCNA crosslinking was accompanied by a distinct electrophoretic mobility shift of the PCNA monomer, as seen with AACLs causing efficient PCNA crosslinking in intact cells. Thr-CL, Glu-CL, (Figure 2D), and Gln-CL, (Figure 4A) were also found to cause PCNA crosslinking in disrupted cells. The PCNA crosslinking in cell lysates, but not in intact cells, suggests that these AACLs are not cell membrane permeant.

PCNA crosslinking by Pro-CL in cells or cell lysates, produced a pattern of high molecular weight PCNA oligomers identical to those caused by glutaraldehyde or singlet oxygen crosslinking of PCNA subunits (23, 42, 43). Long film exposures of the Pro-CL western blots revealed minor bands that include the PCNA dimer (Figure 2D).

Peptide backbone cleavages. PCNA peptide backbone cleavage, indicated by weak PCNA antibody-positive bands migrating faster than the PCNA monomer, occurred in cell lysate experiments for Thr-CL, Glu-CL (both in Figure 2D), and His-CL (Figure 4A). Although His-CL reproducibly caused weak PCNA crosslinking in cells, it was not detected in the cell lysate experiments, probably due to peptide fragmentation and a loss of western blot signal from epitope damage (discussed below). The patterns of peptide backbone cleavage were different, and reproducible for each of these chloramines. Two independent repetitions for Thr-CL and Glu-CL are shown in Figure 2D. In cell lysates, His-CL produced a striking pattern of PCNA backbone cleavage consisting of two strong bands, one at about the position of the PCNA monomer and one just below it, suggesting a single high-efficiency backbone cleavage site in the PCNA monomer. This unique pattern of backbone cleavage was seen in seven independent replicate experiments (three

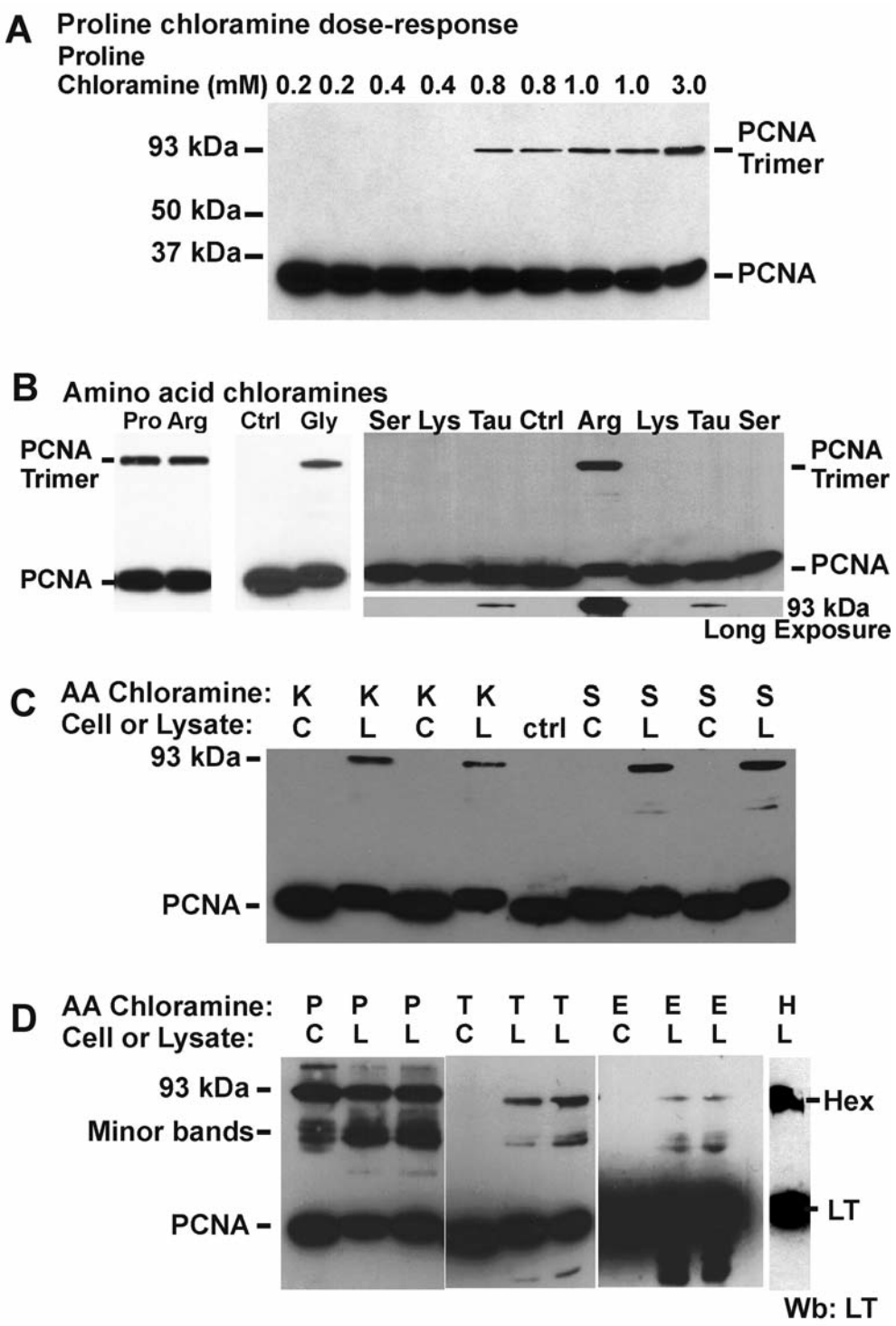


Figure 2. *Proliferating cell nuclear antigen (PCNA) damage by individual amino acid chloramines (AACL). AACL-induced PCNA crosslinking in cells and hypotonic cell lysates. AACLs were prepared and used to treat cells and cell lysates as described in the Materials and Methods, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and anti-PCNA western blotting. AACLs are indicated by either three letter or one letter standard amino acid abbreviations. Treatment of cells ('C') in C-E or cell lysates ('L') was 3 mM AACL, 10 min, 37˚C. A: PCNA crosslinking dose-response for treatment of CV-1 cells with increasing concentrations of Pro-CL in PBS (10 min, 37˚C). B: Effect of Pro-CL, Arg-CL, Gly-CL, Ser-CL, and Tau-CL on PCNA in CV-1 cells. C: Effect of Lys-CL and Ser-CL on PCNA in intact cells or cell lysates. (D) Effect of Thr-CL and Glu-CL on PCNA in cells or lysates (right). His-CL effects on SV40 large T antigen in a GM639 cell lysate: LT, large T antigen monomer; Hex, large T antigen crosslinked hexamer.*

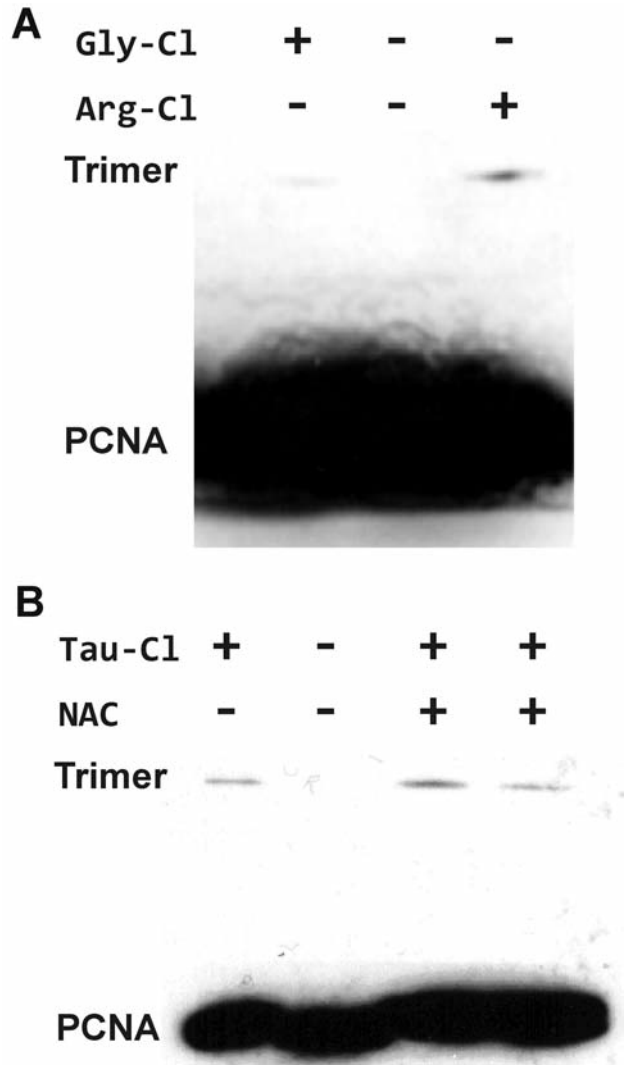


Figure 3. *Proliferating cell nuclear antigen (PCNA) crosslinking in cells by 0.4 mM Gly-CL, 0.4 M Arg-CL and 3 mM Tau-CL. A: Weak PCNA trimer bands at the 93 kDa position revealed by long western blot exposure following treatment of CV-1 cells with Gly-CL and Arg-CL. B: Tau-CL treatment of cells also causes PCNA crosslinking that is not prevented by N-acetyl L-cysteine (NAC) treatment of the cells before lysis.*

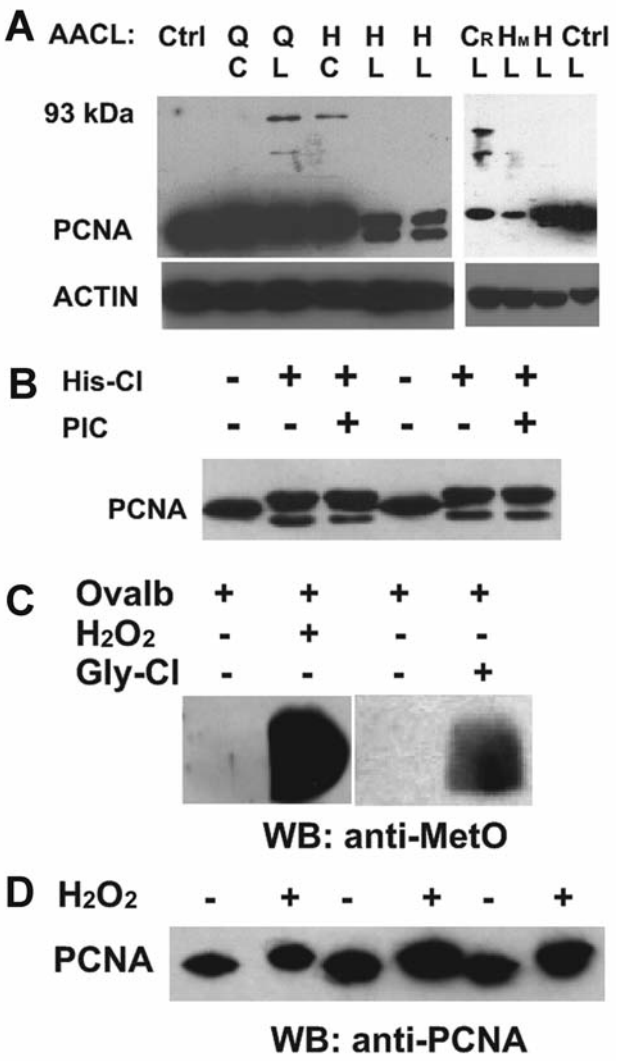


Figure 4. *Proliferating cell nuclear antigen (PCNA) damage by imidazole chloramines, and methionine sulfoxide experiments. A: Treatment of CV-1 cells and cell lysates by Gln-CL (Q) and His-CL (H left). Treatment of cell lysates by carnosine chloramine (C_R), histamine chloramine (H_M), and His-CL (right). Lanes were loaded with 20 μg of protein; film exposure 30 min. B: Proteinase inhibition does not affect the His-CL PCNA peptide backbone cleavages in cell lysates. Cell lysates were treated with His-Cl, with or without protease inhibitor cocktail (PIC). Each experimental lane is a separate experiment. To prevent overexposure, the control lanes were each loaded with 60 μg of protein, and experimental lanes were loaded with 100 μg of protein. Film exposure was performed for 10 min. C: Anti-methionine sulfoxide western blotting of ovalbumin treated with acidified hydrogen peroxide (H_2O_2) or Gly-CL (Gly-Cl). D: Electrophoretic mobility shifting of purified recombinant human PCNA monomer band caused by acidified H_2O_2 treatment (+) and controls not treated (–).*

shown in Figure 4A) and four others in the following protease experiment (Figure 4B).

HOCl damage to protein backbone residues can cause tertiary structural changes that make the proteins more susceptible to proteases (44). To test for the possible role of a protease in the single unique PCNA backbone cleavage caused by His-CL in hypotonic cell lysates, we carried out an experiment with a PIC that inhibits a broad spectrum of proteases, including serine, cysteine and metalloproteases, as well as calpains (Figure 4B). The PIC had no detectable effect on the backbone cleavage caused by His-CL.

There was a noticeable loss of PCNA detection in cell lysate experiments with His-CL relative to untreated controls (Figure 4A), suggesting chemical damage to the epitope recognized by the anti-PCNA antibody, PC-10. In Figure 4B, the controls (no

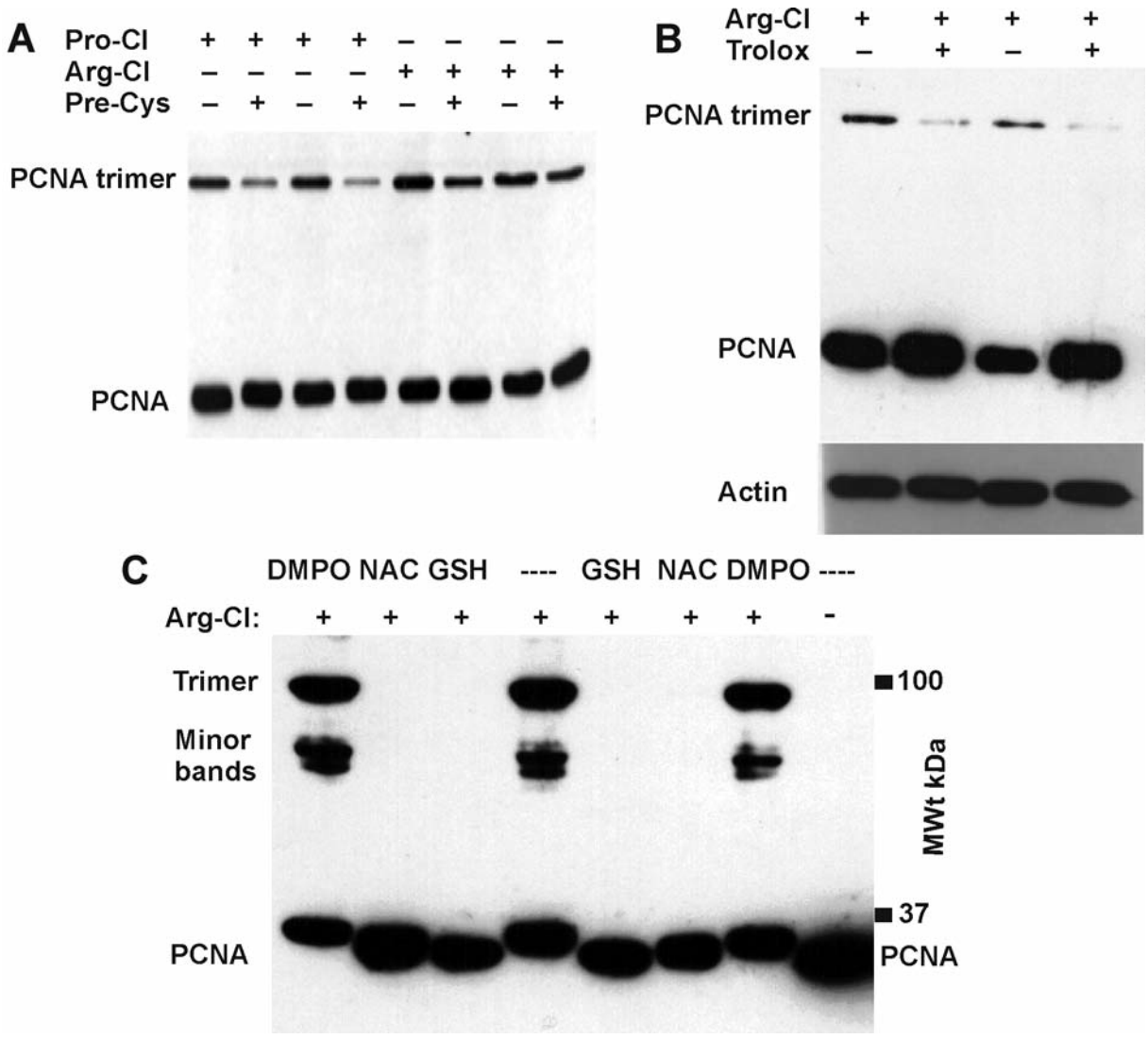


Figure 5. *Antioxidants effects on proliferating cell nuclear antigen (PCNA) damage by amino acid chloramines (AACLs). A: Effect of cysteine pre-treatment on PCNA crosslinking caused by Pro-CL and Arg-CL. CV-1 cells were incubated with 50 mM cysteine for 2 h in phosphate buffered saline (PBS), 37˚C. The cysteine solution was then removed and cells were rinsed once with fresh PBS before incubation with 3 mM Pro-CL or Arg-CL (10 min, 37˚C) then analyzed by anti-PCNA western blotting. B: Effect of pre-treatment with Trolox on PCNA crosslinking caused by treatment with 3 mM Arg-CL. CV-1 cells were incubated with 20 mM Trolox for 30 min at 37˚C before incubation with 3 mM Arg-CL (10 min, 37˚C). C: Effect of CV-1 cell pre-treatment with DMPO, NAC, or GSH (50 mM, 30 min, in PBS) on PCNA crosslinking by 3 mM Arg-CL. The control, untreated by Arg-CL or antioxidants is in the far right lane.*

His-CL, no PIC) were loaded at 60% of the protein concentration loaded for the experimental samples, in order to prevent the overexposure of the PCNA monomer band as seen in the untreated controls in Figure 4A. Since histidine can form an imidazole chloramine that is more reactive than an alpha-amino chloramine (9), we tested carnosine chloramine (C_R) and histamine chloramine (H_M). Both of these imidazole chloramines caused marked loss of PCNA detection by western blotting with PC-10 (Figure 4A, C_R and H_M). Experiments with His-CL and SV40 large T-antigen (Figure 2D, LT) showed covalent crosslinking of the large T-antigen hexamer, similar to that caused by singlet oxygen or glutaraldehyde (23), but no detectable loss of signal. There was also no detectable loss of signal for the actin internal loading control (Figure 4A).

Protection of PCNA from damage caused by AACLs. Cysteine pretreatment of cells reduced PCNA crosslinking by Pro-CL and Arg-CL (Figure 5A). Trolox, a water soluble membrane permeant vitamin E analog and free radical trap, reduced PCNA

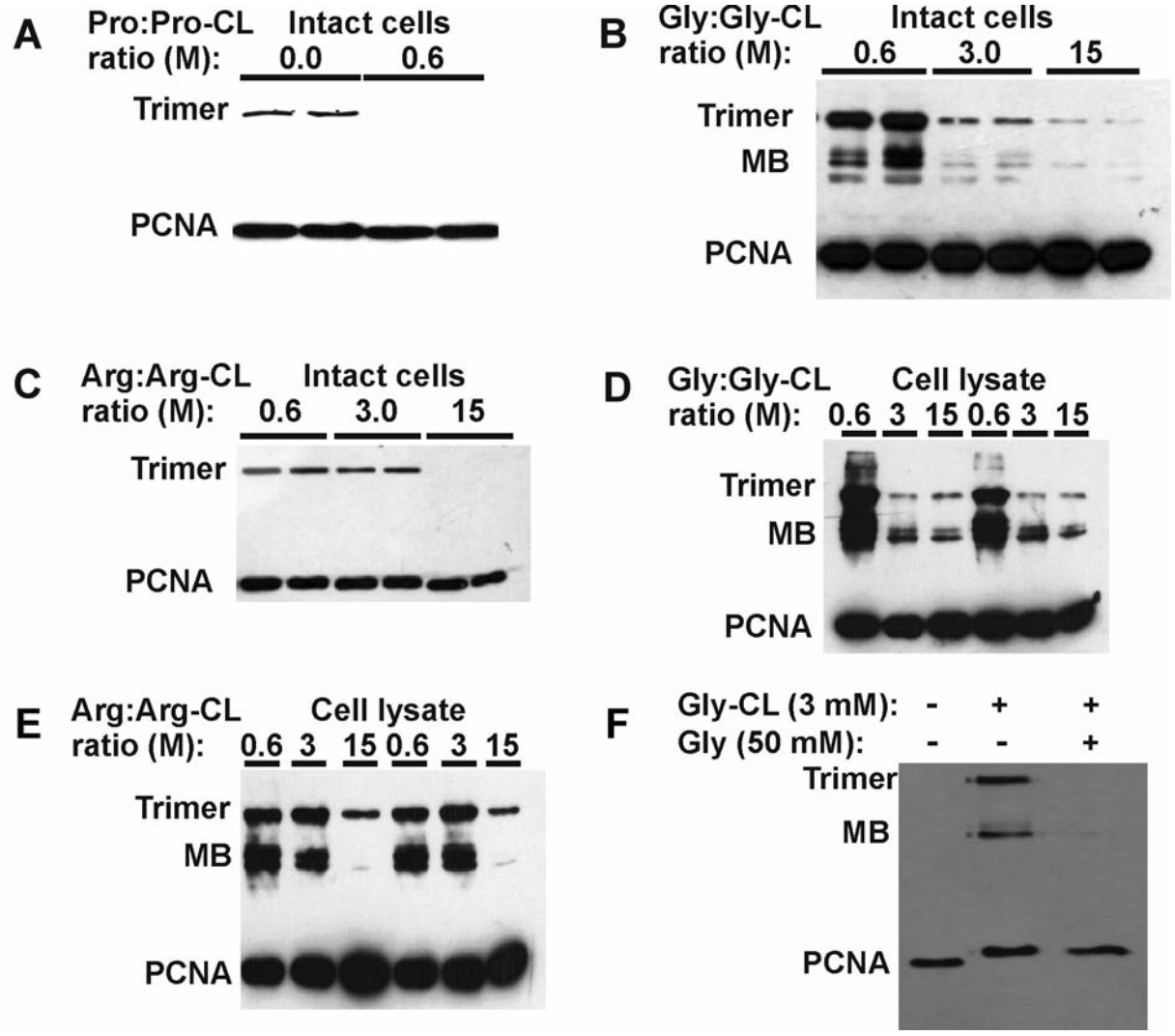


Figure 6. *Excess amino acids suppress proliferating cell nuclear antigen (PCNA) crosslinking by amino acid chloramines (AACLs) in CV-1 cells and lysates. AACLs were prepared, as described, to produce mixtures of 3 mM AACL and 2 mM amino acid (molar ratio M=0.6) as well as other molar ratios. A: Excess proline prevents PCNA crosslinking in cells by Pro-CL. B: Excess glycine reduces PCNA crosslinking in cells by Gly-CL. C: Excess arginine reduces PCNA crosslinking in cells by Arg-CL. D: Excess glycine reduces PCNA crosslinking in cell lysates. E: Excess arginine reduces PCNA crosslinking by Arg-CL in cell lysates. F: Gly-CL crosslinking of purified recombinant PCNA is suppressed by excess glycine. Gly-CL (3 mM) was prepared by mixing 3 mM HOCl with either 5 mM glycine (leaving 2 mM glycine) or 55 mM glycine (leaving 52 mM glycine) and was used immediately to treat 30 ng of purified recombinant PCNA (37°C, 10 min). The reaction was terminated by addition of cysteine (37.5 mM final concentration). Samples were then processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and anti-PCNA western blotting.*

crosslinking caused by Arg-CL (Figure 5B). Trolox treatment also resulted in an increase in the PCNA monomer band in Arg-CL-treated cells, and did not reverse the electrophoretic mobility shift of the PCNA monomer caused by Arg-CL treatment. Pretreatment of the cells with DMPO, a free radical probe, had no effect on PCNA damage caused by Arg-CL, but pretreatment with NAC and GSH prevented PCNA crosslinking and reversed the electrophoretic mobility shift caused by Arg-CL (Figure 5C). All of the treatments with Arg-CL showed a reduced amount of PCNA monomer relative to the untreated control, consistent with damage to the antibody epitope (Figure 5C).

Suppression of PCNA crosslinking by excess amino acids. For Pro-CL, Gly-CL, and Arg-CL, the presence of an excess of the corresponding amino acid-reduced PCNA crosslinking in cells (Figure 6A-C). The same inhibition of PCNA crosslinking was observed in hypotonic cell lysates for Gly-CL and Arg-CL (Figure 6D-E). In both intact cells and cell lysates, the excess

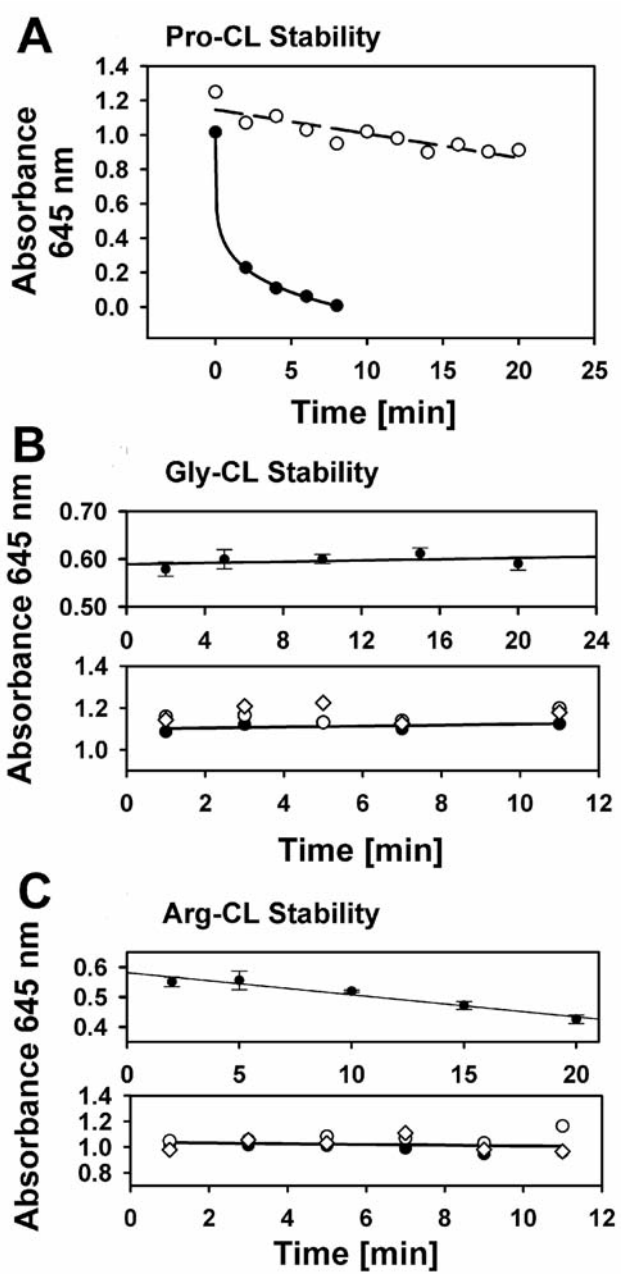


Figure 7. *Excess proline destroys Pro-CL, but Arg-CL and Gly-CLs were stable in the presence of excess of the corresponding amino acid. Chloramines were prepared either in the absence or in the presence of excess amino acids. A: Stability of Pro-CL (3 mM) either in the absence of excess proline (○) or in excess proline (0.6 molar ratio Pro:Pro-CL, ●). B: Upper graph: Stability of Gly-CL (1.5 mM) in the presence of excess glycine [Gly:Gly-CL molar ratio 1, error bars SE, n=3). Lower graph: Stability of Gly-CL (3 mM) in the presence of excess glycine (Gly:Gly CL molar ratios, 0.6 (●), 3 (○) or 15 (◊)]. C: Upper graph: Stability of Arg-CL (1.5 mM) in the presence of excess arginine (Arg:Arg-CL molar ratio 1, error bars SE, n=3). Lower graph: Stability of Arg-CL (3 mM) in the presence of excess arginine [Arg:Arg-CL molar ratios 0.6 (●), 3 (○) or 15 (◊)]. Chloramine concentrations were monitored spectrophotometrically after their preparation at the indicated time points using the TMB reagent (see Materials and Methods).*

amino acids suppressed PCNA crosslinking by the corresponding AACLs without reversing the mobility shift of the PCNA monomer that was also caused by AACLs. An excess of glycine suppressed Gly-CL crosslinking of the subunits of purified recombinant PCNA (Figure 6F).

To understand the suppression of AACL crosslinking of PCNA by excess amino acids, we explored the possible effects of amino acids on the stability of their respective chloramines. Excess proline destabilized Pro-CL (Figure 7A). Pro-CL was only stable when it was prepared by reacting equimolar concentrations of proline and HOCl. Gly-CL and Arg-CL were both stable in the presence of an excess of the corresponding amino acid (Figure 7B and C).

Nature of the covalent PCNA subunit crosslink caused by AACLs. Covalent crosslinks between subunits of the PCNA trimer must occur at the PCNA subunit interface which involves tight packing of the two α-helices, αB1 (residues 72-79) and αA2 (residues141-153) with one another, and the two β-sheets βL1 (residues 109-117) and βD2 (residues 175-183) (45). To test the possible involvement of specific amino acid residues in covalent crosslinking of PCNA subunits, we compared human, *Drosophila melanogaster*, and *Saccharomyces pombe* PCNA for covalent crosslinking by Gly-CL. Histidine in proteins is a target of HOCl and can form a highly reactive imidazole chloramine (9, 10, 46). PCNA His153, located in the αA2 helix, was shown to be involved in singlet oxygen crosslinking of PCNA subunits (47), and it has been suggested that histidine residues in proteins may be involved in protein crosslinking by HOCl (46). Gly-CL was able to crosslink both human PCNA, containing His153, and *Drosophila* PCNA, lacking His153 (Figure 8). Gly-CL was not able to crosslink *S. pombe* PCNA (Figure 8), which uniquely lacks the Cys148 that is present in the αA2 helix of the human and *Drosophila* PCNA sequences. *S. pombe* PCNA also did not show the PCNA monomer band shift that is seen with human, monkey, and *Drosophila* PCNA (Figure 8, and three other independent experiments not shown).

To explore possible crosslink mechanisms involving cysteine residues, we employed the reaction of Gly-CL with GSH as a model system, as has been done with HOCl (48). Since GSH has a sulfhydryl group and a free amino group, we consider the reaction a model for possible chloramine-induced covalent crosslinks between cysteine and lysine residues in proteins and protein subunits. We used LC-MS to investigate the products formed by reaction of Gly-CL with GSH (Figure 9). Masses corresponding to GSH (ES+=308.11 Da), the GSH internal sulfonamide (ES+=338.12 Da), GSH sulfonic acid (ES+=356.11), GSH disulfide (ES+=613.20 Da), GSH thiosulfonate (ES+=645.22 Da), and a GSH-glycine mixed sulfonamide (ES+=412.09 Da) were detected. The GSH thiosulfonate was confirmed by MS/MS since the GSH intermolecular sulfonamide has the same atomic composition and predicted accurate mass.

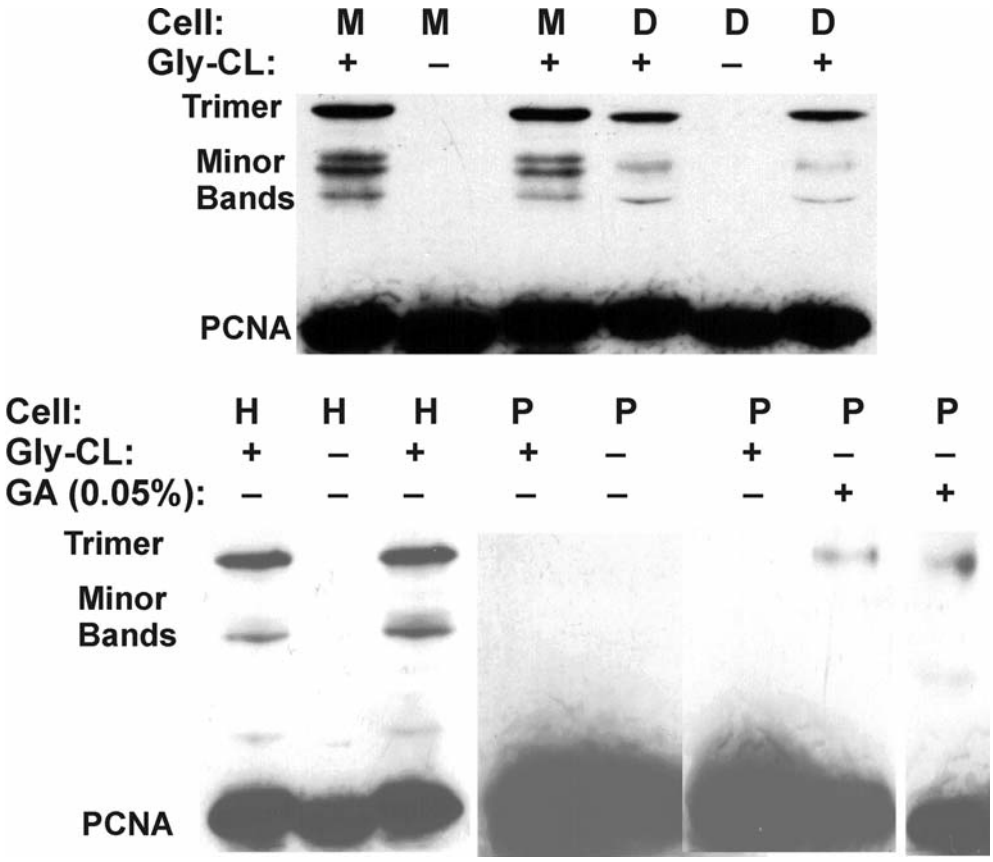


Figure 8. *Gly-CL crosslinking of proliferating cell nuclear antigen (PCNA) in cells from different species. Human GM637 cells (H), Saccharomyces pombe cells (P), Drosophila melanogaster cells (D), and African green monkey cells (M) were either left untreated as controls (–), or were treated with Gly-CL (+). PCNA covalent oligomers are trimers and minor bands. Glutaraldehyde (GA) crosslinking controls are shown for the S. pombe extract (two independent experiments) to show that intact PCNA trimers were present and can be crosslinked. Two independent treatments with Gly-CL are also shown.*

Discussion

AACLs formed in a biological fluid mimicking interstitial fluid can damage nuclear proteins in mammalian cells. We have shown that physiological concentrations of amino acids dramatically change the pattern of HOCl damage to PCNA in mammalian cells (Figure 1A and B), consistent with literature indicating different modes of cell death for cells exposed to HOCl and AACLs, and with the different reactivities of HOCl and AACLs. The concentrations of HOCl used were far higher than those expected to be reached in inflamed tissue, yet there was a dramatic effect by amino acids in cell culture medium. A computational model based on the albumin concentration of the blood plasma predicted that proteins would completely quench HOCl produced by neutrophils to prevent damage to cytoplasmic proteins. In an experiment with serum proteins present at about 50% of the level in blood plasma (mimicking the protein concentration of interstitial fluid), proteins were still able to completely quench HOCl and protect nuclear PCNA from damage (Figure 1C). However, the addition of amino acids to this model interstitial fluid at their plasma and interstitial fluid physiological concentration resulted in clear PCNA covalent crosslinking (Figure 1D). Thus, the presence of amino acids resulted in ROS reaching the cell nucleus, and AACLs are the most likely mediators of this nuclear protein damage.

Cell-permeant AACLs. Studies of cellular GSH oxidation and glyceraldehyde 3-phosphate dehydrogenase inactivation have indicated that Gly-CL, ammonia chloramine, and histamine chloramine are cell membrane permeable, and that Tau-CL is membrane impermeable (12, 13). Larger AACLs and charged AACLs are thought to be membrane impermeable (12). We found that PCNA damage in cells treated with Pro-CL and Arg-CL was surprisingly robust and comparable to that caused by Gly-CL. To our knowledge, the present study

is the first to find high cell permeability for Pro-CL. PCNA crosslinking in cells by Pro-CL (as low as 0.4 mM) was detected by western blotting. His-CL and Tau-CL were also able to damage PCNA in cells, but only weakly, reflecting much lower cell permeability. The similar extent of PCNA crosslinking by Gly-CL, Pro-CL, and Arg- CL in cells and lysates, combined with the relative stability of these AACLs (Figure 7), suggest that they are cell membrane-permeant. Other AACLs were able to damage PCNA only in disrupted cells, indicating inability to cross cell membranes. Our results support high cell membrane permeability for Arg-CL. This is consistent with inhibition of nitric oxide synthase in cells by Arg-CL that has been reported, and argues for the membrane permeability of Arg-CL (15, 16). Although Tau-CL has been reported to be membrane-impermeant, other reports of intracellular protein or organelle damage from Tau-CL suggest at least some membrane permeability (37, 49). Our studies support weak cell permeability for Tau-CL. The finding that a subset of AACLs can readily reach the nucleus and cause damage to nuclear proteins has implications for inflammation-associated chronic diseases such as cancer and heart disease.

Antibody epitope damage by AACLs. The amount of PCNA detected by western blotting with anti-PCNA antibody, PC10, was often decreased in cells or extracts treated with AACLs. This loss of western blotting signal was pronounced for His-CL and other imidazole chloramines (Figure 4A), evident for Arg-CL (Figure 2B) and Pro-CL (Figure 2A), and minimal for other AACLs. Loss of the PCNA western blot signal suggests chemical damage to the epitope recognized by the PC10 antibody. The epitope recognized by PC10 is located in PCNA residues 111-125 (VSDYEMKLMDL DVEQ) (50). The PCNA epitope sequence contains tyrosine and methionine residues that are highly susceptible to damage by AACLs (11, 37, 46, 51). His-CL treatment did not cause loss of western blot signals of SV40 large T-antigen or of actin. The antibody epitopes for these proteins lack tyrosine and methionine residues. The epitope recognized by anti-large T-antigen antibody PAb101 is in SV40 large T-antigen residues 696-708 (FKKPPTPPPEPET) (52), and that recognized by anti-actin antibody AC-40 is in the sequence SGPSIVHRKCF.

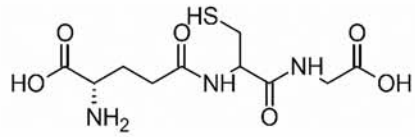
Trolox protected PCNA from epitope damage by Arg-CL (Figure 5B), while NAC and GSH had little or no effect (Figure 5C). AACLs and similar species on proteins that can decay to radicals (53) could account for the efficiency of Trolox in protection from epitope damage. While vitamin E is an efficient one-electron antioxidant that protects molecules from free radical damage, it is a poor two-electron antioxidant (54, 55). Sulfhydryl compounds such as NAC and GSH are very efficient two-electron antioxidants (56). These results suggest that AACLs damage the PCNA antibody epitope due to free radical damage to tyrosine and/or methionine. Antibody epitopes containing susceptible amino acid residues can serve as biomarkers of oxidative protein damage.

Peptide backbone cleavages. The pattern of PCNA peptide backbone cleavage appears to be unique for different AACLs. The single strong PCNA cleavage caused by His-CL was very reproducible and contrasted with the multiple weak backbone cleavages caused by other AACLs. The fact that His-CL did not cause backbone cleavage in SV40 large T-antigen (12 tryptophan residues) suggests that His-CL does not cause backbone cleavage at Trp28, the only tryptophan in PCNA. This single strong cleavage of PCNA was not caused by the chloramines of other imidazole compounds (carnosine and histidine). The peptide sequence has been reported to strongly influence HOCl damage to tryptophan (57), and to tyrosine (9, 51), so sequence-specific peptide backbone cleavage cannot be ruled out. The absence of a weak PCNA crosslinked trimer band in the lysate experiments with His-CL may be due to the overall loss of western blot signal due to epitope damage, in addition to band heterogeneity resulting from partial backbone cleavage. The good spatial separation of PCNA fragments from the monomer, and the ability to take long film exposures of the western blots, allowed the detection of rare protein fragmentation events, although major bands are overexposed.

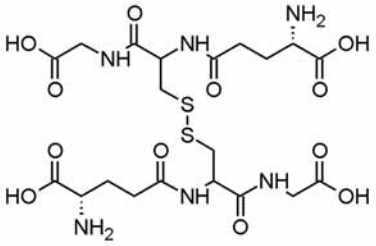
Electrophoretic mobility shift. Oxidation of methionine in proteins is often associated with electrophoretic mobility shifts (38), and is probably due to the decreased lipophilicity of oxidized forms of methionine (58, 59). A scheme for oxidation of methionine to methionine sulfoxide by AACLs has been reported (11). Treatment of proteins with acidified H_2O_2 selectively oxidizes methionine residues to methionine sulfoxide (40) and caused electrophoretic mobility shifting of the PCNA monomer, identical to that caused by AACLs. Treatment of ovalbumin with Gly-CL caused extensive formation of methionine sulfoxide, identical to that formed by acidified H_2O_2. The sulfhydryl antioxidants NAC and GSH protected PCNA from the mobility shift caused by AACLs. These are all consistent with the idea that PCNA mobility shift resulting from exposure to AACLs is due to formation of methionine sulfoxide.

→

Figure 9. *Products of Gly-CL reaction with glutathione (GSH). Products predicted and/or detected from the reaction of Gly-CL with GSH are shown. The LC/MS accurate mass determinations were carried out as described in the Materials and Methods. The identity of GSH thiosulfonate was confirmed by MS/MS (not shown) since the predicted accurate mass was the same as that of GSH sulfonamide. No fragments unique to GSH sulfonamide were found. Those products for which we found evidence by LC/MS accurate mass or MS/MS are indicated by an asterisk.*



Glutathione *



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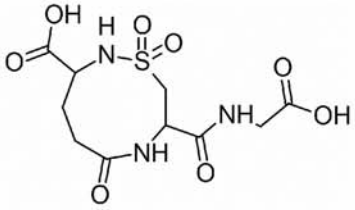
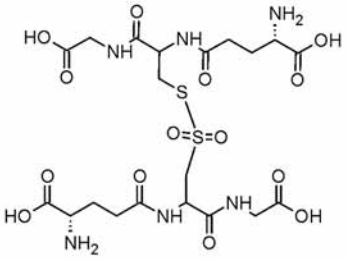
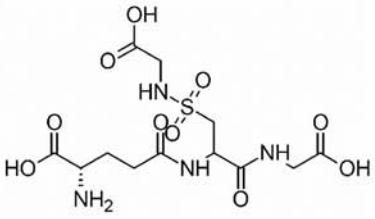


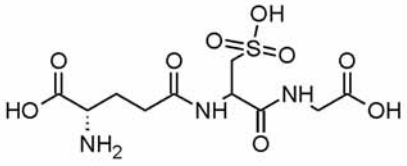
Glutathione internal * sulfonamide



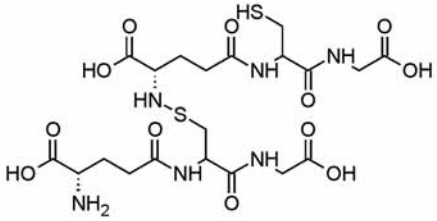
Glutathione thiosulfonate *



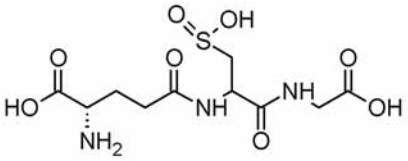
Glutathione-glycine mixed * sulfonamide (trace)



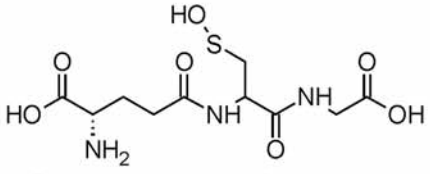
Glutathione sulfonic acid *



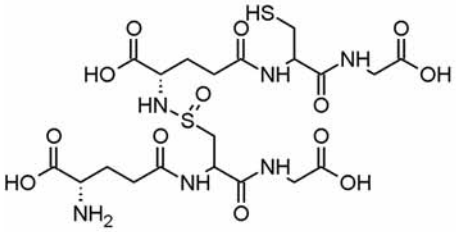
Glutathione intermolecular sulfenamide



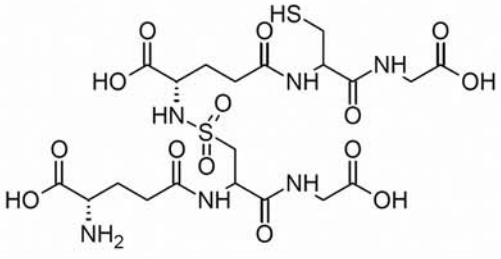
Glutathione sulfinic acid



Glutathione sulfenic acid



Glutathione intermolecular sulfinamide



Glutathione intermolecular sulfonamide

Nature of the covalent PCNA inter-subunit crosslink caused by AACLs. Drosophila PCNA lacking His153 in the subunit interface, was easily crosslinked by Gly-CL, indicating that His153, required for singlet oxygen crosslinking of PCNA, is not involved in covalent crosslinking of PCNA subunits by Gly-CL. *S. pombe* PCNA, which uniquely lacks Cys148, was not crosslinked by Gly-CL. Glutaraldehyde crosslinking of *S. pombe* PCNA demonstrated that intact PCNA trimers were present. This supports an inter-subunit crosslinking mechanism involving Cys148. Possible mechanisms of protein crosslinking by HOCl have been reviewed (60, 61). One possible mechanism of protein crosslinking is by way of aldehydes resulting from decay of chloramines, formed on lysine side chains, followed by formation and reduction of a Schiff base with another lysine side chain (62). However, Fu *et al*. have argued against this mechanism of protein crosslinking by HOCl, as well as any mechanism involving tyrosyl radicals (60). In their study with model peptides, it was shown that HOCl can form a variety of Cys-Lys peptide crosslinks, including intermolecular and intramolecular sulfonamide crosslinks, as well as intramolecular sulfinamide and sulfenamide crosslinks. These mechanisms all involve a cysteine and are consistent with our finding that *S. pombe* PCNA, lacking Cys148 in the PCNA interface, cannot be cross-linked by Gly-CL.

Cysteine sulfhydryl groups from each subunit are present in the PCNA subunit interface (45), and since sulfhydryl groups are easily oxidized, we consider them candidates for PCNA subunit crosslinking. Although cysteine sulfhydryls are easily oxidized to cystine, producing disulfide crosslinks in proteins (63), boiling with dithiothreitol (DTT) during SDS-PAGE sample preparation would reduce disulfide crosslinks back to sulfhydryl groups and reverse the crosslinking. However, other inter-subunit crosslinks involving cysteine are possible.

GSH, having both an amino and a sulfhydryl group, can serve as a model for oxidative peptide damage. The products of the reaction between GSH and HOCl have been well-characterized and include a GSH internal sulfonamide, GSH thiosulfonate, and GSH disulfide (48). Reactions of HOCl with model peptides containing Lys and Cys residues produced Cys-Lys and Cys-Cys crosslinks that represent higher levels of oxidation (60). Tau-CL and ammonia chloramine reactions with GSH were reported to produce very low levels of GSH sulfonamide as measured by an LC-tandem MS assay (64). However, it has been suggested that chloramines might only oxidize GSH to the disulfide (GSH-SS-GSH) (48). To determine if an AACL could produce the higher levels of oxidation needed to produce a DTT-resistant crosslink, we analyzed the reaction of Gly-CL with GSH using LC-MS and MS/MS. Possible products, based on the published studies of HOCl reactions (48, 60), are shown in Figure 9. Those products for which we found evidence by LC-MS accurate mass or MS/MS, are indicated by an asterisk, and included GSH-sulfonic acid, GSH internal sulfonamide, GSH disulfide, GSH thiosulfonate, and GSH-glycine mixed sulfonamide (Figure 9).

Since Gly-CL was a sufficiently strong oxidant to produce the GSH internal sulfonamide, GSH thiosulfonate, and GSH sulfonic acid, a GSH-GSH intermolecular sulfonamide linkage should be possible, but may not be produced in our experiments with GSH due to competing reactions between sulfhydryl groups. In a protein, fixation of amino acid side chains in the peptide backbone could limit competing reactions and could favor sulfonamide crosslinks where cysteine and lysine residues are in close proximity. The fixation of the sulfhydryl and amino groups of GSH relative to one another within the GSH molecule is probably the reason that the GSH internal sulfonamide is produced in significant amounts. The reaction of HOCl with model peptides did produce intermolecular sulfonamide crosslinks (60), and exposure to HOCl caused intra- and intermolecular sulfinamide crosslinks in the cytoplasmic protein S100A8 (65). These crosslinks would not be reduced by DTT, and all of these crosslinks would be consistent with involvement of Cys148 in PCNA crosslinking.

Amino acid suppression of PCNA crosslinking by AACLs. We prepared most AACLs by the standard method, reacting HOCl with a molar excess of the amino acids to ensure complete depletion of the HOCl. However, we found that Pro-CL was only stable if prepared using equimolar concentrations of proline and HOCl, as determined by both PCNA crosslinking in cells and by spectroscopic chloramine measurement using the TMB reagent. Our attempts to determine the structure of the product of Pro-CL with excess proline by mass spectrometry were unsuccessful. Pro-CL has been reported to be highly unstable, although the role of excess proline was not noted (66). Carbon-centered side chain radicals and radical adducts of Pro-CL breakdown were detected by electron paramagnetic resonance (EPR) spin trapping (66). The high ratio of HOCl to proline in the preparation of Pro-CL may also increase the possibility that the efficient PCNA crosslinking is due to proline dichloramine. Although the instability of Pro-CL prevented testing by adjusting these ratios, experiments with Gly-CL did not detect changes in PCNA crosslinking as a result of a 33% decrease in the percentage of glycine dichloramine.

Both PCNA crosslinking and chloramine assays indicated that Arg-CL and Gly-CL were stable for at least 30 min in the presence of 2 mM excess of the corresponding amino acids. The chloramine assay also indicated that larger molar excesses of the respective amino acids had no effect on the stability of Arg-CL and Gly-CL. However, for both of these chloramines-molar excesses of the respective amino acid

decreased PCNA crosslinking in cells in a concentration dependent manner. This is reminiscent of the ability of excess arginine to interfere with the inactivation of nitric oxide synthase in cells by Arg-CL (16). The *in vitro* suppression of purified recombinant PCNA crosslinking by Gly-CL, and the fact that excess glycine does not affect the stability of Gly-CL itself (Figure 7B) suggests that the suppressive effect of excess amino acids may involve a competitive reaction at the amino acid residues involved in crosslinking the PCNA subunits. The suppressive effect of excess amino acids on AACL crosslinking of PCNA may be due to formation of mixed sulfonamides between these amino acids and cysteine side chains, which would compete with formation of sulfonamide crosslinks between these cysteine residues in one PCNA subunit and epsilon amino groups of lysine in another PCNA subunit.

In summary, we have characterized PCNA as a biomarker for AACL damage to nuclear proteins in mammalian cells, and we have used it to gain new insights into the biochemistry and cell biology of AACLs. PCNA damage from AACLs included PCNA subunit crosslinking, peptide backbone cleavage, oxidation, and epitope damage. The AACLs that are membrane permeant and capable of damaging nuclear proteins in mammalian cells were identified, and a suppressive effect of excess amino acids on PCNA crosslinking by AACLs was observed and studied. We were also able to gain insights into the nature of the covalent crosslink between PCNA subunits that was caused by AACLs and the nature of antibody epitope damage by AACLs.

Acknowledgements

We thank Frederick Villamena, and Christopher Hadad for useful discussions, and Edith F. Yamasaki for help with editing the manuscript. We thank Jian-Qiu Wu and Ran Zhao for the *S. pombe* cell extract, and Amanda Simcox for *D. melanogaster* cells. We also thank Kari Green and Liwen Zhang of the OSU CCIC for assistance in planning and interpreting the mass spectrometry experiments. This work was supported by the National Cancer Institute NIH/NCI RO1 CA097107 to RMS, NIH/NCI RO1 CA118208, and the Ohio State University Comprehensive Cancer Center (P30CA16058).

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Received May 15, 2012
Revised June 1, 2012
Accepted June 4, 2012