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Nicotine Mediates Hypochlorous Acid-Induced Nuclear Protein Damage in Mammalian Cells

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Abstract—Activated neutrophils secrete hypochlorous acid (HOCl) into the extracellular space of inflamed tissues. Because of short diffusion distance in biological fluids, HOCl-damaging effect is restricted to the extracellular compartment. The current study aimed at investigating the ability of nicotine, a component of tobacco and electronic cigarettes, to mediate HOCl-induced intracellular damage. We report, for the first time, that HOCl reacts with nicotine to produce nicotine chloramine (Nic-Cl). Nic-Cl caused dose-dependent damage to proliferating cell nuclear antigen (PCNA), a nuclear protein, in cultured mammalian lung and kidney cells. Vitamin C, vitamin E analogue (Trolox), glutathione, and *N*-acetyl-L-cysteine inhibited the Nic-Cl-induced PCNA damage, implicating oxidation in PCNA damage. These findings point out the ability of nicotine to mediate HOCl-induced intracellular damage and suggest antioxidants as protective measures. The results also raise the possibility that Nic-Cl can be created in the inflamed tissues of tobacco and electronic cigarette smokers and may contribute to smoking-related diseases.

KEY WORDS: nicotine chloramine; hypochlorous acid; inflammation; lung diseases; tobacco smoking.

INTRODUCTION

Neutrophil infiltration of lung tissues is a common feature in many obstructive airway diseases, including asthma, chronic obstructive pulmonary disease, bronchiectasis, and bronchopulmonary aspergillosis [1–5]. Neutrophilic airway inflammation is exacerbated by exposure to other pro-inflammatory stimuli such as tobacco smoking [6–9]. Neutrophilia has also been associated with electronic cigarette smoking [10]. Neutrophil infiltration to inflamed kidney, liver, brain, intestine, and other peripheral tissues has been extensively reported [11–21]. During the respiratory burst, neutrophil myeloperoxidase catalyzes the oxidation of chloride ions by hydrogen peroxide to generate hypochlorous acid (HOCl) [22]. The concentration of HOCl in the interstitial fluids of inflamed tissues has been estimated to reach more than 5 mM [23–25]. Because of its high reactivity, HOCl rapidly reacts with a variety of biomolecules and is consumed within 0.46 μm of the site of generation and cannot reach distant intracellular targets [25]. Reaction of HOCl with amines can generate much more stable chloramines that can diffuse greater distances [24, 26, 27]. Many of these chloramines are nonreactive, and their formation is considered as an efficient way for HOCl scavenging and cytoprotection [25, 27, 28]. Only a few low molecular weight amines, including a very small subset of amino acids, have been found to form chloramines that can cross cellular membranes and mediate HOCl-induced intracellular protein damage [27]. Tobacco

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ABBREVIATIONS: DTT, Dithiothreitol; GuHCl, Guanidine hydrochloride; HOCl, Hypochlorous acid; NAC, *N*-acetyl-L-cysteine; Nic-Cl, Nicotine chloramine; Nic, Nicotine; PBS, Phosphate-buffered saline; PCNA, Proliferating cell nuclear antigen; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMB, 3,3′,5,5′-Tetramethylbenzidine

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smoking exposes lung tissues to high levels of xenobiotics including nicotine, a major low molecular weight amine [29–31]. Nicotine comprises about 1.5 % by weight of tobacco and about 95 % of its alkaloid content [32, 33]. Electronic cigarette smoking also exposes lung tissues to considerable amounts of nicotine [34]. It has been reported that residual nicotine concentrations in tissues of heavy smokers reach more than 1 mM [35]. The possibility that nicotine can mediate HOCl-induced intracellular protein damage through the formation of nicotine chloramine (Nic-Cl) has not been previously studied. The present study was initiated to investigate the ability of nicotine to mediate HOCl-induced intracellular protein damage in intact cultured mammalian lung and kidney cells through the formation of Nic-Cl.

Proliferating cell nuclear antigen (PCNA) is a nuclear protein that plays several essential roles in DNA metabolism. PCNA is involved in DNA replication, DNA excision repair, control of sister chromatid cohesion, maintenance of chromatin structure, and RNA transcription [36, 37]. It is also involved in the cell cycle regulation [36]. PCNA is composed of three subunits (homotrimer) with a molecular weight of 29 kDa for each subunit [36]. PCNA subunits interact non-covalently to form a ring-shaped structure that encircles DNA during different DNA-related processes [38]. The non-covalent binding between PCNA subunits allows the PCNA ring to open and encircle DNA, a preliminary step required for PCNA to function properly [38–40]. Covalent cross-linking of PCNA subunit, thus, interferes with PCNA ring opening and, consequently, with its function. Cross-linking of PCNA subunits is thus considered as a critical damage to PCNA [27, 41]. Because PCNA is a nuclear protein, PCNA cross-linking has been used as a sensitive marker for intracellular protein damage induced by different cytotoxic compounds [27, 41]. While covalent cross-linking of PCNA produces mainly trimers with a molecular weight of 93 kDa, other forms such as PCNA dimers and double trimers have also been reported [27, 41, 42]. In the current work, PCNA was used to study the ability of nicotine to mediate HOCl-induced intracellular protein damage in intact cultured mammalian lung and kidney cells.

MATERIAL AND METHODS

Chemicals

Nicotine-free base, hypochlorous acid, reduced L-glutathione, vitamin C (L-ascorbic acid), vitamin E analogue [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox)], and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St Louis, MO, USA). *N*-acetyl-L-cysteine was purchased from Calbiochem (Darmstadt, Germany). Glass microfiber filters (GF/C type, 2.4 cm diameter made by Whatman, UK) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Guanidine hydrochloride (molecular biology grade and DNAse free) was purchased from Promega Corporation (Madison, WI, USA). Tritiated thymidine was purchased from Amersham Biosciences (Piscataway, NJ, USA).

Cells and Cell Culture

Human lung fibroblast cells (IMR90) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA), containing 10 % FBS (Invitrogen) and incubated in 5 % CO_2 incubator. CV-1 kidney fibroblast cells were cultured in MEM containing 10 % calf serum (Invitrogen) and 14 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (Sigma) (MEM/HEPES). Both cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA).

Measurement of Hypochlorous Acid Concentration

The concentration of hypochlorous acid was determined immediately before use by measuring its absorbance at 292 nm in phosphate buffer at pH 12 (E_{292} 350 M^{-1} cm^{-1}) [43].

Preparation of Nicotine Chloramines

Because nicotine is light sensitive, Nic-Cl was prepared under dark conditions in opaque microcentrifuge tubes. Nic-Cl (3 mM) was prepared by mixing 5 mM nicotine in phosphate-buffered saline (PBS) with 3 mM of HOCl (both are final concentrations), vortex mixed for 40 s, and immediately diluted with PBS to the required concentrations. Nic-Cl was added to cells immediately after dilution to the required concentrations. The concentrations of nicotine and HOCl used in this study were chosen based on the estimated HOCl concentration in inflamed tissues and the estimated residual concentrations of nicotine in tissues of smokers [23, 25, 35].

Treatment with Nicotine Chloramine

Cells were washed once with warm PBS after removal of growth media from 35-mm plates of cultured cells. Required concentration of Nic-Cl prepared in PBS was then added to cells in 0.5 ml final volume. Cells were then incubated at 37 °C for 10 min. Nic-Cl-containing PBS was then removed, and cells were washed twice with PBS before lysis and preparation for Western blotting.

Inhibition of Cell Proliferation

Inhibition of cell proliferation by Nic-Cl was evaluated using tritiated thymidine incorporation and glass microfiber filter binding assay [44–46]. Growth media was removed form 35-mm plates of CV-1 mammalian kidney cells. CV-1 cells were then incubated with Nic-Cl (1 mM) at 37 °C for 10 min. Nic-Cl solution was then removed, and cells were washed once with warm PBS. Cells were then incubated in MEM/HEPES media (0.75 ml/plate) containing 10 % fetal bovine serum and tritiated thymidine (5 μCi/ml) at 37 °C for 2 h. Tritiated thymidine-containing media was then removed, and cells were lysed with 0.5 ml/plate of Hirt's lysis buffer (10 mM Tris buffer, 10 mM EDTA, and 0.6 % sodium dodecyl sulfate (SDS)). Samples were then subjected to a modified glass microfiber filter binding assay [47]. Briefly, 20 μl of cell lysate was mixed with 1 ml of 4 M guanidine hydrochloride (GuHCl). Samples were then filtered through glass microfiber filters (GF/C type, 2.4 cm diameter). The glass microfiber filters were then rinsed with 2.75 ml of 4 M GuHCl followed by 5 ml of ice-cold 95 % ethanol. Under these conditions, total cellular DNA in the sample binds the glass microfiber filters. The radioactivity retained on the filters represents the amount of tritiated thymidine that was incorporated into the cellular DNA during the incubation period with tritiated thymidine-containing media. The extent of tritiated thymidine incorporation is proportional to the extent of DNA synthesis and cell proliferation. The radioactivity retained on the filters was counted in disintegration per minute (dpm) using liquid scintillation system (LS 6500, Beckman Coulter).

Treatment with Antioxidants

Cultured cells in 35-mm plates were preincubated with 450 μl/plate of 5 mM (prepared in PBS) of reduced L-glutathione, *N*-acetyl-L-cysteine, L-ascorbic acid, or vitamin E analogue (Trolox) for 30 min at 37 °C. Antioxidant solution was then removed, and cells were washed twice with warm PBS before treatment with Nic-Cl.

Western Blot Analysis

Cells in 35-mm culture plates were lysed with 90 μl of SDS buffer (2 % SDS, 10 % glycerol, and 62.5 mM Tris–HCl, pH 6.8). Protein samples, boiled in Laemmli sample buffer with 100 mM dithiothreitol (DTT) for 5 min, were separated by 10 % SDS-polyacrylamide gel electrophoresis (PAGE) (40 μg per lane) and transferred to a nitrocellulose membrane (Protran, PerkinElmer, Shelton, CT, USA) using a semidry transfer system (Bio-Rad). Blocked membranes were incubated overnight at 4 °C with monoclonal mouse antihuman PCNA primary antibody, PC10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rinsed, and then incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Bio-Rad) for 1 h at room temperature. Protein bands were detected using a Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) using an X-ray film. The apparent molecular weights of PCNA forms induced by Nic-Cl were estimated by comparison to molecular weight markers.

Statistical Analysis

Comparisons between groups' means were analyzed for a statistical significance using Student's *t* test. Differences were considered significant at $p<0.05$. Statistical analysis was performed using SigmaPlot 12 statistics software (Systat Software, Inc., San Jose, CA, USA).

RESULTS

HOCl Reacts Rapidly with Nicotine to Generate Nicotine Chloramine

The reaction of HOCl with nicotine was monitored for complete consumption of HOCl and production of Nic-Cl. The characteristic HOCl peak at 292 nm [43] disappeared upon mixing with molar excess of nicotine, indicating complete consumption of HOCl under our experimental conditions (Fig. 1a). Production of chloramine was tested spectrophotometrically using 3,3′,5,5′-tetramethylbenzidine (TMB) reagent [48]. Nic-Cl was monitored spectrophotometrically for stability, and it was found degradable with time (Fig. 1b).

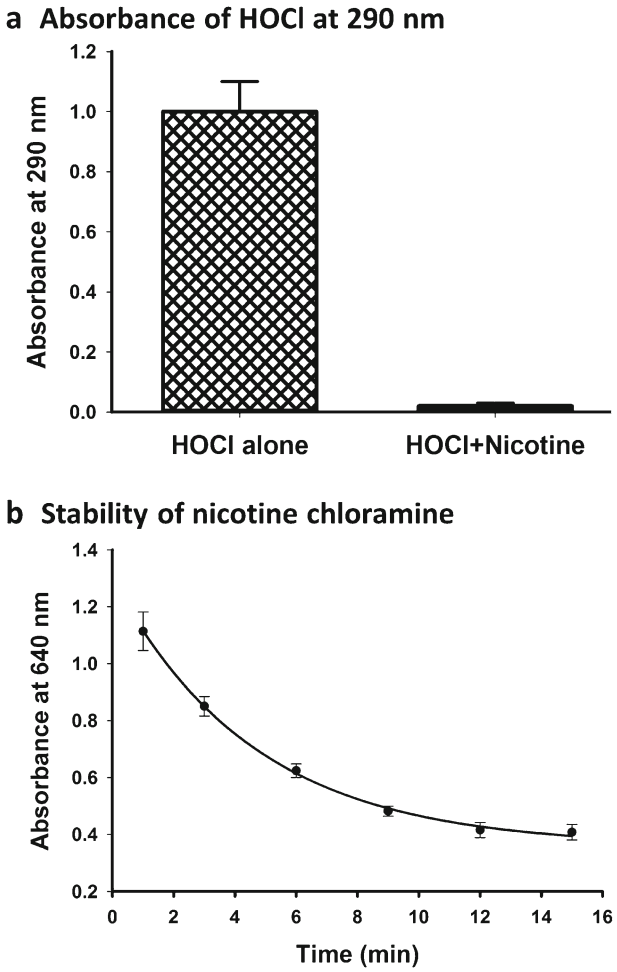

Fig. 1. Reaction of HOCl with nicotine and stability of nicotine chloramine. **a** Absorbance at 290 nm of HOCl alone or HOCl mixed with nicotine (3 mM of HOCl with 5 mM of nicotine, all were final concentrations). The characteristic absorbance of HOCl at 290 nm (HOCl alone) was completely disappeared upon mixing with molar excess of nicotine (HOCl + nicotine), indicating complete consumption of HOCl under our experimental conditions. **b** Stability of Nic-Cl. Nic-Cl was prepared by vortex mixing 3 mM of HOCl with 5 mM of nicotine. The presence and stability of Nic-Cl were monitored spectrophotometrically using TMB reagent by recording the absorbance at 640 nm at the specified time points indicated on the *x*-axis. All results are represented as mean ± standard deviation (n=3).

Nicotine Chloramine Induces PCNA Intersubunit Cross-linking and Inhibits Proliferation in Cultured Mammalian Cells

Tobacco smoke causes lung tissue damage that may lead to a variety of lung diseases including cancer [49]. Nic-Cl was tested for its possible damaging effect. Treatment of CV-1 mammalian cells with Nic-Cl (1 mM) induced a high molecular weight PCNA antibody-reactive band migrating at 93 kDa. This is the well-established molecular weight of the covalently cross-linked PCNA trimer [41]. The formation of the PCNA trimer was robust as detected by Western blotting (Fig. 2a) and was found to be dose dependent (Fig. 3a). Similar results were also observed when IMR90 cultured human lung cells were treated with Nic-Cl (Figs. 2b and 3b). To exclude the possibility that PCNA intersubunit cross-linking is caused by Nic that was present in excess with Nic-Cl, cells were treated with Nic alone. The results indicated that in contrast to Nic-Cl, Nic alone was not able to induce PCNA intersubunit cross-linking (Fig. 2c). To evaluate the ability of Nic-Cl to inhibit cell proliferation, CV-1 cells were incubated with Nic-Cl (1 mM for 10 min at 37 °C) and then subjected to the tritiated thymidine incorporation assay. The results showed that Nic-Cl significantly inhibited cell proliferation (Fig. 4).

Antioxidants Protect Against Nicotine Chloramine-Induced PCNA Intersubunit Cross-linking

Using a model peptide, our previous work showed that PCNA intersubunit cross-linking caused by glycine chloramine is mediated through oxidation of a sulfhydryl group located at the PCNA monomer-monomer interface [27]. To test the possible involvement of oxidation in Nic-Cl-induced PCNA intersubunit cross-linking, different antioxidants including reduced L-glutathione (GSH), *N*-acetyl-L-cysteine (NAC), vitamin C (Vit C), and the water-soluble vitamin E analogue (Trolox) were used. Pretreatment of cells with 5 mM of GSH, NAC, Vit C, or Trolox completely inhibited Nic-Cl-induced PCNA intersubunit cross-linking (Fig. 5), implicating oxidation in the mechanism of PCNA damage by Nic-Cl.

DISCUSSION

HOCl, the product of activated neutrophils, rapidly reacts with a variety of biomolecules in the interstitium of the inflamed tissues and cannot reach distant intracellular targets in the surrounding cells [25, 50]. Using concentrations of hypochlorous acid and nicotine that have been reported in human tissues [23, 25, 35], our *in vitro* experiments showed, for the first time, that the reaction of HOCl with nicotine

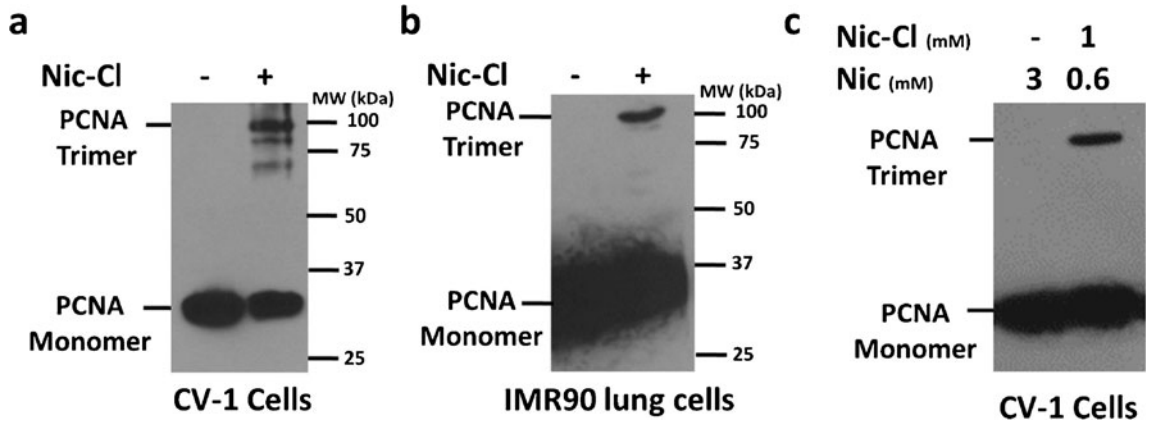

Fig. 2. Nicotine chloramine induces PCNA intersubunit cross-linking in mammalian cells. **a** CV-1 cells were either treated with 1 mM nicotine chloramine (Nic-Cl) for 10 min at 37 °C (*plus lane*) or left untreated as a control (*minus lane*). Cells were then prepared for SDS-PAGE and Western blotting with anti-PCNA antibody. **b** IMR90 lung cells were treated the same way as in **a. c** Effect of nicotine alone (*Nic*) on PCNA intersubunit cross-linking compared to that of Nic-Cl. The experiment was done to show that intersubunit cross-linking of PCNA was not due to excess Nic that is present in Nic-Cl solution.

produced Nic-Cl and that Nic-Cl, or its reactive breakdown product, caused dose-dependent damage to an exclusive nuclear protein, PCNA, in cultured mammalian lung and kidney cells. The reactive product was able to travel to the nucleus crossing both the cell and nuclear membranes. The protein damage detected, covalent cross-linking of PCNA subunits, has been well characterized and is known to be caused by a small set of amino acid chloramines and by a variety of protein-protein cross-linking agents, including singlet oxygen [27, 51]. The covalent cross-linking of PCNA subunits by amino acid chloramines has been shown to require cysteine 148 at the PCNA intersubunit interface, and evidence supports a sulfonamide cross-link between Cys-148, and a lysine side chain has been found [27]. PCNA intersubunit cross-links caused by Nic-Cl were not reversed by boiling with DTT during sample preparation for Western blotting, excluding disulfide cross-linking.

It has been reported that chloramines exhibit oxidant properties [27, 52]. To test the possibility that oxidation is involved in Nic-Cl-induced PCNA damage, different antioxidants were tested for their possible protective effect.

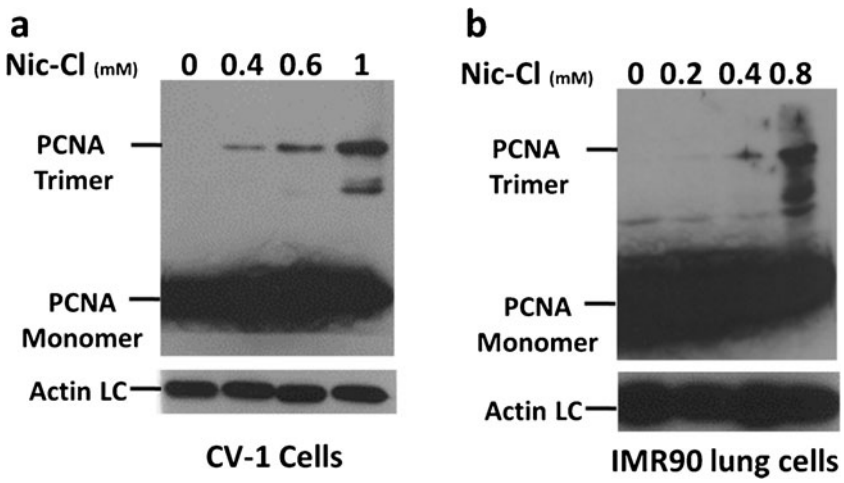

Fig. 3. Dose–response of nicotine chloramine on PCNA intersubunit cross-linking in mammalian cells. CV-1 cells (**a**) or IMR90 lung cells (**b**) were incubated with increasing concentrations of nicotine chloramine (*Nic-Cl*) for 10 min at 37 °C. Cell lysates were then prepared and subjected to SDS-PAGE and Western blotting with anti-PCNA antibody. Actin was used as a loading control (*LC*).

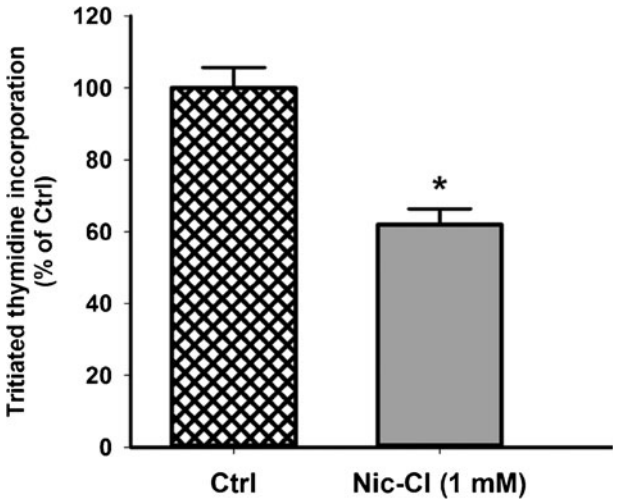

Fig. 4. Inhibition of cell proliferation by nicotine chloramine. Inhibition of cell proliferation was evaluated using tritiated thymidine incorporation assay. CV-1 cells were incubated either with 1 mM of nicotine chloramine (*Nic-Cl*) or kept in PBS as an untreated control (*Ctrl*) for 10 min at 37 °C. Cells were then incubated in MEM/HEPES media containing tritiated thymidine (5 μCi/ml) at 37 °C for 2 h. Tritiated thymidine-containing media was then removed, and cells were lysed and subjected to the modified glass microfiber filter binding assay. The radioactivity retained on the glass microfiber filters that represent the extent of tritiated thymidine incorporation into the DNA was counted using liquid scintillation system (LS 6500, Beckman Coulter). The extent of tritiated thymidine incorporation is proportional to the extent of DNA synthesis and cell proliferation. The data are represented as a percentage of Ctrl. *$p>0.05$, a significant difference from the Ctrl group ($n=3$).

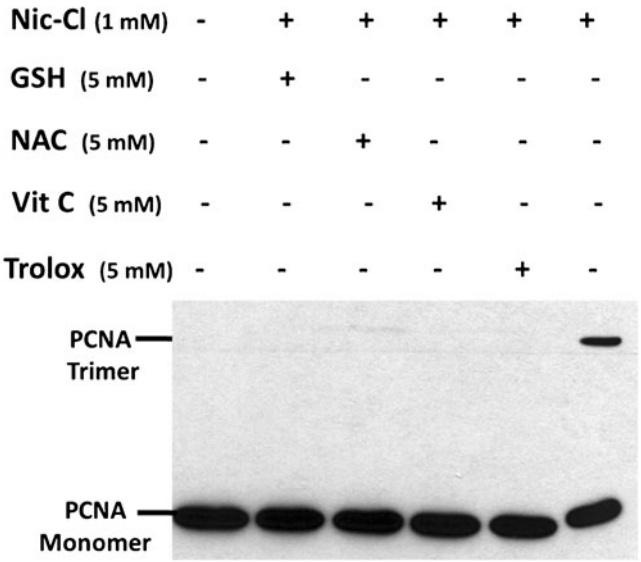

Fig. 5. Effect of sulfhydryl and vitamin antioxidants on nicotine chloramine-induced PCNA intersubunit cross-linking. Preincubation of CV-1 cells with reduced L-glutathione (*GSH*), *N*-acetyl-L-cysteine (*NAC*), vitamin C (*Vit C*), or the vitamin E analogue (*Trolox*) inhibits nicotine chloramine (*Nic-Cl*)-induced PCNA intersubunit cross-linking. CV-1 cells were incubated with 5 mM of GSH, NAC, Vit C, or Trolox for 30 min at 37 °C. Antioxidant solutions were then removed, and cells were washed twice with fresh warm PBS before incubation with Nic-Cl (1 mM) for 10 min at 37 °C. Cell lysates were then prepared and subjected to SDS-PAGE followed by anti-PCNA Western blotting. A negative control (the *first lane*) and a positive control (the *last lane*) were included for comparison.

The results showed that antioxidants including reduced L-glutathione, *N*-acetyl-L-cysteine, vitamin C, and the vitamin E analogue (Trolox) completely inhibited PCNA intersubunit cross-linking by Nic-Cl, implicating oxidation as an important step in Nic-Cl-induced nuclear protein damage. The results also indicated that Nic-Cl was able to inhibit mammalian cell proliferation as evaluated by the tritiated thymidine incorporation assay (Fig. 4), indicating that the intracellular Nic-Cl-induced damage was severe enough to adversely affect the cell proliferation.

Together, our results suggest that in inflamed tissues, HOCl from infiltrating neutrophils has the potential to react with nicotine from tobacco or electronic cigarette smoke to produce Nic-Cl capable of causing molecular protein damage in the nuclei of intact cells either directly due to its membrane permeability or indirectly by way of a membrane permeable Nic-Cl-reactive breakdown product. The ability of Nic-Cl to damage nuclear PCNA suggests that the other biologically important proteins may also be damaged. It is reasonable to speculate that protein damage by Nic-Cl may contribute to cigarette smoking-related diseases.

It is worth noting that tissue damage caused by Nic-Cl may not be limited to lung tissues of smokers. Nic-Cl is expected to be generated at other inflamed body tissues when nicotine from cigarette smoking or other nicotine-containing products such as pipe tobacco and chewing tobacco that efficiently deliver nicotine to the blood and to a variety of tissues, reviewed in [29], reacts with neutrophil-generated HOCl. Tobacco or nicotine use could thus contribute to diseases associated with chronic inflammation [53].

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