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HOCl Exposure of a Human Airway Epithelial Cell Line Decreases Its Plasma Membrane Neutral Endopeptidase

Zhihui Lang and Christopher G. Murlas

Department of Medicine (Pulmonary), Rush University, 1653 West Congress Parkway, Chicago, IL 60612, USA

Abstract. It has recently been demonstrated that luminal exposure of airway segments in vitro to HOCl produces airway muscle hyperresponsiveness to substance P and a decrease in neutral endopeptidase (NEP) activity of tissue segment homogenates, suggesting that HOCl may decrease airway epithelial cell NEP activity. To confirm that this effect occurs in humans and to investigate possible subcellular mechanisms for it, we assessed HOCl exposure of the human airway epithelial cell line Calu-1. These cells, grown to confluency in Dulbecco's modified Eagle medium with 10% fetal bovine serum and penicillin–streptomycin, were exposed in situ for 5 min to 100 μM HOCl in a phosphate-buffered saline solution (PBS; pH 7.0 at 37°C) or to PBS alone. Thereafter, cells were rinsed and assayed for NEP activity employing reverse-phase high-pressure liquid chromatography. This activity was characterized by the generation of phosphoramidon-inhibitable product (ANA) cleaved from the synthetic substrate succinyl-$(ala)_3$-p-nitroaniline during a 30 min incubation at 37°C. Cell viability was assessed by changes in LDH release, trypan blue exclusion, and cell volume. In some experiments, crude plasma membrane and soluble components of exposed cells were isolated and differential NEP activity was assayed. We found that a 5 min exposure to HOCl decreased whole cell NEP activity from 74.1 ± 4.4 (mean ±SE) to 54.3 ± 6.0 pmoles of ANA/min/10^6 cells ($p < 0.05$), while no parameter of cell viability was affected. NEP activity in the crude membrane fraction decreased 36.3 ± 3.1% after exposure ($p < 0.01$), whereas NEP activity in the soluble fraction increased 4.0 ± 0.6%. Isolated membrane NEP exposed by itself was not affected. Subsequent experiments with reducing agents demonstrated that NEP activity of cell cultures pretreated with 100 mM of either beta-mercaptoethanol or dithiothrietol before HOCl exposure was not significantly different

Offprint requests to: C.G. Murlas

from control values. We conclude that whole cell HOCl exposure decreases Calu-1 plasma membrane NEP. This loss appears to occur by internalization of cell membrane NEP.

Key words: Airway cell culture—Hypohalous acid—Neuropeptides—Tachykinins—Oxidant injury.

Introduction

Neutral endopeptidase (NEP) is an ectoenzyme widely distributed in the human body, including cells of the respiratory system [8, 12, 13]. It is known to cleave a variety of bioactive mediators in vitro [3, 18], and to have substantial effects on airway neutropeptide metabolism in vivo [3]. Recent studies have shown that luminal exposure of airway mucosa to HOCl in vitro causes smooth muscle substance P hyperresponsiveness of intact, but not of mucosa-denuded airway segments [14]. Concomitantly, NEP activity in homogenates of HOCl-exposed, intact airway segments was decreased, suggesting that HOCl decreases airway mucosal NEP.

Although a number of biochemical targets of HOCl are known [2, 7, 12, 17, 19, 21], the subcellular mechanisms responsible for the decrease in airway epithelial cell NEP caused by HOCl are not clear. To verify the expression of NEP by, and the effect of this hypohalous acid on, the human airway epithelial cell line Calu-1, we exposed confluent plates of these cells to HOCl. Experiments were then done to characterize the nature of HOCl effect on Calu-1 cell NEP, and to investigate the possible processes involved.

Methods

Cell Culture

Calu-1 cells (obtained from the American Type Cell Collection [ATCC catalog number HTB-54] and derived from an human lung epidermoid carcinoma) were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in 35 × 10 mm tissue culture dishes at 37°C under 5% CO_2. Culture medium was replaced twice weekly. Passages 27–38 were used for the study, and only after they had reached confluency. When larger amounts of cells were needed for preparation of experiments concerning crude plasma membrane and soluble cytoplasmic fractions, cells were grown in 100 × 20 mm dishes. All experiments were performed at 37°C. Cells were exposed in situ at 37°C to various reagents and/or HOCl unless otherwise noted in the text.

Preparation of HOCl

NaOCl (Sigma Chemical Co. St. Louis, MO) was initially diluted 10 times in 0.1 N KOH and this solution was kept at 4°C as stock solution for no more than 2 weeks, during which time the NaOCl solution was stable. This was further diluted 20 times in a phosphate-buffered saline solution (PBS) and adjusted to pH 7.0 using phosphoric acid before use. For each experiment, the solution of HOCl

in PBS, pH 7.0 was prepared fresh every day and used within 30 min after preparation. $HOCl/OCl^-$ concentration was quantified as previously described [20] and assuming an extinction coefficient of $\varepsilon_{291} = 142$ M/cm for $HOCl/OCl^-$.

Exposure of Whole Cells to HOCl and Reducing Agents

After Calu-1 cells reach confluency, the medium was removed and cells were rinsed twice with 2 ml of PBS, pH 7.0 at room temperature. Two ml of differing concentrations of HOCl in PBS, pH 7.0 were carefully added to each dish (containing approximately 1 million cells) so as not to disturb the cell layer in situ. When cells in larger dishes were exposed, the volume of HOCl solution added per million cells was proportional. The dishes were then incubated at 37°C for planned periods of time. After exposure, cells were rinsed immediately with PBS, pH 7.4, and assayed for NEP activity. Exposure of crude Calu-1 membrane and soluble fractions to HOCl was done by incubating these preparations with different concentrations of HOCl at 37°C for 10 min, followed by overnight dialysis in PBS, pH 7.4 at 4°C. For experiments in which treatment of cells with reducing agents was done before or after HOCl exposure, various concentrations of either beta-mercaptoethanol (β-ME) or dithiothrietol (DTT) were used for 10 min at 37°C. The cells were thereafter rinsed with PBS, pH 7.4, and assayed for NEP activity.

Assay of Calu-1 Cell Neutral Endopeptidase Activity in Situ

Confluent layers of Calu-1 cells were rinsed with PBS, pH 7.4. A volume of 0.5 ml of 0.05 mM SA_3NA (a synthetic substrate) in PBS, pH 7.4, containing 10 μM amastatin was added to each dish. The dishes were incubated at 37°C for 30 min with occasional swirling. Thereafter, 0.2 ml aliquots were withdrawn and 50% cold trichloroacetic acid (TCA) was added to the final concentration of 10% to stop reaction. This reaction mixture was incubated in ice for 20 min and spun in an Eppendorf (5415C) microcentrifuge at 15,000 rpm for 20 min at 4°C. Fifty μl of the clear supernatant was injected onto a Waters high-performance liquid chromatography (HPLC) column (μBondapak C-18 [3.9 × 150 mm], Waters Corp., Milford, MA) for analysis. Cells were trypsinized and counted using a hemacytometer. Enzyme activity determined by high-pressure liquid chromatography (HPLC) was expressed in terms of pmoles of ANA produced per minute per million cells. Differences between control and experimental groups (described here and below) were compared using independent t tests and considered significant for values of $p < 0.05$.

Assay of NEP Activity in the Exposure Solution

After Calu-1 cells were exposed to 100 μM HOCl in PBS, pH 7.0, this HOCl solution was lyophilized and reconstituted in 500 μl of PBS, pH 7.4, and then dialyzed against PBS, pH 7.4, overnight at 4°C to remove HOCl. NEP activity of the reconstituted solution was assayed thereafter.

Neutral Endopeptidase Cleavage Product Analysis by HPLC

Enzyme cleavage products were analyzed on a μBondapak C18-column (3.9 × 150 mm) by a Waters HPLC system (model 600E, Waters Corp., Milford, MA) using a 30 min two-step gradient at flow rate of 1 ml/min. Initial conditions consisted of 10% solvent A (methanol) and 90% solvent B (10 mM NaH_2PO_4, pH 2.5). Within 10 min solvent A was increased to 30% and up to 60% in the next 0.5 min. Solvent A was held at 60% for 8 min and then brought back to initial conditions. The eluent was monitored at 314 nm. Each peak of products was integrated and quantitated by comparison to authentic standards. NEP activity was identified as the substrate cleavage product, ala-p-nitroaniline (ANA), which was phosphoramidon (PHA) inhibitable. All the experiments measuring Calu-1 cell

NEP activity were performed in the presence of 10 μM amastatin (AMS) to eliminate the possible influence of aminopeptidases.

Preparation of Crude Cell Membrane and Soluble Cytoplasmic Fractions Using Sonification; and Preparation of Whole Cell Homogenates

A total of 5.0×10^7 cells in culture dishes were rinsed with PBS, pH 7.0, and exposed in situ to 100 μM HOCl in PBS, pH 7.0 or to PBS, pH 7.0, alone (2 ml per million cells) for 5 min at 37°C. The cells were then rinsed, scraped from the dishes, centrifuged, and resuspended in 5 ml of homogenization buffer. This buffer, kept at 4°C, contained 0.34 M sucrose, 10 mM Hepes, 1 mM EGTA, and 0.1 mM $MgCl_2$. After 4 periods of sonification at 100 watts, each of 15 s duration, nuclei and whole cells were separated by centrifugation at 1,000 g for 10 min. Mitochondria and granules were separated by centrifugation at 10,000 g for 30 min. The supernatant was then centrifuged at 134,000 g for 1 h, yielding a crude plasma membrane pellet and a soluble cytoplasmic supernatant fraction. The crude plasma membrane pellet was resuspended in 1 ml of 50 mM Tris-HCl, pH 7.4 containing 0.1% Triton X-100. For whole cell homogenate preparation, cells were scraped from 100 × 20 mm dishes after planned periods of post-HOCl incubation in culture medium. Cells were rinsed twice with cold PBS, pH 7.4, resuspended in the same solution, and then sonicated at 100 watts 4 times for 15 s. NEP activities of these homogenates were assayed immediately without further processing.

Assay of Neutral Endopeptidase Activity of Crude Membrane and Soluble Fractions

The crude plasma membrane pellet was resuspended in 50 mM Tris-HCl, pH 7.4, and 0.1% Triton X-100. Protein concentrations of both fractions were determined using Bio-Rad protein microassay method. A 90 μl aliquot of each fraction was put into Eppendorf microcentrifuge tubes and 10 μl of 10 mM SA_3NA containing 0.1 mM amastatin was added to each tube. After being incubated at 37°C for 10 min, ice-cold 50% trichloroacetic acid was added to each tube to the final concentration of 10% to stop the reaction. The reaction mixtures were then incubated at 4°C for 20 min to precipitate protein and spun in an Eppendorf microcentrifuge at 15,000 g for 20 min at 4°C. Fifty μl of each of the clear supernatant was injected onto a HPLC column for quantification of hydrolysis products (see above). Enzyme activity was expressed as picomoles of ANA production per min per mg of protein.

Immunoprecipitation of Solubilized NEP of Calu-1 Homogenates

Solubilized Calu-1 cell homogenate (56 μl) was added to 1344 μl of solubilization buffer (50 mM Tris HCl, pH 7.4 + 1% Triton X-100 + 250 mM NaCl) with final protein concentration of 0.34 mg/ml. This solution was mixed with 250 μl of 50% protein A-sepharose in solubilization buffer and incubated at room temperature for 30 min on a rotator. After incubation, this was centrifuged at 16,000 g for 3 min and the supernatant was saved. Aliquots of 300 μl of the supernatant each were placed into 3 clean 1.5 ml Eppendorf microcentrifuge tubes and added to this were 20 μl of solubilization buffer, 1 : 5 diluted normal rabbit serum, or 1 : 5 diluted rabbit antihuman NEP serum. These mixtures were incubated at room temperature on a rotator for 30 min and followed by the addition of 50 μl of 50% protein A-sepharose to each tube and incubation for another 1 hr at room temperature on a rotator. After incubation, these were centrifuged at 16,000 g for 3 min and the supernatants were assayed for NEP activity.

Determination of Cell Viability

Both Trypan blue exclusion and lactate dehydrogenase (LDH) release experiments were conducted using previously described methods. For Trypan blue exclusion, cells were trypsinized (trypsinization itself does not affect cell viability) and resuspended in PBS, pH 7.4, after HOCl exposure. Cell suspension (0.2 ml) was mixed with 0.5 ml of 0.4% Trypan blue solution and 0.3 ml of PBS, pH 7.4, was added to make the final volume to 1.0 ml. This was allowed to stand for 5–15 min. Stained and unstained cells were counted separately utilizing a hemacytometer. The percentage of cell viability was expressed as = total viable cells (unstained)/total cells (stained and unstained) × 100%.

LDH release experiments were performed according to procedure provided by Sigma. Cells were incubated in 1 ml of PBS, pH 7.4, for 30 min at 37°C after exposure to HOCl or PBS. Cells were then scraped, centrifuged, and the supernatant was saved as incubating fluid. Both the HOCl- and PBS-exposed cells were resuspended in 1 ml of PBS, pH 7.4, sonificated at 100 watts for 1 min, and centrifuged again to remove particles and whole cells. The supernatants were saved as total cytoplasmic component. Fifty μl each of both the incubating fluid and the total cytoplasmic component was mixed with 1 ml of LDL-20 assay solution and incubated at room temperature for 1 min. Absorbance at 340 nm was monitored at 0, 30, and 60 s using a spectrophotometer (Beckman). Changes of absorbance at 340 nm was used to calculate LDH activity according to formula provided by manufacturer.

Average cell volume, expressed in cubic microns, was measured after HOCl exposure on a Coulter Channelyzer (model ZBI, Coulter Electronics, Inc., Hialeah, FL).

Statistical Analysis

Each experiment was done with an identical number of control samples. Mean values ± SEM for like groups (both experimental and control) were calculated using independent t tests. Differences between experimental and control groups were considered significant for p values less than 0.05.

Reagents

Dulbecco's modified Eagle medium (DMEM), phosphate-buffered saline solution, penicillin, and streptomycin were obtained from Gibco Laboratories (Grand Island, NY). N-Succinyl-Ala-Ala-Ala-p-nitroanilide (SA_3NA), ala-p-nitroaniline (ANA), p-nitroanilide (NA), phosphoramidon, amastatin, fetal bovine serum, β-mercaptoethanol and dithiothrietol, trigon X-100, trichloroacetic acid (TCA), LDL-20 lactate dehydrogenase diagnostic kit, and NaOCl were all obtained from Sigma Chemical Co. (St. Louis, MO). Protein A-sepharose was obtained from Pharmacia LKB Biotechnology Inc. (Pleasant Hill, CA). Bio-Rad dye reagent concentrate for protein assays was purchased from Bio-Rad Laboratories (Richmond, CA). Polyclonal rabbit anti-human NEP antiserum was a generous gift from Drs. R. Skidgel and E. Erdös of the University of Illinois at Chicago.

Results

HOCl Effect on Whole Cell Calu-1 NEP Activity (and Viability)

Calu-1 cells demonstrated NEP activity of 74.1 ± 4.4 pmoles of ANA/min/10^6 cells. To exclude the possibility that this activity was due to nonspecific

Table 1. Effect of inhibitors on Calu-1 NEP

Inhibitors	Calu-1 NEP activity (% of control)
10 μM phosphoramidon	7.7 ± 0.2**
10 μM amastatin	188.5 ± 2.7**
10 μM phosphoramidon + amastatin	5.9 ± 0.4**

Effects are expressed as percentage of control group (no inhibitors added). Values shown are means ± SE (n = 6). Calu-1 cells were preincubated in the presence or absence of inhibitors, and then assayed for NEP activity of whole cells in situ as described in the Methods section. Results are expressed as percent control activity (without inhibitors).

** Significantly different from control values ($p < 0.01$).

hydrolysis of SA_3NA by other peptidases that the cells may contain, we compared activities (Table 1) in the presence or absence of the NEP-specific inhibitor, phosphoramidon (PHA). In the presence of 10 μM PHA, the formation of analine-p-nitroalinine (ANA), a catabolic product of NEP on SA_3NA, was almost totally inhibited. In the presence of amastatin alone, ANA production increased by 88.0 ± 2.7% compared to that of cells alone. This evidences the fact that although aminopeptidases itself does not cleave SA_3NA, the ANA produced by NEP from the substrate is further cleaved by aminopeptidases. The combination of both inhibitors had the same effect as PHA alone (Table 1).

In additional experiments, this phosphoramidon-inhibitable activity on Calu-1 cells was confirmed immunologically to be NEP activity. Solubilized homogenates from these cells were incubated with rabbit antihuman NEP polyclonal antiserum, and precipitated by protein A-sepharose. The supernatant of this immunoprecipitation reaction was assayed for NEP activity. This antiserum totally eliminated NEP activity from the solubilized homogenates whereas normal rabbit serum had no effect (Table 2), thereby confirming that the PHA-inhibitable activity we observed was NEP activity.

Fig. 1 shows the effect of a 5 min HOCl exposure on Calu-1 cell NEP activity. Exposures to 100 μM HOCl decreased NEP activity by 26.7 ± 3.6%. HOCl concentrations of 40 μM or less had no influence on activity. Concentrations greater than 100 μM caused cell lysis. The influence of 100 μM HOCl exposure duration on NEP activity is shown in Fig. 2. There was no significant difference in the effects caused by exposure ranging from 5 to 20 min in duration. However, exposures in excess of 20 min caused cell lysis.

The exposure solutions were also assessed for NEP activity. We were not able to detect any NEP activity in the exposure solutions even after they were concentrated about 1000-fold by lyophilization and dialysis (data not shown).

Table 2. Immunoprecipitation of Calu-1 NEP from solubilized, whole cell homogenates

Antibody	Calu-1 NEP activity (% of control)
Normal rabbit serum	96.7 ± 1.2
Rabbit anti-human NEP serum	0.0 ± 0.1**

Effects are expressed as percentage of control group (buffer only). Values shown are means ± SE (n = 6). Solubilized Calu-1 whole cell homogenates were incubated with normal rabbit serum, rabbit anti-human NEP serum, or solubilization buffer only. This was followed by precipitation with protein A-sepharose, and determination of NEP activities in the supernatants of the immune precipitation reactions as described in the Methods section. Results are expressed as percent control activity (buffer only).

** Significantly different from control value ($p < 0.01$).

Assessment of post-HOCl cell viability by Trypan blue dye exclusion, cell LDH release, and changes in cell volume is shown in Table 3. After Calu-1 cells exposure to 100 μM HOCl, these cells were incubated with 0.2% Trypan blue for 5–15 min at room temperature, and both viable and nonviable cells were counted. The percentage viability was compared to unexposed control cells. Five minute exposures to 100 μM HOCl did not affect cell viability: 89.6 ± 1.0% and 90.6 ± 1.0% of HOCl-exposed and PBS-exposed cells were viable, respectively. In comparison, 60 min exposures decreased viability to 61.0 ± 3.5%. For assessment of LDH release, cells were incubated at PBS, pH 7.4 for 30 min at 37°C after exposure to 100 μM HOCl for 5 and 60 min. The LDH activity of the homogenate of unexposed cells was exposed in relation to total cellular LDH activity. No significant difference was found when the total cellular LDH activities were compared between cells exposed to HOCl and PBS, pH 7.0. As shown in Table 3, the incubating fluids from both control and 5 min HOCl-exposed cells contained less than 1.25 ± 0.4% of total cellular LDH

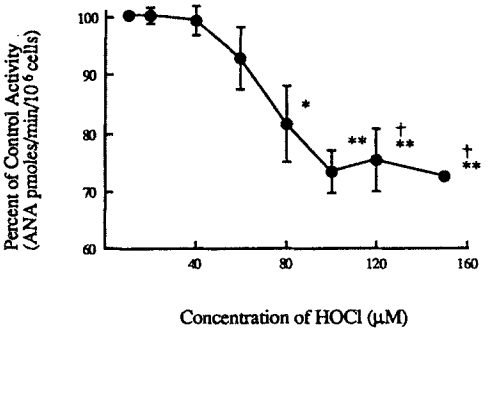

Fig. 1. Effect of various concentrations of HOCl on Calu-1 cell NEP activity. Values shown are means ± SE (n = 6). Approximately 1 × 10^6 Calu-1 cells per dish were exposed to various concentrations of HOCl for 5 min at 37°C and NEP activity was assayed as described in METHODS. Results are expressed as percent of control values (unexposed). Single and double asterisks indicate values significantly different from controls ($p < 0.05$ and 0.01, respectively). †Cell lysis was observed at these concentrations.

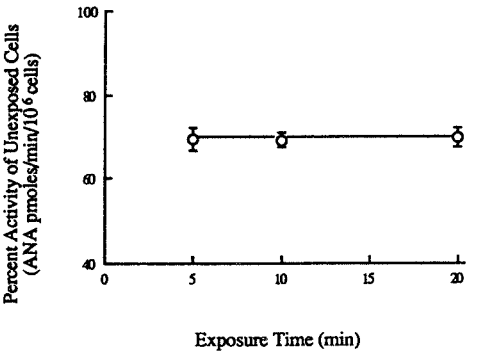

Fig. 2. Effect of various times of HOCl exposure on Calu-1 cell NEP activity. Values shown are means ± SE (n = 6). Approximately 1 × 10^6 cells per dish were exposed to 100 μM HOCl for 5, 10, or 20 min at 37°C and followed by assay of whole cell NEP activity as described in Methods section. Results are expressed as percentage activity of unexposed cells. There was no difference in the effect of 5, 10, and 20 min HOCl exposures on whole cell NEP activity.

activity. In comparison, 60 min exposures caused an increase of 21.0 ± 4.7% in LDH release in the incubating fluid. To determine whether cell volume was affected by 100 μM HOCl exposure, cells were exposed for 5 min and 60 min at 37°C. There was no significant increase in cell volume after HOCl-exposure for 5 min (Table 3). However, 60 min exposures increased cell volume 11.0 ± 2.2%.

Fig. 3 shows the results of experiments in which Calu-1 cells were treated with various concentrations either of β-mercaptoethanol (β-ME) or dithiothrietol (DTT) for 10 min prior to HOCl exposure. It was found that either 100 mM β-ME or DTT protected cells from HOCl damage, although DTT was less protective. Treatment with either agent after HOCl exposure had no protective effect (Fig. 3). Treatment of cells with reducing agents alone for 10 min did not affect Calu-1 NEP activity.

Table 3. HOCl effects on cell viability and volume

Experimental conditions	Experiments		
	Trypan blue exclusion (% of total)	LDH release (% of total)	Cell volume (μm^3)
PBS, pH 7.4, 5 min (control)	90.6 ± 1.0 (n = 8)	1.2 ± 0.0 (n = 6)	1521 ± 13 (n = 6)
100 μM HOCl, 5 min.	89.6 ± 1.0 (n = 14)	1.3 ± 0.4 (n = 6)	1537 ± 21 (n = 6)
100 μM HOCl, 60 min.	61.0 ± 3.5* (n = 6)	21.0 ± 4.7* (n = 6)	1679 ± 34* (n = 6)

Values shown are means ± SE for n indicated. Cell viability in each experimental condition was determined by Trypan blue exclusion and LDH release assays as described in the Methods section. Cell volume measurements were made using a Coulter counter.
*Significantly different from control values ($p < 0.05$).

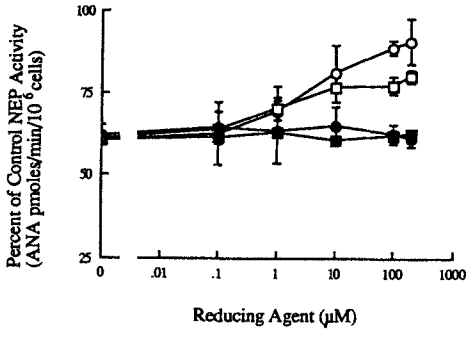

Fig. 3. Effects of reducing agents on HOCl exposure. Values shown are means ± SE (n = 6). Cells were incubated with various concentrations of reducing agents: β-mercaptoethanol (circles); dithiothrietol (squares) at 37°C for 10 min before (open) or after (solid) exposure to 100 μM HOCl. Calu-1 cell NEP activities were then assayed as described in Methods section. In contrast to pre-HOCl treatment, reducing agent treatment post-HOCl had no protective effect.

Recovery in NEP Activity After Sonification of HOCl-Exposed Cells; and Effect of HOCl on Crude Cell Membrane NEP

To assess whether NEP activity was recoverable from cell sonicates after HOCl, cells were exposed to 100 μM HOCl for 5 min, and subsequently harvested 30 min after exposure for sonification and enzyme assay (n = 5). NEP activity of these sonicates was 94.2 ± 5.3%, a value comparable to that for the control group (n = 5). In comparison, as stated above, whole cell enzyme activity was significantly decreased by the same HOCl exposure ($p < 0.05$).

The effect of 100, 200, and 400 μM HOCl on isolated Calu-1 cell crude membrane NEP incubated for 10 min at 37°C is shown in Fig. 4. To eliminate the possibility that Tris buffer or detergent could affect the oxidizing potential of HOCl, the crude membrane was suspended in PBS, pH 7.4, rather than being solubilized with Triton X-100. HOCl was removed by dialysis overnight in PBS, pH 7.4. Activity of both the HOCl-exposed and PBS-exposed enzymes was assayed. In comparison to the inhibitory effect of HOCl on Calu-1 cell NEP, NEP activity in the isolated crude membrane preparation was not affected by exposure to the oxidant.

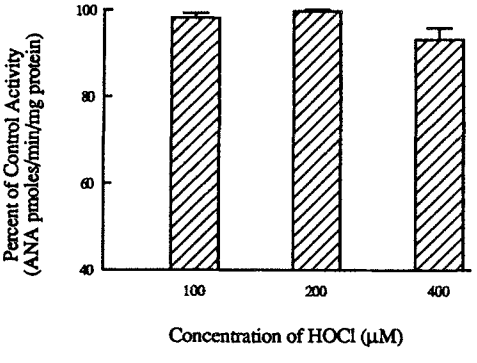

Fig. 4. HOCl influence on crude membrane Calu-1 NEP. Values shown are means ± SE (n = 3). Isolated, crude membrane NEP was incubated with various concentrations of HOCl for 10 min at 37°C. After exposure, HOCl was removed by overnight dialysis in PBS, pH 7.4. NEP activities were determined as described in Methods section. Results are expressed as percentage of control activity (unexposed). HOCl did not decrease isolated plasma membrane NEP activity.

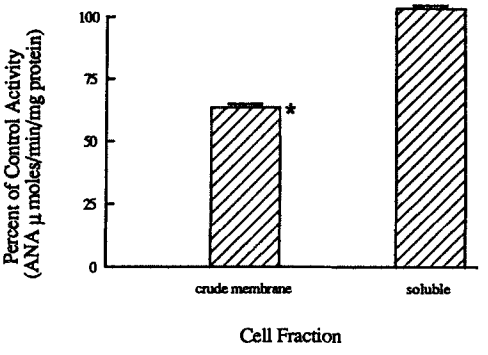

Fig. 5. Influence of HOCl on different fractions of cell NEP activity. Values shown are means ± SE (n = 3). Calu-1 cells were exposed to 100 μM HOCl or PBS, pH 7.0 for 5 min at 37°C. NEP activity of the whole cell homogenates were measured as described in Methods section. Results are expressed as μmoles of ANA produced per min per mg protein. *Significantly different from control values ($p < 0.01$).

Localization of HOCl Effect on Calu-1 NEP

After HOCl exposure, cell membrane and cytoplasmic components were separated as described in the Methods section. Normally, the crude cell membrane and soluble cytoplasmic fractions contain approximately 10% and 90% of the total cellular protein, respectively. NEP activity of the membrane fraction derived from HOCl-exposed cells was decreased by 36.3 ± 3.1% compared to control values (Fig. 5). This percentage decrease in NEP activity was similar to that seen in whole cell assays. NEP activity in the cytoplasmic fraction was increased by 4.0 ± 0.6% after HOCl exposure (Fig. 5). Because NEP activities of both fractions was expressed as pmoles of ANA produced per min per mg protein, this means that the total loss in NEP activity of the membrane fraction was similar in amount to the total increase in NEP activity seen in the soluble cytoplasmic fraction after HOCl exposure.

Discussion

As in normal respiratory epithelial cells from other species in our experience [12, 14], our study demonstrates that plasma membranes of the Calu-1 cell line are rich in NEP activity. We have also shown that HOCl exposure of whole Calu-1 cells in situ leads to a substantial decrease in whole cell NEP activity. This effect occurs within 5 min after oxidant exposure, although cell viability within 30 min of exposure is not affected. The degree of decrease in activity of crude membrane NEP exposed to HOCl was similar to that seen in whole cells exposed in situ. Having separated the crude cell membrane and soluble cytoplasmic fractions after exposure, we also found that the decrease in NEP activity of the membrane fraction caused by HOCl is accompanied by a commensurate increase in the cytoplasmic fraction. In other words, the loss in cell membrane NEP activity caused by HOCl exposure could be recovered after cell sonification. These results suggest to us that HOCl induces the internalization of NEP from the plasma membrane surfaces of Calu-1 cells.

In comparison to the effect of HOCl on whole cell NEP activity,

concentrations of HOCl as high as 400 μM did not significantly decrease NEP activity in the membrane fraction derived from these cells. This suggests that the effect of HOCl on Calu-1 cell NEP is not due to simple chemical modification of the enzyme itself. Although HOCl is an extremely powerful oxidant that may attack a wide range of molecular targets (including amines, amino acids, thiols, thioethers, nucleotides, hemo-proteins, and polyenoic acids [22–24]), it appears that oxidation of NEP itself by as much as 400 μM HOCl does not affect its catalytic activity.

The possibility that HOCl exposure leads to the direct or indirect proteolytic cleavage of NEP also seems unlikely in that one would have expected evidence that ectoenzyme was separated from the cell surface [8, 11] if this was true. The fact that we found no NEP activity in cell supernatants argues against this possibility. Studies by other investigators also argue against this contention. Drozdz [2] and colleagues showed that HOCl did not split peptide bonds of the pentapeptide Leu-Trp-Met-Arg-Phe-COOH, although individual amino acids of the molecule were oxidized by it.

In comparison to the Calu-1 cell line, studies of the marine macrophage-like tumor cell line P388D1 indicate that low concentrations of HOCl (10–20 μM) have more profound cellular consequences in these cells [17]. The reported plasma membrane-associated effects include oxidation of plasma sulfhydryls, glucose and aminoisobutyric acid uptake inactivation, intracellular K^+ loss, and cell volume increase. It was also found that higher doses of HOCl (>50 μM) led to the oxidation of sulfhydryl groups, of methionine and tryptophan residues, and to the formation of protein carbonyls, and cell lysis [20].

Calu-1 cells appear to be much more resistant to HOCl. Concentrations of this acid less than 80 μM had no significant inhibitory effect on Calu-1 cell NEP. Furthermore, there was no difference in cell viability between HOCl-exposed and unexposed within 30 min after exposure as assessed by either trypan blue exclusion, lactate dehydrogenase (LDH) release, or cell volume changes. These results may result from the relatively short exposure time chosen, or from the heartiness of the cells used.

Whereas treatment before exposure with either 100 mM beta-mercaptoethanol or dithiothreitol prevented NEP inactivation, treatment with either after exposure had no protective effect. Similar observations have been made by other investigators [17]. Although we thoroughly rinsed the cells after pretreatment and prior to HOCl exposure, it is still likely that this protective effect of reducing agents was due to a direct effect on HOCl as reported by others [17].

Our results lead us to speculate that the decrease in NEP activity on Calu-1 plasma membranes after HOCl exposure is caused by HOCl-induced cell endocytosis. Oxidation of certain enzyme determinants, such as the sulfhydryl groups of cysteine and methionine, may be a signal for this process to ensue. Cells may respond to such damage by turning over the oxidized ectoenzyme. Analogous processes in monoclonal antibody-modulated normal adult bone marrow cells, fibroblasts, and cells of several nonhematopoietic human tumor cell lines have been reported [15, 16]. In these studies, CD10 (which has been shown to be identical to NEP [13] and to the common acute lymphoblastic

leukemia antigen (CALLA [9, 13]) appeared to be rapidly internalized and degraded by cells in culture. Such antigenic modulation of CD10 has been likened to the down-regulation or loss of certain cell surface receptors that can be induced by some peptide hormones [1, 4–6, 10, 15, 16]. In human neutrophils, other investigators have shown that both anti-NEP antibody and protein kinase C activators, such as phorbol ester or diacylglycerol, can induce the endocytosis of plasma membrane NEP [4].

Although the molecular mechanisms remain unknown, our data suggest that Calu-1 cells sense HOCl attack and respond by internalizing NEP, possibly by endocytosing it in an active form initially. NEP activity is fully recoverable from the cell cytoplasm within 30 min after HOCl exposure and cannot be detected in cell culture supernatants. This argues against the possibility that HOCl causes the release or shedding of NEP from cell surfaces. In Calu-1 cells, the subsequent fate of NEP internalized after HOCl exposure appears to differ from that caused by other agents' action on different cell types that lead to NEP inactivation shortly after its endocytosis [4, 15, 16]. Because of its potential relevance to the pathogenesis of various airway diseases, the regulation and ultimate fate of NEP oxidized on the surface of respiratory epithelial cells merit further investigation.

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