Published in final edited form as:

Inflammation. 2014 June ; 37(3): 785–792. doi:10.1007/s10753-013-9797-6.

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

Samir A. Salama^{1,2,*}, Hany H. Arab^{1,3}, Hany A. Omar⁴, Ibrahim A. Maghrabi⁵, and Robert M. Snapka^{6,7}

¹Division of Biochemistry and GTMR Unit, College of Clinical Pharmacy, Taif University, Taif 21974, KSA

²Department of Biochemistry, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt

³Department of Biochemistry, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt

⁴Department of Pharmacology, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62511, Egypt

⁵Department of Clinical Pharmacy, College of Clinical Pharmacy, Taif University, Taif, 21974, KSA

⁶Department of Radiology, Faculty of Medicine, The Ohio State University, Columbus 43201, Ohio, USA

⁷The Ohio State University Comprehensive Cancer Center, Columbus 43202, Ohio, USA

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, 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.

Keywords

Nicotine chloramine; Hypochlorous acid; Inflammation; Lung diseases; Tobacco smoking

*To whom correspondence should be addressed. Samir A. Salama, Ph.D., Division of Biochemistry, College of Clinical Pharmacy, Taif University, Al-Haweiah, Taif, 21974, Saudi Arabia. salama.3@buckeyemail.osu.edu; Phone: +9665935913345.

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 μ m of the site of generation and cannot reach distant intra-cellular 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 non-reactive 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 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 1mM [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 crosslinking of PCNA subunit, thus, interferes with PCNA ring opening and, consequently, with its function. Crosslinking of PCNA subunits is thus considered as a critical damage to PCNA [27, 41]. Because PCNA is a nuclear protein, PCNA crosslinking has been used as a sensitive marker for intracellular protein damage induced by different cytotoxic compounds [27, 41]. While covalent crosslinking 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 (Trolox: (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), 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, N.J.). Guanidine hydrochloride (molecular biology grade and DNase free) was purchased from Promega Corporation (Madison, WI). Tritiated thymidine was purchased from Amersham Biosciences (Piscataway, NJ).

Cells and cell culture

Human lung fibroblast cells (IMR90) were cultured in high glucose DMEM (Invitrogen, Carlsbad, CA), containing 10% FBS (Invitrogen) and incubated in 5% CO₂ incubator. CV-1 kidney fibroblast cells were cultured in MEM containing 10% calf serum (Invitrogen) and 14 mM 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 ($E_{292} 350 \text{ M}^{-1} \text{ cm}^{-1}$) [43].

Preparation of nicotine chloramines

Because nicotine is light sensitive, Nic-Cl was prepared under dark conditions in opaque microcentrifuge tubes. Nic-Cl (3mM) was prepared by mixing 5 mM nicotine in PBS with 3 mM of HOCl (both are final concentrations), vortex mixed for 40 second, 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 from 35 mm plates of CV-1 mammalian kidney cells. CV-1 cells were then incubated with Nic-Cl (1mM) 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 uCi/ml) at 37 °C for 2 hours. 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% SDS). Samples were then subjected to a modified glass microfiber filter binding assay [47]. Briefly, 20µL of cell lysate was mixed with 1ml 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 pre-incubated 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: (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) 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 DTT for 5 min, were separated by 10% SDS-PAGE (40 µg per lane) and transferred to a nitrocellulose membrane (Protran, PerkinElmer, Shelton, CT) using a semi-dry transfer system (BioRad). Blocked membranes were incubated overnight at 4 °C with monoclonal mouse anti human PCNA primary antibody, PC10, (Santa Cruz Biotechnology, Santa Cruz, CA), rinsed, and then incubated with horseradish peroxidase conjugated goat anti mouse secondary antibody (BioRad) for 1 hour at room temperature. Protein bands were detected by Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL) using 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 mean were analyzed for 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).

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 1 a). 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 1 b).

Nicotine chloramine induces PCNA inter-subunit crosslinking 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 (1mM) induced a high molecular weight PCNA antibody-reactive band migrating at 93 kDa. This is the well-established molecular weight of the covalently crosslinked PCNA trimer [41]. The formation of the PCNA trimer was robust as detected by Western blotting (Fig 2 a) and was found to be dose-dependent (Fig 3 a). Similar results were also observed when IMR90 cultured human lung cells were treated with Nic-Cl (Fig 2 b and 3 b). To exclude the possibility that PCNA inter-subunit crosslinking is caused by nicotine (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 inter-subunit crosslinking (Fig 2c). To evaluate the ability of Nic-Cl to inhibit cell proliferation, CV-1 cells were incubation 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 inter-subunit crosslinking

Using a model peptide, our previous work showed that PCNA inter-subunit crosslinking 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 inter-subunit crosslinking, 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. Pre-treatment of cells with 5 mM of GSH, NAC, Vit C, or Trolox completely inhibited Nic-Cl-induced PCNA inter-subunit crosslinking (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 intra-cellular 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 reaction of HOCl with nicotine 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 crosslinking 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 crosslinking agents, including singlet oxygen [27, 51]. The covalent crosslinking of PCNA subunits by amino acid chloramines has been shown to require cysteine 148 at the PCNA inter-subunit interface, and evidence supports a sulfonamide crosslink between Cys-148 and a lysine side chain has been found [27]. PCNA inter-subunit crosslinks caused by Nic-Cl were not reversed by boiling with DTT during sample preparation for Western blotting, excluding disulfide crosslinking.

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. The results showed that antioxidants including reduced L-glutathione, N-acetyl-L-cysteine, vitamin C, and the vitamin E analogue (Trolox) completely inhibited PCNA inter-subunit crosslinking 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 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 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].

Acknowledgments

This work was supported by Taif University (2260-434-1 to SAS); and National Institutes of Health (RO1-CA097107 to RMS).

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
SFM	serum free media
TMB	3,3',5,5'-tetramethylbenzidine

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a: Absorbance of HOCl at 290 nm **b: Stability of nicotine chloramine**

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 nicotine chloramine (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 $\pm$ standard deviation (n = 3).

Fig. 2. Nicotine chloramine induces PCNA inter-subunit crosslinking in mammalian cells

(a) CV-1 cells were either treated with 1 mM nicotine chloramine (Nic-Cl) for 10 min at 37 °C (+ lane) or left untreated as a control (– 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 inter-subunit crosslinking compared to that of Nic-Cl. The experiment was done to show that inter-subunit crosslinking of PCNA was not due to excess Nic that is present in Nic-Cl solution.

Fig. 3. Dose-response of nicotine chloramine on PCNA inter-subunit crosslinking 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 group) or kept in PBS as an untreated control (Ctrl group) for 10 min at 37 °C. Cells were then incubated in MEM/HEPES media containing tritiated thymidine (5 uCi/ml) at 37 °C for 2 hours. 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 percentage of control (Ctrl). (*) means significant difference from the control group (Ctrl), p 0.05, (n = 3).

Nic-Cl (1 mM)	-	+	+	+	+	+
GSH (5 mM)	-	+	-	-	-	-
NAC (5 mM)	-	-	+	-	-	-
Vit C (5 mM)	-	-	-	+	-	-
Trolox (5 mM)	-	-	-	-	+	-

Fig. 5. Effect of sulfhydryl and vitamin antioxidants on nicotine chloramine-induced PCNA inter-subunit crosslinking

Pre-incubation 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 inter-subunit crosslinking. 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.