Inhibition of hypochlorous acid-induced cellular toxicity by nitrite

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Contributed by Hilary Koprowski, August 1, 2002

Chronic inflammation results in increased nitrogen monoxide (·NO) formation and the accumulation of nitrite (NO_2^-). Neutrophils stimulated by various inflammatory mediators release myeloperoxidase to produce the cytotoxic agent hypochlorous acid (HOCl). Exposure of chondrocytic SW1353 cells to HOCl resulted in a concentration- and time-dependent loss in viability, ATP, and glutathione levels. Treatment of cells with NO_2^- but not nitrate (NO_3^-) substantially decreased HOCl-dependent cellular toxicity even when NO_2^- was added at low (μM) concentrations. In contrast, NO_2^- alone (even at 1 mM concentrations) did not affect cell viability or ATP and glutathione levels. These data suggest that NO_2^- accumulation at chronic inflammatory sites, where both HOCl and ·NO are overproduced, may be cytoprotective against damage caused by HOCl. We propose that this is because HOCl is removed by reacting with NO_2^- to give nitryl chloride (NO_2Cl), which is less damaging in our cell system.

inflammation | cell toxicity | nitryl chloride | nitric oxide | arthritis

At sites of chronic inflammation, activated neutrophils release the enzyme myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2) to catalyze the formation of hypochlorous acid (HOCl; Eq. **1**).

$$H_2O_2 + Cl^- \xrightarrow{\text{MPO}} HOCl + OH^- \qquad [1]$$

It is estimated that between 25 and 40% of the H_2O_2 generated by activated neutrophils is used to form HOCl (1, 2). Throughout this paper we use the term HOCl (pKa = 7.46) to refer to the ≈50% ionized mixture of HOCl and OCl^- species that exists at pH 7.4 (3).

HOCl oxidizes many important biomolecules such as plasma membrane ATPases, collagen, ascorbate, proteins including α_1-antiproteinase, nucleotides, sulfhydryls, thioethers, DNA, and DNA-repair enzymes (2, 4–11), depletes intracellular ATP and reduced glutathione (GSH), and causes cell death (12). HOCl is a reactive chlorine species (RCS), capable of chlorinating protein tyrosine residues to form the proposed biomarker for RCS, 3-chlorotyrosine (13–16). Levels of 3-chlorotyrosine are increased in the intima and circulating low-density lipoprotein of some atherosclerosis patients (17–19), bronchoalveolar lavage fluid of lung-transplant patients (20), and expectorated sputum specimens of cystic fibrosis patients (21). HOCl also chlorinates cholesterol in cell membranes (22) as well as the cytosine and adenine residues in DNA (23–26).

An additional reaction of HOCl is with nitrite (NO_2^-) to form NO_2Cl (Eq. **2**; refs. 27–29).

$$HOCl + NO_2^- + H^+ \rightarrow NO_2Cl + H_2O\ (26) \qquad [2]$$

This reaction is favored with decreasing pH, such as may occur during chronic inflammation, and its second-order rate constant has been estimated at pH 7.2 and 25°C as $7.4 \pm 1.3 \times 10^3$ $M^{-1}{\cdot}s^{-1}$ (30).

Levels of NO_2^- found in plasma taken from healthy human volunteers range between 0.5 and 21.0 μM (31, 32), and these levels are elevated significantly during inflammation, e.g., up to 36 μM in patients with HIV infection (33). Serum NO_2^- levels in patients with systemic sclerosis are reported to be much higher (34). In the synovial fluid of patients with rheumatoid arthritis, NO_2^- levels are reported to range from 0.3 to as high as 15 μM (26, 35–37). NO_2^- has been used for decades in the food industry as a preservative and for curing meat. It is also estimated that 5% of ingested nitrate (NO_3^-) is reduced to NO_2^- by oral microflora where it enters the gastrointestinal tract (reviewed in refs. 38 and 39). Furthermore, dietary NO_2^- has been proposed as an oral and gut antimicrobial agent (38, 39). Salivary levels of NO_2^- of up to 98 μM have been reported (40).

NO_2Cl formation by activated human neutrophils in the presence of added NO_2^- has been demonstrated (29). NO_2Cl is capable of nitrating, chlorinating, and dimerizing phenolic compounds such as tyrosine (27–29), and exposure of isolated human low-density lipoprotein to NO_2Cl results in the depletion of β-carotene and α-tocopherol as well as protein modification (30). The addition of HOCl to isolated DNA or cells in the presence of NO_2^- results in increased cytosine chlorination and DNA oxidation compared with DNA or cells in the absence of added NO_2^- (26, 41). Bacteria phagocytosed by polymorphonuclear cells contain nitrated and chlorinated protein tyrosine residues, suggesting an important host–defense mechanism for reactive nitrogen and chlorine species such as HOCl, NO_2Cl, and peroxynitrite (42, 43). These studies have emphasized the potential deleterious effects of NO_2^-/HOCl-reaction products. However, NO_2^- has been reported also to inhibit the antimicrobial activity of HOCl (44–46), isolated MPO (47), and MPO-mediated low-density lipoprotein oxidation (48).

Although NO_2^-, NO_3^-, and HOCl are formed in substantial amounts during inflammation and are present normally in saliva and the gut at high micromolar concentrations, there are few data on the effects of HOCl on human cells in the presence of these nitrogen monoxide (·NO) metabolites (24). Therefore, in this paper we describe the effects of physiologically relevant concentrations of NO_2^- on HOCl-induced cell toxicity in chondrocytic SW1353 cells as a model of cartilage cells exposed to HOCl/NO_2^- during inflammatory joint disease (49, 50).

Experimental Procedures

Materials. DMEM, Earle's balanced salt solution (EBSS), trypsin-EDTA solution, GSH, oxidized glutathione, *Aspergillus* nitrate reductase (EC 1.6.6.2), firefly lantern extract, sodium nitrite ($NaNO_2$), sodium nitrate ($NaNO_3$), and all other reagents were purchased from Sigma–Aldrich (Poole, Dorset, U.K.). Sodium arsenite was obtained from BDH (Poole). All cell-culture flasks and microplates were obtained from Greiner (Greiner, Nurtingen, Germany).

Abbreviations: MPO, myeloperoxidase; HOCl, hypochlorous acid; GSH, reduced glutathione; NO_2^-, nitrite; NO_3^-, nitrate; LDH, lactate dehydrogenase; NO_2Cl, nitryl chloride.

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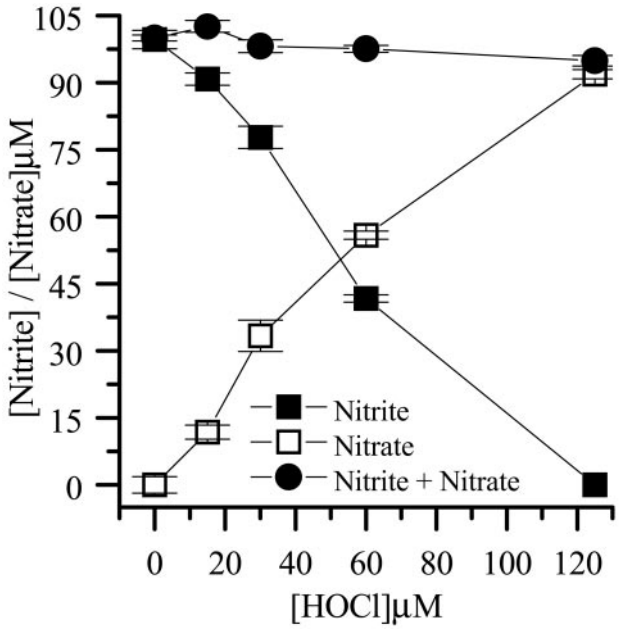

Fig. 1. Reaction of NO_2^- with HOCl. EBSS was incubated with $NaNO_2$, and HOCl was subsequently added. Residual NO_2^- was measured by the Griess assay as described in *Experimental Procedures*. Data are expressed as mean ± standard deviation of six or more separate experiments.

Cell Culture. Human chondrosarcoma cells were obtained from American Type Culture Collection (SW1353) and cultured in DMEM with 1% (vol/vol) penicillin, 10% (wt/vol) FCS, and 5% CO_2/95% O_2 with ≈95% humidity to 90% confluency before use (49, 50).

Measurement of HOCl. HOCl concentration was quantified immediately before use spectrophotometrically at 290 nm (pH 12.0, $\varepsilon = 350\ M^{-1}\cdot cm^{-1}$) (3). HOCl was diluted in ice-cold EBSS to a concentration of 10 mM and stored on ice for no longer than 1 min. After this time, the solution was discarded, and fresh HOCl (10 mM) was prepared. Monitoring HOCl concentration (as OCl^-) spectrophotometrically at 290 nm showed that there was no significant loss of HOCl over this time.

Exposure of SW1353 Cells to HOCl. Cells were washed twice with PBS and once with EBSS warmed to 37°C. Fresh EBSS then was added followed by HOCl addition. Cells then were incubated at 37°C, and the reaction was terminated by the addition of 10 mM oxidized glutathione. Adding HOCl did not alter the pH of the reaction mixture significantly.

Exposure of Cells to NO_2^-/NO_3^-. Cells were washed as described above and incubated in EBSS. $NaNO_2$ or $NaNO_3$ solutions were prepared (10 mM) in EBSS and added to the cells for 10 min. HOCl then was added, and the cells were incubated further at 37°C for up to 30 min when the reaction was terminated by the addition of 10 mM oxidized glutathione. The addition of $NaNO_2$ or $NaNO_3$ did not alter the pH of the reaction mixture significantly. NO_2^- and NO_3^- remaining in the culture medium were measured by the Griess assay after reduction of NO_3^- with NO_3^- reductase as described (51).

Assessment of Cell Viability. Cells were seeded overnight at a density of 3.5×10^4 cells per well in 96-well plates. After exposure to HOCl/NO_2^-/NO_3^-, cells were washed gently twice in warm (37°C) EBSS, and then 200 μl of warm DMEM plus 50 μl of 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (0.5

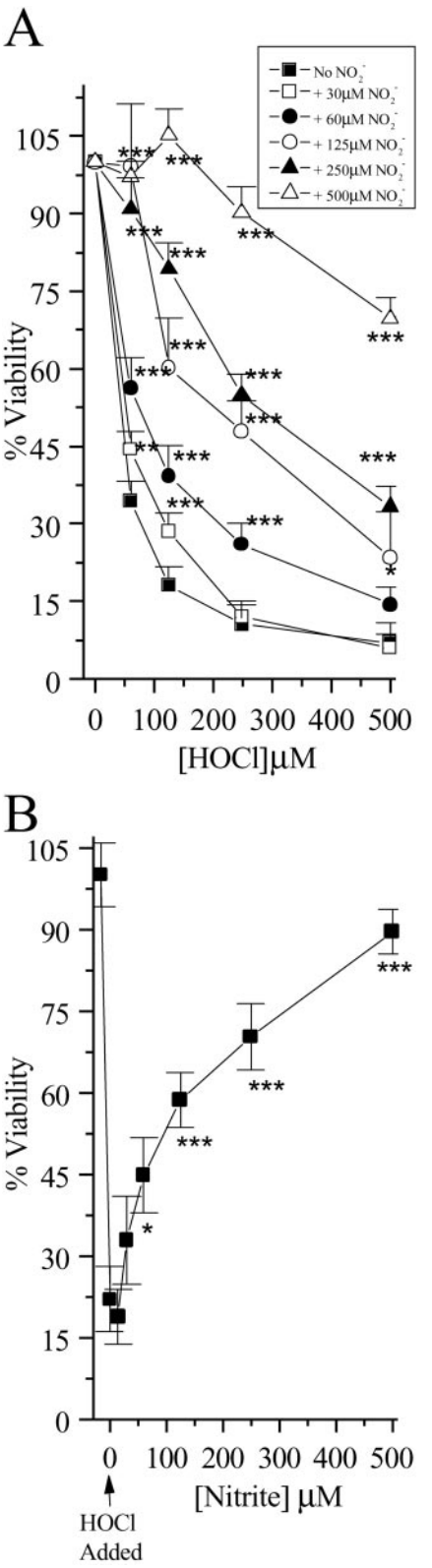

Fig. 2. HOCl-mediated loss of cellular viability: Effect of NO_2^-. NO_2^- was added to cells in EBSS 10 min before HOCl (*A*) or NO_2^- premixed with HOCl (125 μM) for 5 min and then added to cells for 10 min (*B*). Cells were washed in DMEM, and cellular viability was measured by using the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide assay as described in *Experimental Procedures*. Data are expressed as mean ± standard deviation of six or more separate experiments. *, $P < 0.1$; **, $P < 0.05$; and ***, $P < 0.01$ compared with HOCl addition alone.

mg/ml dissolved in DMEM) was added. Cells were incubated at 37°C in the dark for 1 hr and then washed twice with PBS, and 200 μl of DMSO was added to solubilize the formazan dye. Absorbance at 550 nm then was read on a Molecular Devices Spectramax190 plate reader after gentle shaking in the dark for 30 min. Where required, NO_2^- or NO_3^- was premixed for 5 min with HOCl in warm EBSS before addition to cells. Control experiments showed that there was no significant HOCl loss over this time.

Measurement of Cellular Glutathione and ATP. Cells were seeded overnight at a density of 0.25×10^6 cells per well in 24-well plates. After washing twice with EBSS, 2.0 ml of fresh EBSS was added and HOCl added. The reaction was terminated by the addition of 10 mM oxidized glutathione. Cells then were washed twice in ice-cold PBS to remove any residual NO_2^- or NO_3^-. This was followed by the addition of 250 μl of ice-cold trichloroacetic acid 6.5% wt/vol) and left on ice for 10 min. The trichloroacetic acid extract then was removed and either stored at −80°C or used immediately for analysis. NaOH (200 μl of 1M solution) then was added to solubilize cellular protein. Protein concentration then was measured by the Bradford method. The trichloroacetic acid extract then was used to assess cellular GSH and ATP.

Analysis of cellular GSH was performed as described (52). Briefly, 7.5 μl of trichloroacetic acid extract was added to 96-well fluorescence plates followed by the addition of 227.5 μl of 100 mM KH_2PO_4-KOH buffer, pH 10.0, and 15 μl of *o*-phthaldialdehyde (10 mg/ml freshly prepared in methanol). Samples were stored in the dark at room temperature for 25 min and measured by fluorescence (excitation = 350 nm, emission = 420 nm) using a Gemini Fluorescence plate reader (Molecular Devices). Concentrations of GSH then were determined by comparing the values obtained with a freshly prepared standard curve of GSH.

Loss of cellular ATP was assessed by using firefly lantern extract as described (53). Briefly, 3 μl of sample was incubated with 200 μl of sodium arsenite buffer (comprising 26.67 mM $MgSO_4 \cdot 7H_2O$/3.33 mM KH_2PO_4/33.33 mM $Na_2HASO_4 \cdot 7H_2O$, pH 7.4). After the addition of 10 μl of filtered firefly lantern extract per sample, light emission then was measured for 10 sec per sample by using a LUMI-ONE portable luminometer (Trans Orchid Enterprises, Tampa, FL). Concentrations of ATP then were determined by comparing the values obtained with a freshly prepared standard curve of ATP. Where required, NO_2^- or NO_3^- was premixed for 5 min with HOCl in warm EBSS before addition to cells.

Assessment of Lactate Dehydrogenase (LDH) Release. Cells were seeded overnight at a density of 0.25×10^6 per well in 24-well plates, washed, and exposed to NO_2^-, NO_3^-, and HOCl as described above. After 10 min of exposure, the cells were washed gently in warm PBS, and DMEM was added. Preliminary experiments showed that HOCl inactivated LDH; therefore, cells were washed and HOCl was removed before measuring LDH leakage after 24 hrs of incubation in DMEM at 37°C. LDH activity was measured by using a commercially available kit (Sigma, LD-50) and a Molecular Devices SpectraMax 190 microplate reader and compared as a percentage of LDH activity from cells lysed with 0.1% Triton X for 20 min at 37°C.

Where required, NO_2^- or NO_3^- was premixed for 5 min with HOCl in warm EBSS before addition to cells.

Data Analysis. All graphs are plotted with mean ± standard deviation of the mean. In all cases the mean values were calculated from data taken from at least six separate experiments performed on separate days by using freshly prepared reagents. Where significance testing was performed, an independent

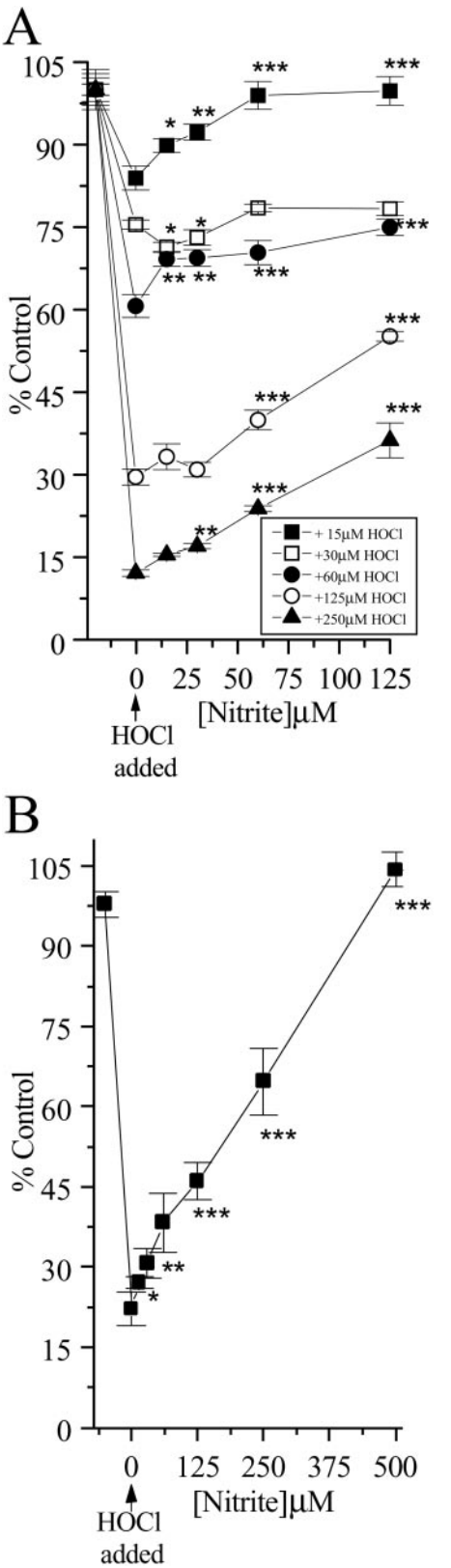

Fig. 3. HOCl-mediated loss of cellular ATP: Effect of NO_2^-. NO_2^- was added to cells in EBSS 10 min before HOCl (*A*) or NO_2^- premixed with HOCl (125 μM) for 5 min and then added to cells for 10 min (*B*). Cells were washed in DMEM, and cellular ATP was measured by using firefly lantern extract as described in *Experimental Procedures*. Data are expressed as mean ± standard deviation of six or more separate experiments. *, $P < 0.1$; **, $P < 0.05$; and ***, $P < 0.01$ compared with HOCl addition alone.

Student's t test (two populations) was used (*, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.01$).

Results

Effect of HOCl, NO_2^-, and NO_3^- on Cell Viability. Incubation of 100 μM NO_2^- with increasing concentrations of HOCl in EBSS resulted in the oxidation of 1 mol of NO_2^- to NO_3^- per mole of HOCl added (Fig. 1). The addition of HOCl to SW1353 cells substantially reduced cell viability as measured by 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide in a time- and concentration-dependent manner. Preliminary experiments (data not shown) established that low concentrations of HOCl (30 μM) were sufficient to significantly reduce cell viability after a 10-min incubation time at 37°C, pH 7.4. From these data, an incubation time of 10 min was chosen for subsequent studies. However, the presence of $NaNO_2$ before HOCl addition substantially inhibited HOCl-induced cytotoxicity with marked protection observed with 30 μM NO_2^- (Fig. 2*A*). The same degree of inhibition was observed if HOCl (125 μM) was premixed with NO_2^- for 5 min before its addition to cells (Fig. 2*B*). This effect was not observed with NO_3^- even when tested at 1 mM (data not shown). However, if the cells were incubated first with HOCl for 10 min followed by NO_2^- addition, NO_2^- was unable to prevent HOCl-mediated loss of viability (data not shown), which indicates that NO_2^- cannot reverse damage that has been caused by HOCl. Control experiments showed that incubating SW1353 cells with up to 1 mM $NaNO_2$ or $NaNO_3$ for 1 hr did not cause significant loss of cell viability (0.98 ± 0.5 and 1.02 ± 0.6% reduction in viability, respectively).

Effect of HOCl, NO_2^-, and NO_3^- on Intracellular ATP Depletion. To characterize further the effects of HOCl and NO_2^- on cellular metabolism, levels of ATP were examined (Fig. 3 *A* and *B*). HOCl caused a rapid depletion of ATP when added to SW1353 cells at low (<30 μM) concentrations. The presence of NO_2^- (but not NO_3^-, tested up to 1 mM) before HOCl addition significantly inhibited HOCl-dependent ATP loss even in the presence of high HOCl concentrations (250 μM; Fig. 3*A*). The same degree of inhibition was observed if HOCl (125 μM) was premixed with NO_2^- for 5 min before the addition to cells (Fig. 3*B*).

However, if the cells were incubated first with HOCl for 10 min followed by NO_2^- addition, NO_2^- was unable to prevent HOCl-mediated ATP (data not shown). Control experiments showed that incubating SW1353 cells with up to 1 mM $NaNO_2$ or $NaNO_3$ for 1 hr did not affect cellular ATP levels significantly.

Effect of HOCl, NO_2^-, and NO_3^- on Intracellular GSH. Preliminary experiments also showed that HOCl caused a rapid depletion of cellular GSH. Increasing the time of incubation increased the extent of GSH loss, and a 10-min incubation time was selected for subsequent studies. The addition of NO_2^- (but not NO_3^-, tested up to 1 mM) to the cells before HOCl addition significantly prevented HOCl-dependent GSH loss even in the presence of high HOCl concentrations (250 μM; Fig. 4*A*). A similar degree of inhibition was observed if HOCl (125 μM) was premixed with NO_2^- for 5 min before the addition to cells (Fig. 4*B*). Again, if the cells were incubated first with HOCl for 10 min followed by NO_2^- addition, NO_2^- was unable to prevent HOCl-mediated GSH loss (data not shown). Control experiments showed that incubating SW1353 cells with up to 1 mM $NaNO_2$ or $NaNO_3$ for 1 hr did not affect cellular GSH levels significantly.

Effect of HOCl, NO_2^-, and NO_3^- on LDH Release. Fig. 5 shows the effect of high concentrations of HOCl (200 μM) on LDH release from SW1353 cells. HOCl induced substantial cellular LDH release 24 hr after exposure. This effect was inhibited significantly when cells were incubated with NO_2^- (>30 μM), but not NO_3^- (up to 1 mM), for 10 min before HOCl addition. A similar degree of

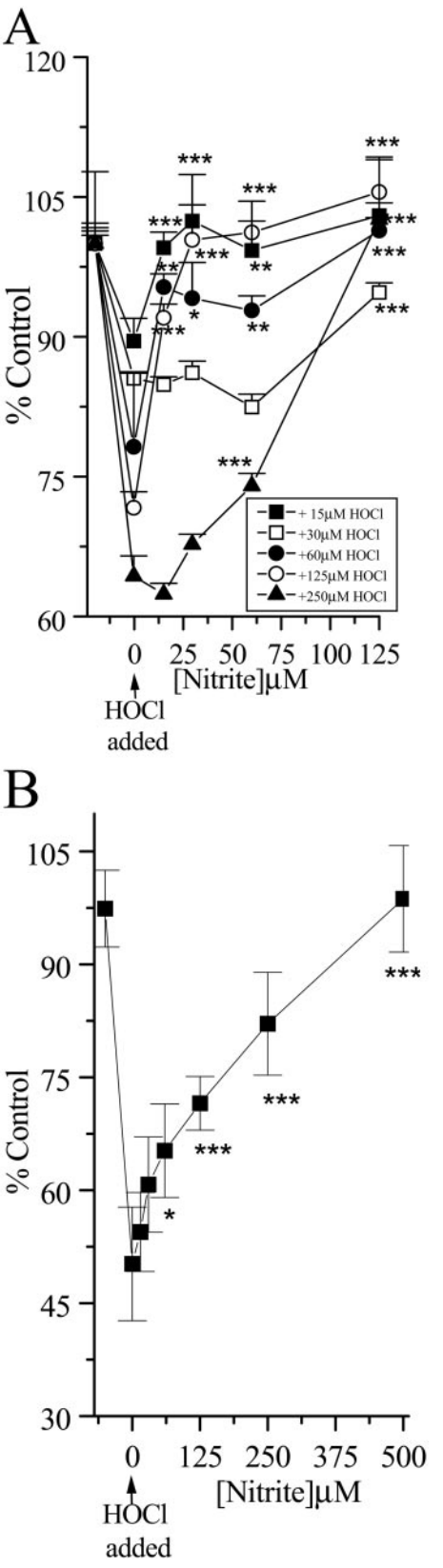

Fig. 4. HOCl-mediated loss of cellular GSH: Effect of NO_2^-. NO_2^- was added to cells in EBSS 10 min before HOCl (*A*) or NO_2^- premixed with HOCl (125 μM) for 5 min and then added to cells for 10 min (*B*). Cells were washed in DMEM, and cellular GSH was measured as described in *Experimental Procedures*. Data are expressed as mean ± standard deviation of six or more separate experiments. *, $P < 0.1$; **, $P < 0.05$; and ***, $P < 0.01$ compared with HOCl addition alone.

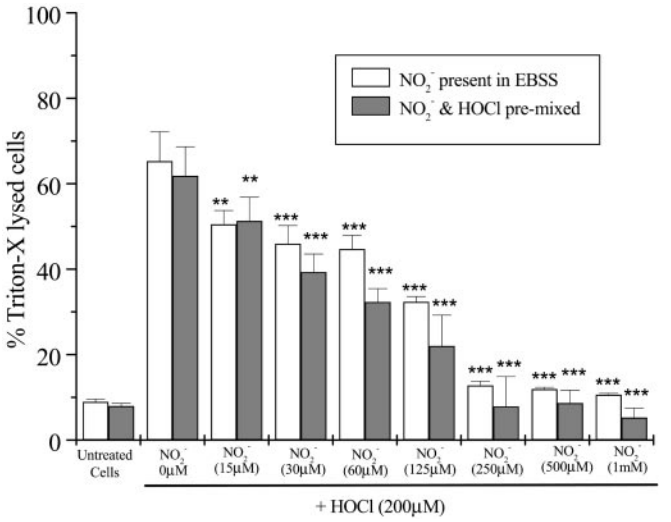

Fig. 5. HOCl-mediated LDH leakage: Effect of NO_2^-. NO_2^- was added to cells in EBSS 10 min before HOCl (■) or NO_2^- premixed with HOCl (200 μM) for 5 min and then added to cells for 10 min (□). After treatment cells were washed in DMEM, and LDH leakage was measured in the medium after 24 hrs as described in *Experimental Procedures*. Data are expressed as mean ± standard deviation of six or more separate experiments. *, $P < 0.1$; **, $P < 0.05$; and ***, $P < 0.01$ compared with HOCl addition alone.

inhibition of LDH release was observed if HOCl (200 μM) was premixed with NO_2^- for 5 min before the addition to cells (Fig. 5). However, treatment of cells with HOCl followed by NO_2^- or NO_3^- addition did not inhibit LDH release significantly (data not shown).

Discussion

There is considerable evidence that HOCl and the end products of ·NO metabolism (NO_2^- and NO_3^-) accumulate at sites of chronic inflammation (1, 2, 33–36). HOCl is known to oxidize many important classes of biomolecules (1–12) as well as chlorinate protein, DNA, and cholesterol (13–26) and cause cell death (12). Although there is a wealth of information on HOCl-mediated processes at sites of chronic inflammation, HOCl is coproduced with ·NO, and relatively little information is available on the consequences to cells of HOCl generated in the presence of NO_2^- (24). The relatively fast second-order rate constant of reaction for NO_2^- and HOCl (pH 7.2, 25°C, 7.4 ± $1.3 \times 10^3\ M^{-1}{\cdot}s^{-1}$; ref. 30), the high concentrations of HOCl, and the high concentration of NO_2^- at sites of chronic inflammation (36–39) or present in saliva (40) and the gut (38) make this reaction plausible *in vivo*. Therefore, it was pertinent to examine the effects of HOCl on cell function in the presence of physiologically relevant concentrations of NO_2^-.

HOCl caused rapid depletion of cellular ATP and GSH as well as reduced cell viability. However, when HOCl was added in the presence of physiological concentrations of NO_2^-, the latter was oxidized to NO_3^-, and HOCl-induced cytotoxicity was inhibited substantially (Figs. 2–5), presumably because of removal of the HOCl. This was observed even at high concentrations of HOCl (>200 μM). Such high concentrations of HOCl are unlikely to occur *in vivo*, but the fact that NO_2^- can protect cells from toxicity at low concentrations even when HOCl is in molar excess is notable. We speculate that the NO_2Cl formed from the reaction of HOCl with NO_2^- is less cytotoxic than HOCl in our cell system. Perhaps it does not penetrate the cell as readily as HOCl. Indeed, preincubating HOCl with NO_2^- also removed the cytotoxic activity of HOCl (Figs. 2*B*, 3*B*, 4*B*, and 5).

Thus under our reaction conditions at physiologically attainable concentrations, NO_2^- but not NO_3^- seems to act as an antioxidant in that it reacts preferentially with HOCl by a two-electron process to form NO_3^- in a 1:1 molar ratio (Fig. 1; ref. 29) to prevent HOCl-mediated cytotoxicity. Indeed, NO_2^- substantially inhibits the bactericidal activity of HOCl toward *Escherichia coli* (44–46) and prevents HOCl-mediated inactivation of α_1-antiproteinase, the major inhibitor of serine proteases in human body fluids (25), as well as HOCl-mediated loss of 8-hydroxyguanine and FAPy guanine in isolated calf thymus DNA (41). NO_2^- also inhibits MPO to limit tyrosine nitration and chlorination as well as oxidation reactions (47, 48).

Based on studies of nitration of phenolics and proteins, oxidation of lipids, depletion of α-tocopherol, and chlorination of DNA and proteins, previous reports have suggested that NO_2^- might enhance HOCl-mediated toxicity at sites of inflammation (13–26). The current findings indicate that this is unlikely to be the case, because NO_2^- decreases the toxicity of HOCl for cells *in vitro*. Nevertheless, NO_2Cl, the product of HOCl and NO_2^-, is likely to cause some cell damage (12–23). The present studies indicate that, in our cell system, NO_2Cl is far less damaging than HOCl. The removal of HOCl by NO_2^- could represent an additional endogenous defense mechanism to limit HOCl-mediated oxidative damage of cellular targets. Therefore it is plausible that some of the antiinflammatory and cytoprotective actions of ·NO could involve NO_2^- (54, 55).

In summary, we have shown that low and physiologically relevant concentrations of NO_2^- substantially inhibit HOCl-mediated cellular toxicity to human SW1353 chondrosarcoma cells. This may be of great importance in diseases that are associated with oxidative stress such as rheumatoid arthritis (56) and inflammatory bowel disease (57), whereby endogenous HOCl-removing antioxidants such as GSH and ascorbate are depleted substantially and ·NO, NO_2^-, and HOCl formation are elevated substantially. Further work is needed to address these issues.

We thank Dr. Jeremy P. E. Spencer (King's College, London) for his critical discussion of this manuscript. We are grateful also to the National Medical Research Council of Singapore (NMRC/0474/2000 and NMRC/0481/2000) and the National University of Singapore Academic Research Fund (R183000053214) for their generous research support.

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