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Research Article

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Mechanisms of Hypochlorite Injury of Target Cells

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Abstract

HOCl, which is produced by the action of myeloperoxidase during the respiratory burst of stimulated neutrophils, was used as a cytotoxic reagent in P388D1 cells. Low concentrations of HOCl (10–20 μM) caused oxidation of plasma membrane sulfhydryls determined as decreased binding of iodoacetylated phycoerythrin. These same low concentrations of HOCl caused disturbance of various plasma membrane functions: they inactivated glucose and aminoisobutyric acid uptake, caused loss of cellular K^+, and an increase in cell volume. It is likely that these changes were the consequence of plasma membrane SH-oxidation, since similar effects were observed with *para*-chloromercuriphenylsulfonate (*p*CMBS), a sulfhydryl reagent acting at the cell surface. Given in combination *p*CMBS and HOCl showed an additive effect.

Higher doses of HOCl (> 50 μM) led to general oxidation of -SH, methionine and tryptophan residues, and formation of protein carbonyls. HOCl-induced loss of ATP and undegraded NAD was closely followed by cell lysis. In contrast, NAD degradation and ATP depletion caused by H_2O_2 preceded cell death by several hours. Formation of DNA strand breaks, a major factor of H_2O_2-induced injury, was not observed with HOCl.

Thus targets of HOCl were distinct from those of H_2O_2 with the exception of glyceraldehyde-3-phosphate dehydrogenase, which was inactivated by both oxidants. (*J. Clin. Invest.* 1990. 85:554–562.) hypochlorite toxicity • plasma membrane protein oxidation • sulfhydryls

Introduction

When neutrophils are stimulated they release oxidants that serve as antimicrobial weapons but at the same time may cause injury of the surrounding tissue (1–4). Both H_2O_2 (5–8) as well as neutrophil derived proteases (9, 10) have been shown to induce injury in various target cells. HOCl formed from H_2O_2 by the action of myeloperoxidase has similarly been shown to be cytotoxic (11, 12). Because a minimum of 25–40% (13, 14) of the H_2O_2 formed by stimulated human PMNs is halogenated into HOCl, it seemed important to define the mechanisms involved in HOCl-induced impairment of cell function.

Bacteria exposed to HOCl are killed within milliseconds after the addition of the oxidant (15, 16). Inhibition of bacterial membrane transporters (17) as well as damage to their respiratory enzymes (18, 19) have been described as possible pathways for HOCl-mediated bacterial killing. Although a number of biochemical targets of HOCl are known (thioethers, amino-, heme-groups, etc.) (20–23), the pathways leading to cell injury in mammalian cells are poorly understood. The current studies were therefore undertaken to define the biochemical targets of HOCl and to analyze the effect of their oxidation on cellular functions.

Methods

Cell culture. P388D1 murine macrophage-like tumor cells were grown in RPMI 1640 as previously described (24). The cells were centrifuged at 400 *g* for 5 min, washed two times in modified Gey's buffer (MGB,[1] 147 mM NaCl, 5 mM KCl, 1.9 mM KH_2PO_4, 1.1 mM Na_2SO_4, 5.5 mM glucose, 1.5 mM $CaCl_2$, 0.3 mM $MgSO_4$, and 1 mM $MgCl_2$, pH 7.4), and resuspended in that buffer at a concentration of 4×10^6 cells/ml. To avoid high local concentrations of HOCl, HOCl was added to MGB, immediately followed by the addition of cells to a final concentration of 2×10^6 cells/ml. All experiments were performed at 37°C. In some experiments MGB was replaced with 150 mM choline chloride (Calbiochem-Behring Corp., La Jolla, CA), 5.5 mM glucose, pH 7.4.

Cell lysis was determined by trypan blue exclusion or LDH release.

Preparation of HOCl. NaOCl (Mallinckrodt, Inc., St. Louis, MO) was adjusted to pH 7.4 with H_2SO_4. The sum of $HOCl/OCl^-$ was quantified by assuming an extinction coefficient of $e_{235} = 100\ M^{-1}\ cm^{-1}$ for HOCl, which accounts for 50% at the pH of 7.4 (16) or by iodometric titration (14).

Determination of protein sulfhydryls and disulfides. 2×10^6 cells were exposed to HOCl for 15 min and then processed for sulfhydryl determination as follows.

Cellular protein was precipitated with 400 μl 50% TCA, samples were left on ice for 10 min, microfuged for 1 min, and washed twice with 7% TCA. For reduced sulfhydryl determination, the precipitate was resuspended by sonication in 2 M guanidine thiocyanate, 500 mM Tris, 10 mM EDTA, pH 7.6, 100 μM dithionitrobenzoic acid (DTNB; Sigma Chemical Co., St. Louis, MO) (25, 26); sulfhydryls reduce DTNB forming yellow colored TNB. Alternatively, resuspension was in 2 M guanidine thiocyanate, 50 μM glycine, 100 mM sodium sulfite, 3 mM EDTA, 100 μM 2-nitro-5-thiosulfobenzoate (NTSB), pH 9.5 (27). The sulfite cleaves disulfide bonds, such that the sum of -SHs and disulfides is measured by reduction of NTSB.

The samples were incubated for 25 min at room temperature and the $OD_{415/550}$ nm was measured on titertek plates on 200-μl aliquots (Autoreader, EL 310; Biotek, Inc., Burlington, VT). Molarities were calculated assuming an extinction coefficient of $e_{415} = 12{,}200\ M^{-1}\ cm^{-1}$ for TNB.

Disulfides were calculated as the difference between the two determinations.

Determination of extracellularly accessible sulfhydryls. For ^{203}Hg-*p*CMBS binding, 2×10^6 cells/ml were incubated with 5 nmol *p*CMBS

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1. *Abbreviations used in this paper:* Di-O-C_5, 3,3'dipentyloxacarbocyanine; DOG, 2-deoxyglucose; DNTB, dithionitrobenzoic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; I-PE, iodoacetylated phycoerythrin; MGB, modified Gey's buffer; MPO, myeloperoxidase; NTSB, 2-nitro-5-thiosulfobenzoate; poly-ADPRP, poly-ADP-ribose polymerase; *p*CMBS, *p*-chloromercuriphenylsulfonate.

containing 50 nCi ^{203}Hg-*p*CMBS (Amersham Corp., Arlington Heights, IL; 10–100 mCi/g Hg). After a 5-min incubation at 37°C, 200-μl aliquots of cell suspension were centrifuged through silicone oil as described for amino acid uptake. *p*CMBS as a charged molecule (28) does not penetrate into uninjured cells.

Since *p*CMBS binding is affected by plasma membrane permeability changes, a larger size sulfhydryl reagent was needed to quantify extracellular -SH groups in HOCl damaged cells: iodoacetylated R-phycoerythrin (I-PE, mol wt 240,000; Molecular Probes, Eugene, OR) was used for this purpose. 1×10^6 P388D1 cells/500 μl MGB were incubated with HOCl for 10 min at 37°C. 5 μl 0.5 M Tris, pH 8.5 was added to bring the pH to 7.9, followed by 11.1 μg/sample of I-PE. This concentration of phycoerythrin label had been titrated to yield maximal binding. The cells were incubated for 30 min at room temperature, and phycoerythrin binding was determined by FACS analysis (FACS IV, Becton Dickinson Co., Mountain View, CA) using rhodamine filters. To subtract background fluorescence, cells were exposed to 16 mM β-mercaptoethanol before the addition of I-PE, which completely prevented labeling of the cells.

Glutathione determination. Total and oxidized glutathione were determined as previously described except that the samples were assayed on microtitertek plates (29). GSH was calculated as the difference between the two determinations.

Determination of protein methionine sulfoxide formation. P388D1 cells were labeled with 10 μCi [^{35}S]methionine (600 Ci/mmol; New England Nuclear, Boston, MA) for 18 h. After two washes in MGB, the cells were exposed to HOCl for 15 min at 37°C, and protein was precipitated by TCA. The samples were boiled for 10 min, microfuged, and washed twice with TCA to remove free methionines. Cell precipitates were solubilized in 70% formic acid and digested with 30 mM cyanogen bromide for 20 h. Cyanogen bromide cleaves proteins at methionine residues forming volatile isothiocyanate, whereas oxidized methionine is not reactive with cyanogen bromide. ^{35}S-label was determined before and after removing [^{35}S]isothiocyanic acid by volatilization under N_2, and label in methionine was calculated from the difference. Label in methionine sulfoxide was calculated from the amount of label that was not volatilized (20).

Determination of tryptophan fluorescence. Cells were TCA precipitated as described for sulfhydryl determination, solubilized in SDS, and fluorescence (280 nm excitation, 345 nm emission) (30) was determined on a fluorometer (Perkin-Elmer Corp., Norwalk, CT). Tryptophans were quantitated by comparison with a bovine albumin standard, assuming 2 tryptophans/albumin molecule.

Determination of protein carbonyls. Protein carbonyls were determined as described by Starke et al. (31) except that whole cell sonicate rather than only the supernatant fraction was TCA precipitated. In this assay dinitrophenylhydrazine forms hydrazone products with aldehydic protein groups.

Determination of amino acid uptake. [^{3}H]aminoisobutyric acid was used as a nonmetabolized amino acid analogue. Cells were exposed to HOCl for 15 min, microfuged, and resuspended in 10 μM aminoisobutyric acid containing 2 μCi [^{3}H]aminoisobutyric acid (New England Nuclear) and incubated for 30 min at 37°C. 200-μl cell aliquots were microfuged through 150 μl silicone oil (four parts Dow Corning 550, one part Dow Corning 200; Dow Corning Co., Midland, MI) into 10 μl 0.5 M sucrose. The cell pellets were scintillation counted. The same procedure was followed for [^{3}H]leucine uptake, replacing aminoisobutyric acid with leucine and stopping the incubation with label at 8 min.

Measurement of glucose transporter activity. Glucose transporter activity was determined with [^{3}H]2-deoxyglucose±cytochalasin B in the presence of 100 μM glucose as previously described (32).

Determination of cell volume. P388D1 cells were exposed to HOCl for various periods of time, 10 μg/ml propidium iodide were added, and the cell volume/viability was determined on a FACS analyzer using a 75-μm orifice with a current setting of 0.71 mA for the volume measurement. The cell volume was assessed by calibration against 4.4-, 6.0-, 9.0-, and 15.67-μm diam beads (Microbeads; Coulter, Hialeah, FL).

^3H_2O space was determined as previously described (32).

Measurement of cellular ion content and fluxes. Total cellular K^+, Na^+ and Ca^{2+} content were determined by atomic absorption as previously described (33).

Cellular Na^+ content was measured with the fluorescent probe SBFI-AM (34) (1 mM in DMSO; Molecular Probes). 1×10^7 cells/ml of P388D1 cells were incubated with 10 μM SBFI-AM for 30 min at 37°C. After two washes in MGB, 2×10^6 cells/ml were incubated with HOCl for 30 min at 37°C. SBFI fluorescence ratios were determined on a fluorometer (650-15; Perkin-Elmer) with excitation at 340 and 380 nm and emission at 510 nm. Free SBFI and various ratios of MGB and its Na^+-free K^+ equivalent were used for calibration.

K^+ efflux was determined with ^{86}Rb (New England Nuclear). 100 μCi ^{86}Rb was added to a 150-ml flask of P388D1 cells 24 h before harvest. The cells were centrifuged at 4°C, washed twice with MGB at 4°C, and used immediately. They were incubated at 37°C, 200-μl aliquots were layered over silicone oil (see above), and microfuged for 30 s; 100 μl of supernatant as well as of cell suspension were counted in a scintillation counter.

Preparation of plasma membranes. 10^8 cells were exposed to various concentrations of HOCl for 15 min, the cells were centrifuged and resuspended in 5 ml 0.34 M sucrose, 10 mM Hepes, 1 mM EGTA, 0.1 mM $MgCl_2$, and 1 mM ATP, and kept at 4°C throughout the preparation. After N_2 cavitation for 15 min at 600 psi, nuclei, and whole cells were removed 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 cytoplasmic supernatant fraction.

Determination of plasma membrane ATPase activity. 50 μg plasma membrane protein determined with the BCA reagent (Pierce Chemical Co., Rockford, IL) was incubated with 6 mM ATP±0.2 mM ouabain (Sigma Chemical Co.), ±5 mM EGTA for 1 h at 37°C. Inorganic PO_4 concentrations were determined (35) and compared to a lyophilized K_2HPO_4 standard.

Determination of various cellular moieties. ATP and NAD were quantified by HPLC as previously described (36, 37). When extracellular nucleotides were determined, the supernatant after removal of the cells by centrifugation was spiked with [^{3}H]adenine, the samples were lyophilized, resuspended in H_2O in 1/10 their original volume, recovery was determined by scintillation counting, and the samples were used in HPLC analysis.

Mitochondrial respiration was measured with a Clark type O_2 electrode (32).

Lactate formation was assessed by determination of [^{14}C]lactate produced over a 30-min period at 37°C from a medium containing 5 μCi/ml U[^{14}C]glucose (32) and 100 μM glucose. At the end of this period cells were permeabilized with 0.01% digitonin, microfuged at 4°C, and an aliquot of the medium was applied to silica 60 F254 TLC plates (EM Science, Gibbstown, NJ), separated in H_2O/ethanol/NH_4OH (4:20:1) (38), and quantitated on the AMBIS beta scanning system (AMBIS Systems, San Diego, CA).

GAPDH and hexokinase activities were determined as described (32, 39). When GAPDH activity was determined in *p*CMBS-treated cells, the cells were washed twice to remove traces of extracellular *p*CMBS. DNA strand breaks were measured by alkaline unwinding (37, 40), and poly-ADP-ribose polymerase activity was measured as TCA precipitable [^{3}H]NAD-derived label of digitonin-treated cells (36, 41).

Ammonia was determined with a Sigma kit (Sigma Chemical Co.; No. 70-UV).

Results

HOCl-induced cell lysis

A dose-response assay of the quantity of HOCl inducing cell lysis as a function of time is presented in Fig. 1. Doses as low as

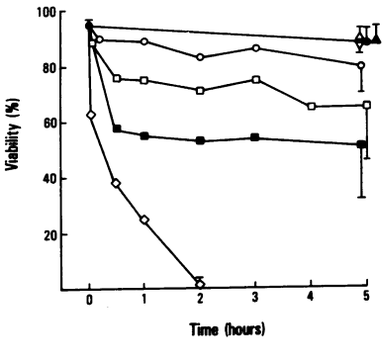

Figure 1. Time-course of cell lysis—as determined by trypan blue exclusion—in 2×10^6 P388D1 cells/ml exposed to various concentrations of HOCl. Mean±SD of at least three experiments. For reasons of clearness, SDs are shown for selected time-points only. ●, control cells; ○, 25 μM HOCl; □, 35 μM HOCl; ■, 50 μM HOCl; ◇, 75 μM HOCl; ▲, 50 μM DTT + 50 μM HOCl; △, 50 μM glycine + 50 μM HOCl; ▽, 50 μM methionine + 50 μM HOCl.

75 μM HOCl were found to lyse all cells. No cell lysis was observed with < 20 μM HOCl over a period of up to 6 h.

Addition of 50 μM DTT, glycine or methionine added before a bolus of 50 μM HOCl completely prevented HOCl-induced cell lysis. Addition of the same scavenging species as little as 30 s after the exposure to HOCl had no protective effect.

For comparison, 200 μM NH_2Cl caused 5% and 300 μM 32% cell lysis at 6 h, ~ 10% of the effect of HOCl. Up to 300 μM taurine chloramine (42) did not induce any lysis over a period of 6 h.

LDH release was always less sensitive than trypan blue exclusion: a concentration of HOCl that caused trypan blue uptake in 60% of the cells, only led to a 25% loss of cellular LDH.

Oxidation of protein moieties

Sulfhydryl oxidation. To define biochemical targets of HOCl toxicity, various protein moieties of HOCl-treated cells were analyzed.

Protein sulfhydryls were particularly sensitive to oxidation by HOCl. When total cellular sulfhydryls were determined, a decrease of -SH groups was observed with a threshold of 50–60 μM HOCl (Fig. 2). The loss of -SH groups could be completely accounted for by disulfide formation up to HOCl concentrations of 120–150 μM. With higher concentrations of HOCl these disulfides, too, disappeared. Addition of 1 mM methionine before the HOCl completely prevented -SH oxidation.

Glutathione became oxidized with the same concentrations of HOCl as total acid precipitable sulfhydryls (Fig. 2).

Due to the high reactivity of HOCl it was assumed that -SH groups of the plasma membrane were oxidized with lower concentrations of HOCl. Extracellularly accessible -SHs, measured by binding of I-PE, decreased with as little as 10 μM HOCl, and a maximal decrease was seen at 20–30 μM HOCl (Fig. 2). *p*CMBS decreased binding of I-PE in a similar concentration range as HOCl.

The absolute amount of extracellularly accessible -SHs in control cells was determined as 25.0±4.9 pmol/10^6 cells by [^{203}Hg]*p*CMBS binding.

Oxidation of methionine

The dose response for methionine sulfoxide formation was identical to that for cysteine oxidation (Fig. 3). In control cells 8.1±0.35% of [^{35}S]methionine incorporated into protein was present as methionine sulfoxide. This percentage increased to

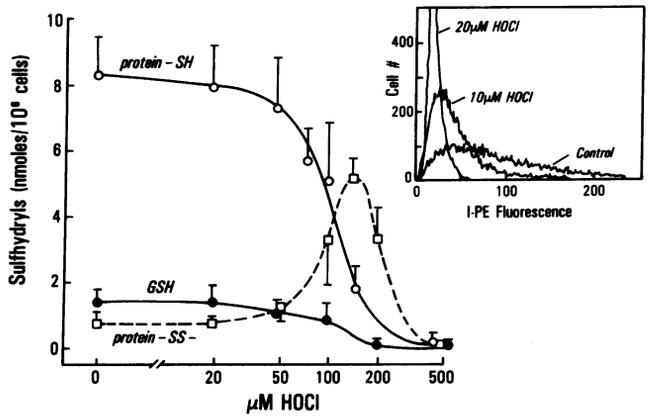

Figure 2. Sulfhydryl oxidation in P388D1 cells by HOCl. 2×10^6 P388D1 cells were exposed to doses of HOCl as shown on the abscissa for 15 min at 37°C. The cells were then assayed (*a*) for total protein sulfhydryls ○, (*b*) for total protein sulfhydryls and disulfides; the difference between the two assays represents disulfides □, disulfide concentrations are represented as -SH equivalents, and (*c*) for GSH ●. (*Inset*) Determination of extracellularly accessible -SH groups: histogram of I-PE binding as described in Methods.

13.0±0.53% after a 15-min exposure to 20 μM HOCl, and to 45.0±1.2% with 100 μM HOCl.

Plasma membrane methionines were oxidized with smaller concentrations of HOCl than the bulk methionine content of the cell (Table I).

Oxidation of tryptophans

Tryptophans were also oxidized by HOCl, although slightly higher concentrations were needed (Fig. 3). Assuming that 280 nm fluorescence of acid cell precipitates is tryptophan specific, control cells contained the equivalent of 2.27±0.21 nmol tryptophans/10^6 cells, whereas cells exposed to 100 μM HOCl contained 1.74±0.45 nmol tryptophan after 15 min exposure.

Formation of protein carbonyls. Formation of protein carbonyls was observed with similar concentrations (Fig. 3) of HOCl, presumably due to deamination after chloramine formation of NH_2-terminal amino acids and lysine residues (43).

Effect of HOCl on plasma membrane transporter function. Since HOCl is a potent -SH oxidizing reagent and since reduced thiols are essential for the function of various cellular transport systems (44–46), the effect of HOCl on some of these transporter activities was assessed.

Table I. Protein Methionine Sulfoxide Formation Induced by HOCl in Various Cellular Fractions

μM HOCl	p.m.	Cytoplasm	mito.	Nuclei
0	16.2±1.9	14.0±2.6	12.8±3.8	13.3±3.7
35	27.7±8.3	16.7±4.7	21.6±8.0	20.0±7.4
50	32.5±11.2	21.5±7.8	26.9±8.9	22.6±6.3

Plasma membranes (p.m.), cytoplasm, mitochondria and granules (mito.) and nuclei (+ some whole cells); 5×10^7 [^{35}S]methionine-labeled P388D1 cells were exposed to HOCl for 15 min, followed by cell fractionation and cyanogen bromide digestion as described in Methods. Results are expressed as percent [^{35}S]methionine not cleaved by cyanogen bromide. Mean±SD for four experiments in duplicate.

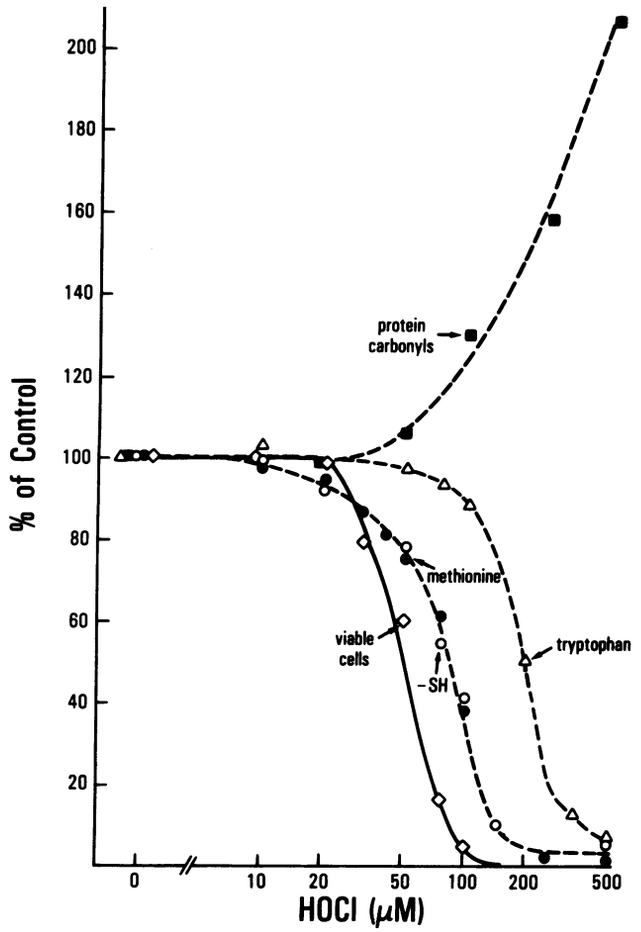

Figure 3. HOCl-induced oxidation of various cellular protein targets. Sulfhydryls were determined as described for Fig. 2. Methionine sulfoxides, tryptophans, and protein carbonyls were assayed as described in Methods. Mean of at least three experiments in duplicate, all determined after 15 min exposure to HOCl. For comparison cell lysis data (trypan blue exclusion) at 30 min are shown in the same figure.

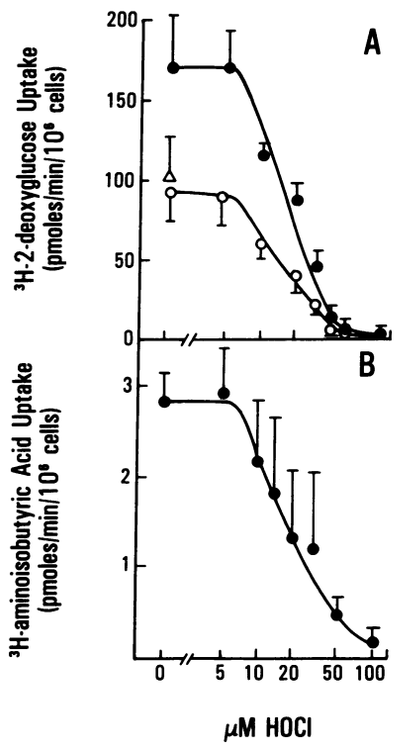

Figure 4. Inhibition of [^{3}H]2-deoxyglucose and [^{3}H]aminoisobutyric acid uptake by HOCl. (*A*) Inhibition of [^{3}H]2-deoxyglucose uptake by HOCl, determined as described in the method section. ●, Cells treated with HOCl only, ○, cells pretreated with 0.2 μM cytochalasin B for 15 min before the addition of HOCl, △ cells incubated with 10 μM *p*CMBS for 30 min. (*B*) Inhibition of [^{3}H]-aminoisobutyric acid uptake by HOCl. Mean±SD of four experiments in duplicate.

Effect of HOCl on glucose uptake. When [^{3}H]2-deoxyglucose (DOG) uptake was determined in P388D1 cells exposed to HOCl for 15 min, a dose-dependent inhibition of uptake was observed with complete inhibition at 50 μM HOCl (Fig. 4 *A*). When a dose of cytochalasin B that half-maximally inhibits glucose uptake (0.2 μM) (32, 47) was added before the HOCl, a parallel inhibition curve was obtained (Fig. 4 *A*). This suggests that decreased [^{3}H]2-DOG uptake at low concentrations of HOCl was due to transporter inactivation, although it is possible that inhibition of glucose uptake was a secondary effect, e.g., due to the loss of Na^+ and K^+ gradients across the plasma membrane (see below). With > 40 μM HOCl leakage of 2-DOG-phosphate may have contributed to the low levels of intracellular [^{3}H]DOG measured since cells prelabeled with [^{3}H]DOG lost the label upon exposure to > 40 μM HOCl (data not shown).

*p*CMBS caused a similar inhibition of [^{3}H]DOG uptake (Fig. 4 *A*). Methionine prevented inhibition of glucose transport only if added before the HOCl.

Effect of HOCl on [^{3}H]aminoisobutyric acid uptake. Aminoisobutyric acid is taken up by the "A" transport system for amino acids, but is then not incorporated into protein. [^{3}H]Aminoisobutyric acid uptake was inhibited by the same concentrations of HOCl as the glucose transporter (Fig. 4 *B*). *p*CMBS again inhibited this transport system. When 5 μM *p*CMBS and 10 μM HOCl, either one of which caused about a 20% inhibition of transport on its own, were added together, this resulted in a 43% inhibition of aminoisobutyric acid uptake.

[^{3}H]Leucine uptake which is transported by the "L"-system and is not Na^+-dependent, was similarly inhibited by HOCl, showing a 50% inhibition at 24 μM HOCl.

Effect of HOCl on cell volume. The same concentration range of HOCl led to an increase of cell volume. The observation of cell swelling was originally made when examining cells exposed to 20–40 μM HOCl under the microscope. Fig. 5 *A* shows the increase of cell volume 30 min after the addition of a bolus of HOCl as measured on a FACS analyzer. ^3H_2O space increased in parallel (Fig. 5 *A*), but to a lesser degree. *p*CMBS, but not other sulfhydryl reagents (1 mM iodoacetate, 200 μM *n*-ethylmaleimide, or 1 mM DTNB) caused a similar increase in cell volume (Fig. 5 *A*). When cells were exposed to HOCl in the presence of *p*CMBS, an additive effect on cell volume was observed (Fig. 5 *B*).

The increase in volume of P388D1 cells could be largely prevented, when 1 mM DTT was added 30 s after the addition of HOCl, suggesting a sulfhydryl oxidation mediated mechanism (Fig. 5 *B*).

When the Na^+ buffer was replaced by choline chloride, a much bulkier molecule, the increase in cell volume was similarly prevented (Fig. 5 *B*). The same replacement of MGB by choline chloride did not prevent cell lysis nor did it prevent the efflux of ^{86}Rb from cells exposed to HOCl (see below). Thus the increase in cell volume was associated with, but not causative of HOCl induced cell lysis.

Effect of HOCl on cation fluxes. Since the volume increase and its inhibition by choline chloride suggested that HOCl caused an ionic imbalance, cellular cations were determined.

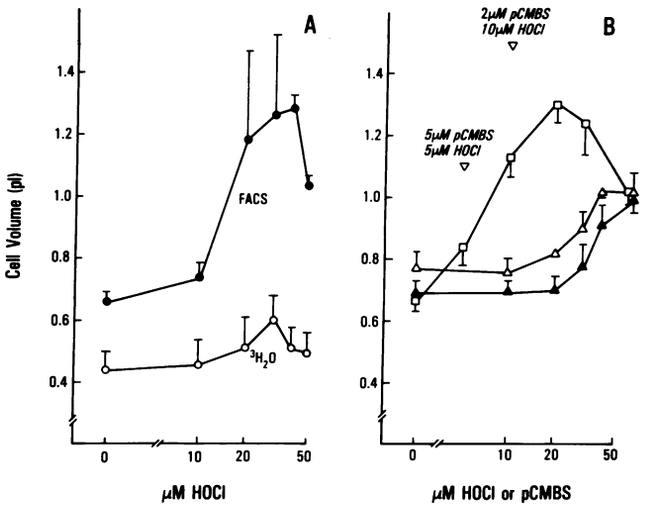

Figure 5. Effect of HOCl on cell volume. Cell volume was measured on a FACS analyzer or by uptake of ^3H_2O as described in Methods. 2 × 10^6 cells were incubated with HOCl for 30 min at 37°C. (*A*) ●, FACS volume of P388D1 cells exposed to HOCl for 30 min; ○, ^3H_2O space of P388D1 cells exposed to HOCl for 30 min. (*B*) Modulation of cell volume by various means as analyzed by FACS: ▲, effect of 1 mM DTT 30 s after the addition of HOCl; △, effect of replacement of NaCl by choline chloride before the addition of HOCl; □, effect of the sulfhydryl reagent *p*CMBS on cell volume in the absence of HOCl, molarities are the same as for HOCl, ▽, effect of combined HOCl/*p*CMBS incubation. Mean±SD of at least three experiments for FACS analysis; n = 18 for ^3H_2O space.

When intracellular cations (K^+, Ca^{2+}, Na^+) were measured by atomic absorption, a fast decrease of K^+ was observed (Fig. 6). Ca^{2+} and Na^+ concentrations increased over time, but with poor reproducibility (data not shown). Presumably cations leaked out of the cells again during the period of time necessary for the choline chloride washes. To circumvent this problem cells were labeled with the fluorescent Na^+-probe SBFI-AM. Intracellular Na^+ determined by this method increased from < 10 mM to apparently 20 mM with 10 μM HOCl at 30 min.

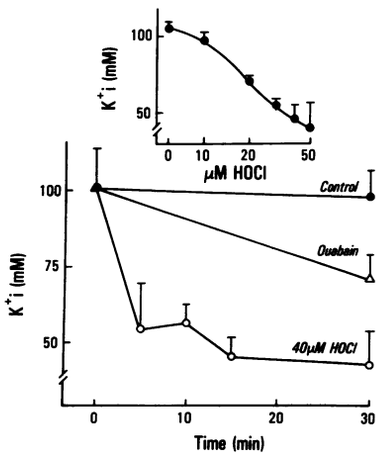

Figure 6. Effect of HOCl on cellular K^+ concentrations. The potassium content of 3 × 10^7 P388D1 cells was determined by atomic absorption as previously described (33). Cells were exposed to 40 μM HOCl for 5 to 30 min at 37°C. For comparison K^+-concentrations were also determined in cells incubated with 0.2 mM ouabain to inhibit Na^+-K^+-ATPase. K^+ molarities were calculated assuming a cell volume of 0.7 pl, obtained from the FACS analysis. The insert shows a dose response to HOCl induced K^+-loss determined after a 30-min incubation at 37°C.

Compatible results were observed using ^{86}Rb as an indicator of K^+ fluxes. 10 μM HOCl was sufficient to induce an instant increase in the rate of ^{86}Rb efflux from P388D1 cells (Fig. 7 *A*). ^{86}Rb-efflux stayed increased in HOCl-injured cells incubated at 4°C, suggestive of involvement of a passive leak. A similar increase in ^{86}Rb efflux was observed with the cell-impermeable sulfhydryl oxidant *p*CMBS (10 μM), while 1 mM iodoacetic acid (data not shown) had no effect. Addition of 5 μM *p*CMBS, which did not cause an increased ^{86}Rb efflux by itself, and 10 μM HOCl caused a measurable enhancement of ^{86}Rb efflux when added together (Fig. 7 *A*).

5 mM $BaCl_2$, an inhibitor of K^+ channels (48), prevented HOCl induced increased ^{86}Rb efflux at low concentrations of HOCl (5–20 μM), but only slightly inhibited the ^{86}Rb efflux at higher concentrations (Fig. 7 *B*).

Since plasma membrane ATPases are important for the regulation of cellular ion homeostasis, P388D1 cells were exposed to HOCl for 15 min and plasma membranes were then isolated. Inhibition of both Na^+-K^+-ATPase and Ca^{2+}-ATPase activity were observed (Table II). However, the concentrations of HOCl (30–40 μM) required to inhibit the ATPases, were higher than those sufficient to cause marked ionic disturbances in the cells. The greater loss of K^+ in HOCl injured cells as compared to ouabain-treated cells (Fig. 6) also indicates that ATPase inactivation is not sufficient to explain the observed ionic disturbance. No effect of HOCl on membrane potential could be observed with either 3,3'dipentyloxacarbocyanine (Di-O-C_5) and FACS analysis (49) or [^{3}H]tetraphenylphosphonium bromide distribution (50). With the latter method a membrane potential of ψ = −71±9 mV was calculated for control cells, while cells exposed to 30 μM HOCl showed a ψ = −73±7 mV. The ionic redistribution in the presence of HOCl thus did not appear electrogenic in these cells.

Effect of HOCl on lipid peroxidation. Up to 200 μM HOCl added to whole cells did not cause lipid peroxidation as determined by the malonaldehyde assay (51), but could induce lipid peroxidation in liposomes (unpublished observation).

Effect of HOCl on intracellular targets. The data presented thus far indicate that HOCl in low concentrations (10–20 μM) rapidly induces oxidative changes in proteins of the plasma

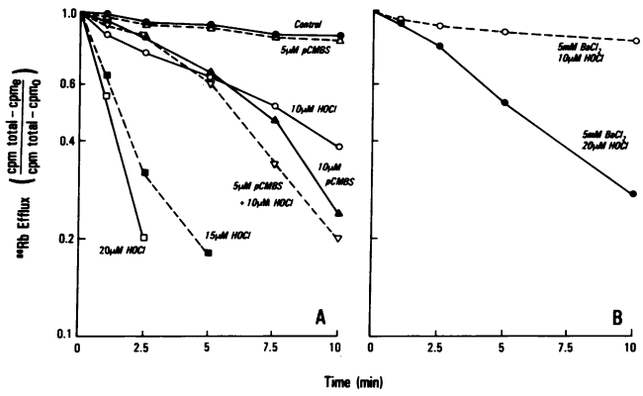

Figure 7. (A and B) ^{86}Rb-efflux in P388D1 cells exposed to HOCl: ^{86}Rb-efflux was determined as described in Methods. The fraction of ^{86}Rb retained by the cells was calculated from cpm_{max}-cpm_e/cpm_{max}-cpm_0, where cpm_{max} represents the maximal cpm released by the cells (~ 98% of total cpm), cpm_e the counts of the experimental sample, and cpm_0 the cpm released at 0 time. For comparison cells were treated in parallel with 10 μM *p*CMBS.

Table II. Inactivation of Plasma Membrane ATPase by HOCl

μM HOCl	Total ATPase	Na^+/K^+ATPase	Ca^{2+}ATPase
		μmol PO4/h per mg protein	
0 (n = 5)	1,673±213	218±09	540±12
20 (n = 2)	1,573	200	533
40 (n = 5)	1,210±169	68±55	347±143
60 (n = 5)	629±272	70±37	196±111

1×10^8 P388D1 cells/50 ml were exposed to HOCl for 15 min. Plasma membranes were prepared by differential centrifugation and ATPase activity was determined as described in Methods. Na^+/K^+ATPase and Ca^{2+}ATPase activities were calculated from the total activity minus the activity in the presence of 0.2 mM ouabain or 5 mM EGTA, respectively.

membrane. We then examined the possibility that HOCl could traverse the plasma membrane to induce oxidative changes intracellularly. Several intracellular targets were examined: GAPDH, hexokinase, DNA, NAD, and ATP, and mitochondrial O_2 consumption.

GAPDH of the glycolytic pathway is very sensitive to oxidative inactivation (16, 32) and was indeed inhibited with concentrations of HOCl that did not cause cell lysis (Fig. 8 *A*). If cells were lysed with 0.01% digitonin before the addition of HOCl, 90% inactivation of GAPDH was seen with 10 μM HOCl, indicating that the cytoplasmic location protects GAPDH from inactivation in intact cells to a considerable degree, but not completely. *p*CMBS similarly inactivated GAPDH (59% inactivation at 10 μM) in spite of its poor membrane permeability (44).

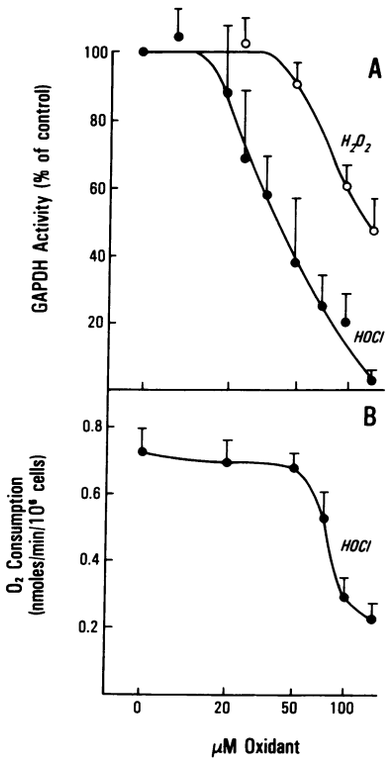

Figure 8. Effect of HOCl on GAPDH activity and mitochondrial respiration: O_2 consumption as well as GAPDH activity were determined after a 15 min exposure to H_2O_2.

HOCl was able to inactivate other enzymes of the glycolytic pathway such as hexokinase. The concentrations needed (120 μM for 50% inhibition) were, however, higher than those inducing cell death.

Lactate formation from U[^{14}C]glucose was similarly decreased by HOCl (Table III), but may have been due to inhibition of glucose uptake rather than GAPDH inactivation.

Mitochondrial respiration was also inhibited by HOCl, but again fairly high concentrations of the oxidant were necessary: half-maximal inhibition of basal O_2 consumption in whole P388D1 cells was observed with 80 μM HOCl (Fig. 8 *B*).

Cellular ATP concentrations fell after the addition of HOCl (Fig. 9 *A*). No extracellular ATP was observed under these conditions. This loss of ATP with low concentrations of HOCl thus was presumably due to changes in ATP metabolism, such as glucose transport inhibition. With more severe injury it became impossible to differentiate between inhibition of ATP synthesis and leakage of ATP into the extracellular medium, followed by extracellular degradation: When cells lost 89% of their ATP, 60% of the remaining ATP was found in the extracellular medium, indicating that it was not degraded, but lost due to permeability changes in the plasma membrane.

In contrast to H_2O_2, HOCl in concentrations up to 200 μM did not induce DNA strand breaks in P388D1 cells or isolated PM2 phage DNA. Nor could the sequelae of DNA strand break formation seen in H_2O_2 injured cells (8) including activation of poly-ADP-ribose-polymerase and early NAD depletion be observed. Fig. 9 *B* shows the effect of HOCl on cellular NAD levels. Lost intracellular NAD could be quantitatively recovered in the extracellular fluid, indicating that it was not degraded, but lost due to permeability changes in the plasma membrane.

Discussion

Injurious effects of HOCl on cells at low concentrations of HOCl. These results suggest that HOCl in concentrations as low as 10–20 μM rapidly oxidizes proteins but not lipids—in the external membrane of cells as shown by binding of I-PE. The damage to membrane proteins leads to loss of homeostatic control of ions especially K^+ across the plasma membrane and causes cell swelling. Table IV summarizes and compares the effect of HOCl on various cellular targets: plasma membrane protein functions (I-PE accessible -SH groups, K^+ efflux, DOG and aminoisobutyric acid uptake, cell volume) are half-maximally affected by ~ 20 μM HOCl. The

Table III. Lactate Formation from U[^{14}C]Glucose in P388D1 Cells Exposed to HOCl for 30 min in MGB Containing 100 μM Glucose

μM HOCl	Lactate formation
	pmol/10^6 cells per min
0	153±43
10	140±35
20	99±46
30	67±53
40	55±49
50	45±40

Lactate was determined as described in Methods. Mean±SD of four experiments.

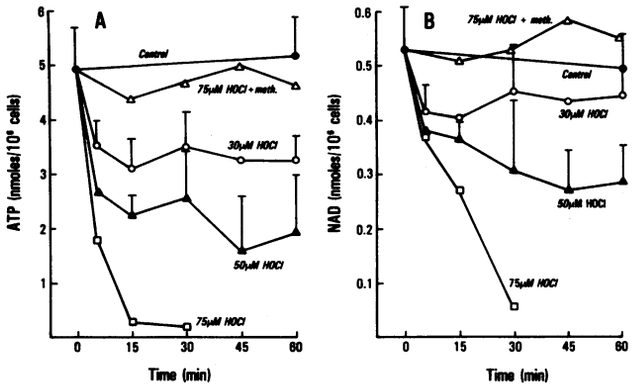

Figure 9. Effect of HOCl on cellular ATP and NAD concentrations determined by HPLC (36): mean and SD of three experiments. Meth, methionine.

same changes were observed in the presence of *p*CMBS, which acts primarily with plasma membrane -SHs (44, 45, 52). The additive effect of HOCl and *p*CMBS on ^{86}Rb efflux, aminoisobutyric acid uptake and increase in cell volume suggests that the two reagents act upon functionally similar cellular targets, presumably extracellularly accessible -SH groups.

Under conditions where glucose uptake is inhibited by ~ 75%, ATP formation and lactate production are inhibited by ~ 50%. GAPDH is inactivated in the same concentration range. It is not clear at this point whether the inactivation of GAPDH was caused by penetration of a small amount of HOCl into the cell, or whether GAPDH is inactivated due to -SS- exchange between extra- and intracellular moieties; on the one hand HOCl can penetrate lipid vesicles and bleach 5-carboxyfluorescein inside these vesicles leading to decreased fluorescence after Triton X-100 lysis of these liposomes (53) exposed to HOCl (data not shown). On the other hand it has been suggested that there is a shuttling mechanism between extracellular and cytoplasmic -SS- groups (54) such that the inactivation of GAPDH could be secondary to plasma membrane oxidation. This mechanism would also explain that *p*CMBS, acting only at the surface, inactivated GAPDH.

Table IV. Comparison of Effect of HOCl on Various Cellular Parameters

Parameter determined	Concentration of HOCl inducing half-maximal effect
	μM
Loss of I-PE binding	12
K_i^+ loss	18
Inhibition of [^{3}H]DOG uptake	20
Inhibition of [^{3}H]AIBA uptake	20
FACS vol. increase	21
ATP loss	29
Inhibition of lactate formation	30
GAPDH inactivation	36
NAD loss	50
Trypan blue uptake	50
LDH_i loss	79
Inhibition of O_2 consumption	98

All parameters were determined after a 30-min exposure of 2×10^6 P388D1 cells/ml at 37°C. Concentrations of HOCl inducing a half-maximal effect were extrapolated from dose-response curves. For K_i^+ loss the amount of K_i^+ retained in cells lysed with 0.01% digitonin (40 mM) was subtracted, since it presumably represented K^+ contained in organelles. LDH_i stands for intracellular LDH, [^{3}H]AIBA for [^{3}H]aminoisobutyric acid. ATP, NAD, trypan blue exclusion, and LDH data were obtained in parallel on the same samples to allow direct comparison.

Injurious effects of HOCl at high concentrations of HOCl. Slightly higher concentrations of HOCl (50 μM) led to cell lysis assessed by trypan blue exclusion as well as to leakage of NAD into the extracellular medium. In HOCl-mediated injury loss of trypan blue exclusion was a more sensitive measure of cell lysis than LDH release (Table IV), suggesting that HOCl induced cell death was due to perturbation of plasma membrane integrity with preferential leakage of small molecules.

The nonspecific protein oxidation caused by HOCl made it difficult to disect malfunctioning of specific pathways as causative of cell death. Various SH-oxidizing agents (e.g., *p*CMBS, cystamine) (55) cause cell death, and it is likely that HOCl induces cell death due to sulfhydryl oxidation. However, sublytic concentrations of HOCl may be sufficient to cause cellular dysfunction (56).

Comparison between cellular targets of HOCl and H_2O_2. H_2O_2 is capable of oxidizing -SHs but did not cause general protein -SH oxidation in P388D1 cells when as much as 5 mM H_2O_2 was added. This was presumably due to the very active glutathione cycle and HMPS present in these cells, which keep intracellular sulfhydryls reduced (29). Conditions of peroxide induced protein -SH oxidation in hepatocytes were always preceded by complete GSH depletion (57), while protein -SHs and GSH disappeared concomitantly in the presence of HOCl. H_2O_2 induced -SH oxidation was very specific. When analyzing the effect of 5 mM H_2O_2 on the K_m and V_{max} of every enzyme of the glycolytic pathway, only GAPDH was affected. No such specificity was seen with HOCl (16), which rather indiscriminately oxidized -SHs.

Similarly, when sulfhydryl-dependent plasma membrane functions were determined only HOCl, but not H_2O_2 affected these parameters in our system: glucose transport, amino acid transport, and plasma membrane ATPases were all inhibited by HOCl, but not by H_2O_2 (8, 32). H_2O_2 had no effect on cell volume (34) and K^+ loss was a late event that presumably followed Na^+K^+-ATPase inactivation due to > 90% depletion of cellular ATP (33, 58). While HOCl preferably oxidized targets of the plasma membrane, H_2O_2 diffuses into cells similar to H_2O. It reacts directly with very few targets, e.g., GAPDH, but forms $^{\cdot}OH$ when reacting with transition metal. H_2O_2 induced damage to DNA, lipids (59) or ATPases (60) is dependent on the presence of free transition metals.

Table V summarizes the effect of HOCl and H_2O_2 on various cellular targets.

H_2O_2-induced cell death appears to be a late event following > 90% depletion of ATP (8, 59), disturbance of cellular Ca^{2+} homeostasis (33, 57), and cytoskeletal derangement (55). DNA damage although induced by very small concentrations of H_2O_2 is not necessarily followed by loss of cell integrity. HOCl-induced cell death, on the other hand, occurs rapidly. Looking at cell membrane integrity, it is ~ 10 times more cytotoxic than H_2O_2, and since at least 30–40% of the H_2O_2 produced by stimulated PMNs is converted into HOCl (13, 14), local HOCl formation may well be in the 100-μM range. HOCl did not induce DNA strand breaks and while it is likely that HOCl will react with DNA bases (16), it is doubtful that it can reach nuclear sites except after cell lysis.

Table V. Comparison of Targets of H_2O_2 and HOCl

H_2O_2	HOCl
Degradation	
Degraded enzymatically, (catalase, GSH cycle)	Degraded by reaction with amino acids, proteins, etc.
Location of targets	
Diffuses freely into cells, site-directed damage due to metal-dependent 'OH formation	Reacts with closest target
Cell death	
Lysis after several hours; threshold dose 250–300 μM	Lysis within 1 h; threshold dose 25–35 μM
Sulfhydryl oxidation	
Total -SH: no general -SH oxidation at millimolar concentrations, very specific targets (e.g., GAPDH)	50% oxidation at 100 μM, Multiple molecular targets
Extracellular -SH: 25% oxidized at 700 μM H_2O_2, no functional consequences	50% oxidation at 12 μM dysfunction of -SH dependent plasma membrane proteins
Cause of ATP depletion	
Inactivation of GAPDH	Inactivation of GAPDH
Inhibition of mitochondrial ADP-phosphorylation preceding cell death by hours	Inhibition of mitochondrial ADP-phosphorylation Inhibition of glucose transport barely preceding cell death
DNA	
DNA strand breaks at <100 μM, base hydroxylations, activation of poly-ADPRP causing severe NAD depletion	No DNA strand breaks at 200 μM, base oxidation products unknown NAD loss at time of cell lysis

Molarities refer to results obtained in P388D1 cells.

Comparison between HOCl and chloramines. Chloramine toxicity has been investigated in much more detail at this point (21–23), and there are problems in differentiating the effects of one from the other. While amino acids as well as NH_4^+ are abundant in vivo, direct effects of HOCl cannot be excluded since MPO binds avidly to target cells thus directing HOCl formation to the plasma membrane. Since our buffer was protein and amino acid-free and the release of NH_4^+ from 2×10^6 P388D1 cells did not exceed 14.0±3.2 nmol over a period of 30 min at 37°C, chloramines were no primary source of oxidants in our system, although secondary chloramine formation from cellular proteins cannot be excluded. Indeed, in vivo the formation of less reactive chloramines may serve a protective role.

In summary, HOCl proved to be cytotoxic to P388D1 cells with a threshold dose of 20–30 μM. Even lower doses of HOCl inhibited various sulfhydryl dependent plasma membrane functions.

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