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Critical Review

Living with a Killer: The Effects of Hypochlorous Acid on Mammalian Cells

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Summary

The production of hypochlorous acid (HOCl) by the myeloperoxidase-H_2O_2-Cl^- system of phagocytes plays a vital role in the ability of these cells to kill a wide range of pathogens. However, the generation of a potent oxidant is not without risk to the host, and there is evidence that HOCl contributes to the tissue injury associated with inflammation. In this review, we discuss the biological reactivity of HOCl, and detail what is known of how it interacts with mammalian cells. The outcome of exposure is dependent on the dose of oxidant, with higher doses causing necrosis, and apoptosis or growth arrest occurring with lower amounts. Glutathione (GSH) and protein thiols are easily oxidized, and are preferred targets with low, sublethal amounts of HOCl. Thiol enzymes vary in their sensitivity to HOCl, with glyceraldehyde-3-phosphate dehydrogenase being most susceptible. Indeed, loss of activity occurred before GSH oxidation. The products of these reactions and the ability of cells to regenerate oxidized thiols are discussed. Recent reports have indicated that HOCl can activate cell signaling pathways, and these studies may provide important information on the role of this oxidant in inflammation.

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INTRODUCTION

Phagocytic white blood cells, particularly neutrophils, are a major source of oxidants in mammalian systems. These cells ingest and destroy invading pathogens, and are the predominant cell present in the early stages of acute inflammation. Phagocytosis of bacteria is accompanied by the activation of an NADPH oxidase complex in the cell membrane, resulting in generation of superoxide (O_2^-) and the release of cytoplasmic granule proteins into the phagosome [reviewed in (*1*)]. One of the major granule proteins is the enzyme myeloperoxidase, a heme protein that accounts for 5% of the total neutrophil protein. The combination of O_2^- production and myeloperoxidase release gives neutrophils a broad and unique oxidative potential (Fig. 1). Hydrogen peroxide (H_2O_2), generated by dismutation of O_2^-, is utilized by myeloperoxidase to oxidize halides to generate hypohalous acids. At physiological concentrations chloride is the most likely substrate, and hypochlorous acid (HOCl) has been shown to be a major end-product of the neutrophil respiratory burst (*2–5*).

The powerful antimicrobial nature of HOCl and its conjugate base, hypochlorite (OCl^-) has been well documented (*6–8*). It is the active ingredient in household bleach and the species responsible for the microbicidal properties of chlorinated water supplies. The production of HOCl by neutrophils is an integral part of the ability of these cells to kill a wide range of pathogens (*1*). However, the properties that make it such a useful antimicrobial agent also place the host at considerable risk, because HOCl has the potential to damage host tissue through the same processes used in the destruction of invading microorganisms. Neutrophil oxidants have been implicated in the tissue injury associated with inflammatory diseases, including respiratory distress, ischemia-reperfusion injury, acute vasculitis, arthritis, and glomerulonephritis (*9*). Many in vitro studies have shown that HOCl is able to mediate tissue injury (*1*, *10–12*), and recent work has provided direct evidence for the production of HOCl in various pathological disease states. Using an antibody raised against HOCl-modified protein, the generation of HOCl in vivo has been demonstrated in diseased kidney tissue and in human atherosclerotic plaques (*13*, *14*). This material was recently found to colocalize with myeloperoxidase in atherosclerotic lesions (*15*). Elevated levels of 3-chlorotyrosine, a marker of HOCl generation, have also been demonstrated in LDL molecules from human atherosclerotic intima (*16*).

Taken together, these studies confirm that HOCl is released from phagocytic cells in vivo and suggest that HOCl produced by neutrophils and other phagocytic cells could form a significant

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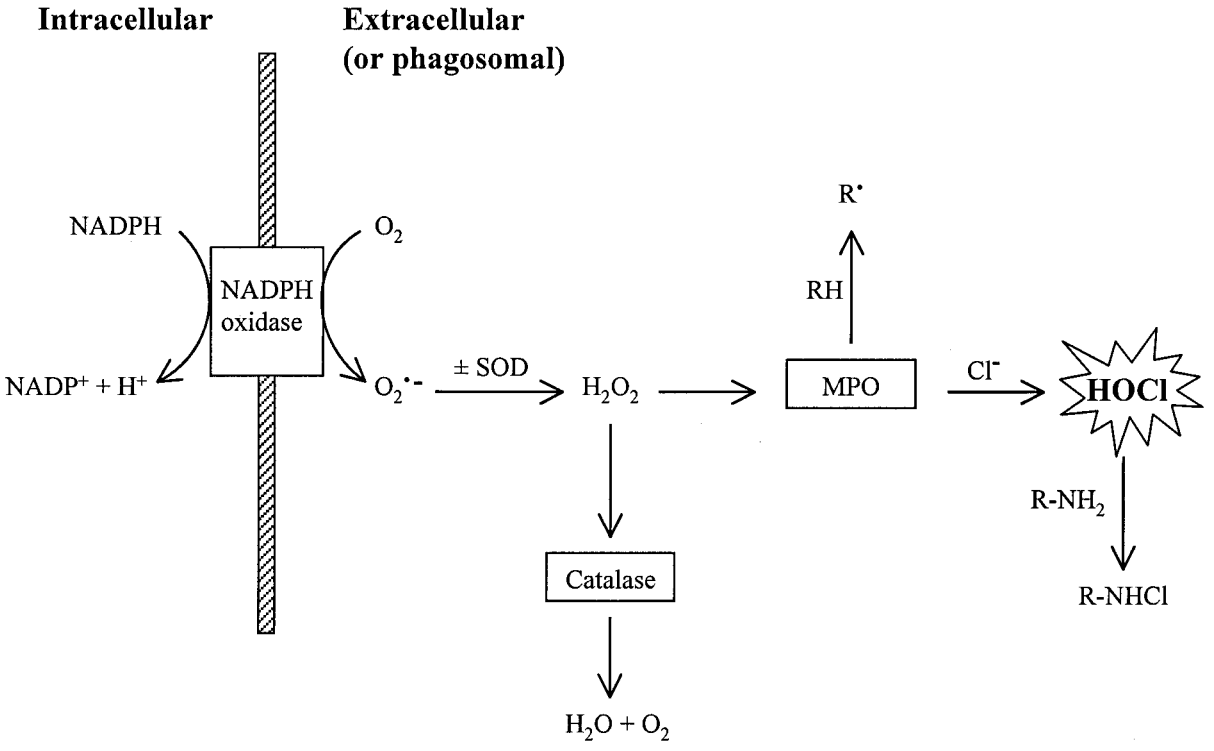

Figure 1. Neutrophil oxidative products.

oxidative stress on host cells and tissues. In this paper, we present background information on the chemical reactivity of HOCl with biological compounds and review what is known about how this translates into the effects of HOCl in cells and tissues.

BIOLOGICAL REACTIONS OF HOCl

HOCl exists in equilibrium with OCl^- and chlorine gas (Cl_2). Because HOCl has a pK_a of 7.5, it is present as an equal mixture of HOCl and OCl^- physiologically, and Cl_2 is present in significant amounts only at much lower pH (*17*). The 'active' chlorine in these species is in a formal oxidation state of +1. It may act as either a one-electron or two-electron oxidizing agent, but reduction potentials favor two-electron oxidation (*18*). HOCl is a powerful oxidising agent that can react with many biological molecules. Thiol groups and thioethers such as methionine are the most readily oxidized, at a rate approximately 100 times that of amine groups (*19*). Rate constants for the reaction of HOCl with reduced glutathione (GSH) and ascorbate are $>10^7\ M^{-1}\ s^{-1}$ and $\sim 6 \times 10^6\ M^{-1}\ s^{-1}$ respectively (*20*). Heme groups and iron–sulfur centers also react readily (*21*).

HOCl can also halogenate cell constituents. The most favored chlorinating reaction of HOCl is with amines to form monochloramines and dichloramines.

$$RNH_2 + HOCl \rightarrow RNHCl + H_2O \quad \text{(Reaction 1)}$$
$$RNHCl + HOCl \rightarrow RNCl_2 + H_2O \quad \text{(Reaction 2)}$$

Chloramines, while longer-lived and less reactive than HOCl, retain the two oxidising equivalents. They can oxidize thiols, thioethers and heme proteins, and thus extend the reactivity of HOCl (*22*, *23*). Chloramines can also be toxic to cells and bacteria, the extent of which depends on their structure and their ability to cross the plasma membrane (*24*, *25*). Additionally, chloramines, particularly those of α-amino acids, are susceptible to breakdown to aldehydes, which themselves are cytotoxic (*26*).

HOCl can react with nucleotides and with DNA. It reacts rapidly with NADH and the NH-groups of pyrimidines, and slow denaturation of DNA has been observed (*27*, *28*). A recent study has demonstrated DNA double-strand breaks and the formation of modified nucleotides in an epithelial cell line exposed to HOCl (*29*).

Chlorination of unsaturated fatty acids and cholesterol to chlorohydrin derivatives has been demonstrated, which suggests that the lipid component of cell membranes could be susceptible to attack by HOCl. However this reaction is relatively slow, and chlorohydrins have only been detected in cells after exposure to cytotoxic concentrations of HOCl (*30–33*). Several studies have shown that HOCl does not cause lipid peroxidation (*34*, *35*).

Cell proteins are likely to be a major target for HOCl and varied products are possible. Cysteine and methionine residues are readily oxidized (*36*). The amino groups of lysine and the N-terminal amines react to form chloramines, and these can

undergo secondary reactions with thiols and thiol-containing proteins (*37*). Another possible consequence of chloramine formation is the generation of radicals that may result in protein fragmentation or lipid peroxidation (*38*, *39*). The formation of carbonyls in proteins exposed to HOCl can also occur via a chloramine intermediate (*40–42*). Irreversible protein crosslinks have been observed in cell membranes exposed to HOCl and are associated with cell lysis (*32*). Although the mechanism for crosslinking is unclear, this reaction is rapid and is seen with low doses of oxidant (*43*). Free tyrosine and the tyrosine residues of protein can also be chlorinated to form the mono- or dichloro derivatives (*44*). This reaction of HOCl is being successfully used as a marker of neutrophil activation in vivo. Other susceptible protein targets include tryptophan, histidine, arginine, and the amide peptide bond (*45*).

EFFECT OF HOCl ON CELL VIABILITY

Studies with stimulated neutrophils and the cell-free myeloperoxidase-H_2O_2-chloride system provided the initial evidence for the toxicity of HOCl to mammalian cells. Inhibition of cytotoxicity with myeloperoxidase inhibitors and by the HOCl scavenger methionine strongly implicated HOCl as the mediator of these reactions (*46–50*). Similarly, neutrophils lacking myeloperoxidase or the membrane oxidase complex were not cytotoxic to target cells unless myeloperoxidase or H_2O_2 levels were restored (*46*). Also, reagent HOCl can kill a wide range of cell types including red cells (*34*, *51*, *52*), endothelial cells (*24*), epithelial cells (*53*, *54*), fibroblasts (*55*), T-cell lines (*48*), and tumor cells (*35*). These studies have used membrane integrity and cell lysis as a measure of cell death and consequently provide evidence for necrotic cell death.

The cellular changes responsible for necrosis have not been fully elucidated. Work investigating the bactericidal effects of HOCl first emphasized the importance of protein components of the bacterial cell wall and cytoplasmic membrane as sensitive targets (*56*, *57*). The mammalian cell membrane is similarly susceptible. In tumor cells, low concentrations of HOCl caused disruption of various plasma membrane transport functions, a decline in cellular K^+ and an increase in cell volume. There was a concurrent loss of membrane thiol groups and a similar loss of membrane functions, caused by a thiol binding agent, led the authors to suggest that membrane thiol oxidation mediated the above changes (*35*). In red cells, membrane protein modification closely correlated with lysis, and the formation of pores due to protein crosslinking was proposed as the mechanism responsible for lysis (*32*).

The ability of HOCl to induce apoptotic cell death has recently been described (*58*). Treatment of cultured endothelial cells with intermediate concentrations of HOCl caused the cells to undergo apoptosis, while higher concentrations induced necrotic cell death (Fig. 2). Apoptosis was characterized by phosphatidylserine exposure, changes in nuclear morphology, DNA fragmentation and caspase activity. In HL-60 cells, apoptosis initiated by H_2O_2 was shown to be dependent on myeloperoxidase activity in the cells, and HOCl was implicated (*59*).

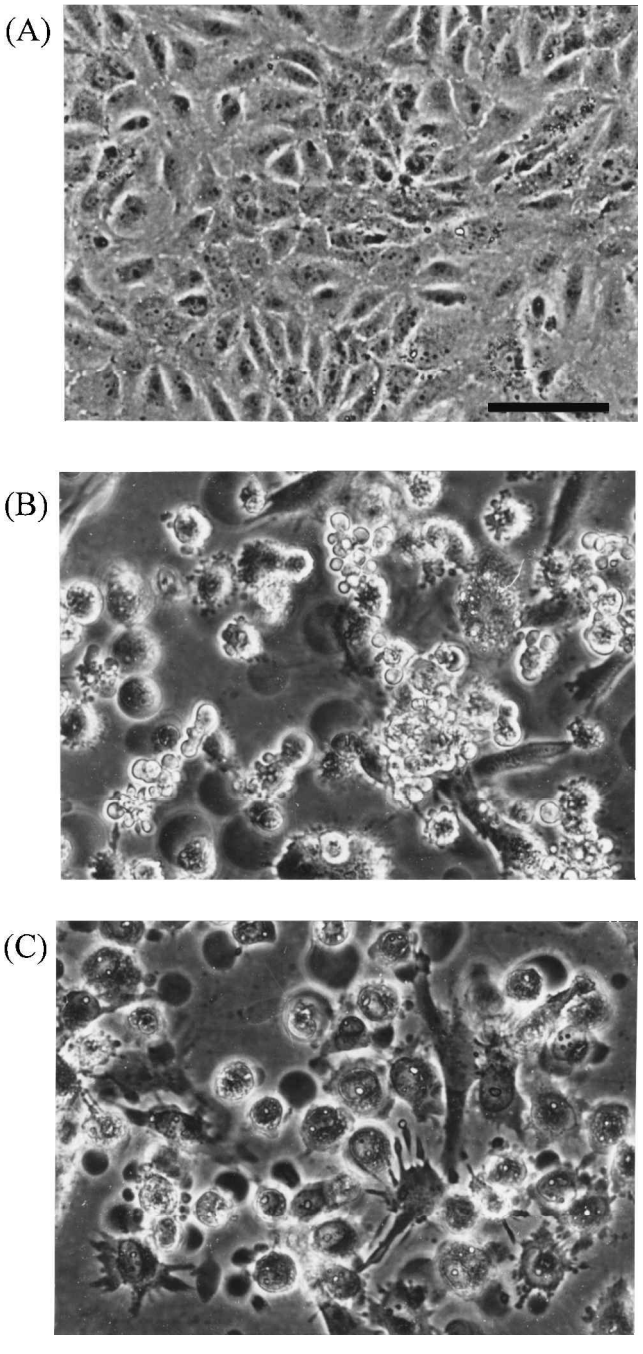

Figure 2. Morphological changes to human endothelial cells exposed to HOCl. (A) Control monolayer of endothelial cells. (B) After exposure to intermediate concentrations of HOCl, many cells detach and exhibit typical apoptotic blebbing. (C) With higher doses of oxidant, cell swelling and necrosis predominate. Bar represents 100 μm. Figure from Vissers, Pullar, and Hampton, 1999 (*58*)].

REACTION OF HOCl WITH GSH AND THIOL PROTEINS

The very fast reaction of HOCl with thiols suggests that these would be major cell targets. Several studies have investigated the relative susceptibilities of different cell constituents to HOCl, and have shown that GSH and protein thiols are preferred targets for oxidation (*60*, *61*). In red cells, when 80% of the GSH was reacted, only about 20% of membrane thiols were lost (*61*). Changes in cell parameters such as cell swelling and formation of membrane cross-links, were only observed once all the GSH was oxidized. Protein thiol loss was also demonstrated in neutrophils (*62*) and in a tumor cell line (*35*). With endothelial cells (*24*), about 30% of cellular GSH and 13% of total protein thiols were lost with a sublethal dose of HOCl. These studies helped establish that HOCl can penetrate the cell membrane and react with intracellular constituents. Previously, it had been thought that HOCl reacted almost exclusively at the membrane and that only derivative lipophilic chloramines reached the interior of the cell (*22*, *63*).

The effectiveness of antioxidant defenses against HOCl will depend on the products formed on reaction with thiols and whether the parent thiols can be regenerated. Several studies have suggested that higher oxidation states are produced (*19*, *27*). The reaction of HOCl with GSH is complex, and in vitro generates two novel products in addition to GSSG (*64*). One has been preliminarily identified as an internal sulfonamide of GSH (Fig. 3), and the other as a further oxidation product of GSSG, glutathione thiolsulfonate. Few studies have investigated the products of the reaction in cells. When red cells were exposed to HOCl the GSH was initially converted to GSSG and at low oxidant concentrations could be regenerated (*61*). In contrast, with neutrophils (*62*) and endothelial cells (Fig. 3 and J. M. Pullar, M. C. M. Vissers, and C. C. Winterbourn, submitted for publication), very little GSSG was formed and only a small fraction of the GSH loss was accounted for as protein mixed disulfides. The remaining GSH products were rapidly exported from the cells, and were detected in the extracellular medium. They appear to include the sulfonamide as well as other yet to be identified products. These findings have major implications for the maintenance of the cell's redox state. The sulfonamide could also serve as a useful marker of neutrophil oxidative injury in inflammation.

Protein thiols may vary in reactivity with HOCl depending on their accessibility and pK_a. In human endothelial cells susceptibility to inactivation by low amounts of HOCl varied between three thiol-containing enzymes (*60*). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was much more susceptible to oxidation than were creatine kinase or lactate dehydrogenase. It was also more readily oxidized than GSH (*60*). GAPDH has an essential cysteine at the active site (Cys 149) that is

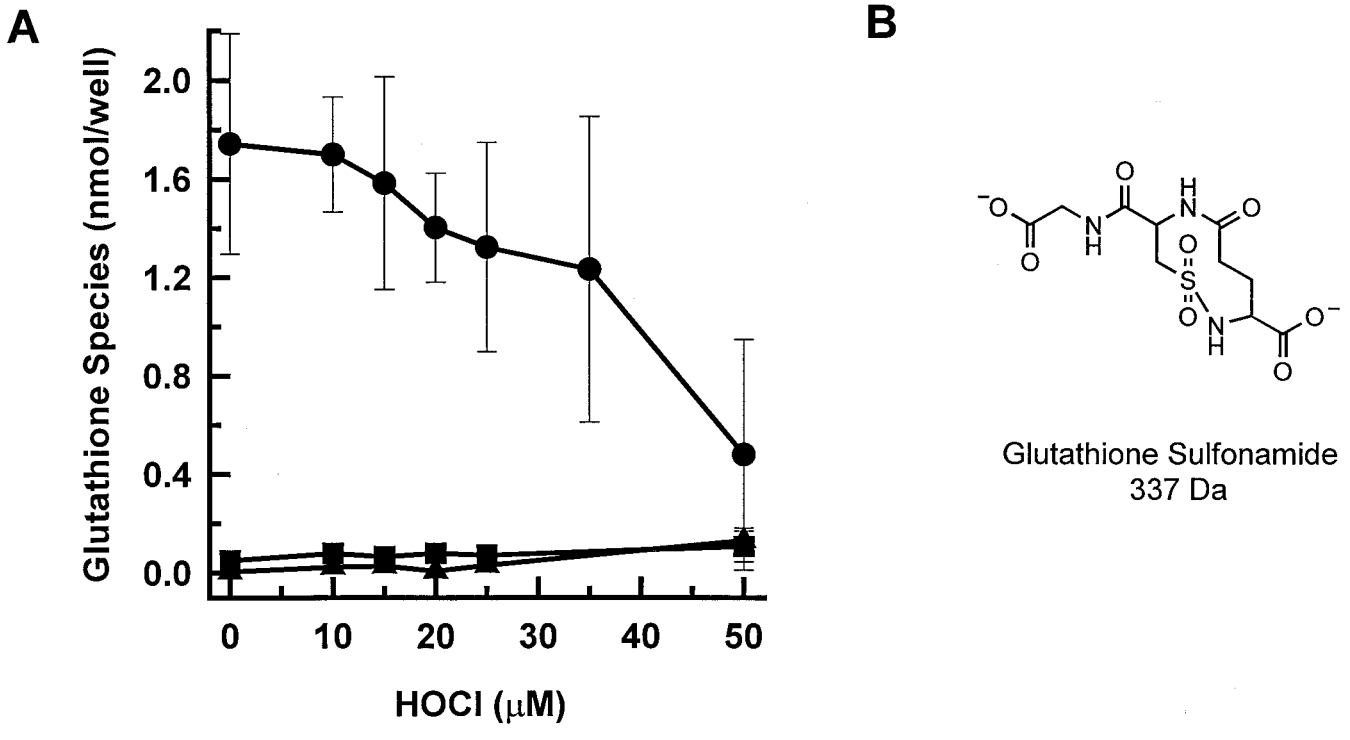

Figure 3. Loss of GSH and formation of products after exposure of endothelial cells to HOCl (A). Total intracellular and extracellular GSH (●), GSSG (■) and glutathione sulfonic acid (▲) were measured in endothelial cells 10 minutes after exposure to HOCl. As shown, there was minimal formation of GSSG or the sulfonic acid. (B) Proposed structure of glutathione sulfonamide, a novel product of the reaction of HOCl with GSH.

present as the thiolate anion at neutral pH and is particularly reactive (*65*). Thiol-labeling of tissue samples from patients with inflammatory bowel disease has provided strong evidence that GAPDH oxidation occurs at sites of inflammation and suggests that this is a particularly sensitive marker for oxidative stress (*66*). In endothelial cells, oxidation of GAPDH was reversible but only at lower concentrations of HOCl (*60*). Higher amounts of oxidant may convert the essential cysteine to a sulfonic acid or some other higher oxidation state that cannot be regenerated.

Isolated rat hearts perfused with HOCl showed impaired contractile function that occurred concurrently with protein thiol loss (*67*). Dithiothreitol could restore both function and protein thiol levels, suggesting that HOCl induced injury through the oxidation of protein thiol groups (*68*). In a follow-up study, the impaired contractile performance was associated with a decline in sarcoplasmic reticulum Ca^{2+}-ATPase activity, Ca^{2+} uptake, and protein thiol levels (*69*). Dithiothreitol significantly reversed the above changes, implicating thiol oxidation of the Ca^{2+}-ATPase. This enzyme plays a major role in the regulation of intracellular Ca^{2+} levels and the generation of force in the cardiomyocytes, and it can be inhibited by HOCl (*70*). Treatment of isolated cardiac myocyte sarcolemmal membranes with HOCl also caused inhibition of Na^+-K^+-ATPase (*71*, *72*), an enzyme that regulates the intracellular Na^+ concentration. Inhibition may alter Ca^{2+} levels via Na^+/Ca^+ exchange reactions, and hence affect myocyte function.

HOCl can cause the release of zinc from zinc finger proteins in which the metal is bound to the sulfydryl group of cysteines by thiolate bonds (*73*). Treatment of cultured cells with HOCl causes the mobilization of intracellular zinc, most likely from these metalloproteins (*74*, *75*). In endothelial cells Zn^{2+} mobilization was accompanied by a loss of cell thiols, shortening of actin microfilaments, retraction of cells, and an increase in endothelial cell permeability that is related to oxidative damage to the cytoskeleton proteins (*24*, *76*).

HOCl has been shown to cause alterations in the levels or activity of a number of cell constituents important in maintaining cellular function. ATP levels are decreased by treatment of cells with HOCl, and this can occur at sublethal doses (*35*, *54*, *60*, *77*). The reactions causing a decline at ATP levels have not been established, but inhibition of GAPDH, mitochondrial respiration and glucose transport have all been shown to occur with HOCl treatment of cells (*35*, *78*). HOCl can also react directly with ATP (*27*). Treatment of tumor cells with HOCl induced a decrease in NAD and an inhibition of cellular respiration (*35*). These changes occurred at reasonably high concentrations of HOCl.

HOCl AS A SIGNALING MOLECULE

The finding that HOCl can react with intracellular components, combined with its high reactivity with thiols, has led to speculation that it can regulate specific cell processes. Several recent studies suggest that this could occur. Exposure to low doses of HOCl initiates a transient growth arrest in endothelial cells (*58*) and human skin fibroblasts (*79*). In fibroblasts this was associated with an increase in the levels of the transcription factor p53, and the p53-dependent gene product WAF1/CIP1 was upregulated, confirming that the increased p53 was transcriptionally active (*79*). HOCl has also been shown to activate the transcription factor NF-κB in a T-lymphocyte cell line (*80*). In this study, the HOCl was added in the presence of whole medium, and this effect is likely to be caused by the action of secondary chloramines.

Apurinic endonuclease (APE) is a DNA repair enzyme that also regulates the redox state of several transcription factors. Pretreatment with a sublethal dose of HOCl reduced the frequency of chromosomal aberrations caused by subsequent exposure to H_2O_2 (*81*). This was attributed to transcription of the APE gene. Thus, HOCl induced an adaptive response in mammalian cells that protected cells from further oxidant-mediated chromosomal aberrations. Many signaling pathways involve the activation of MAP kinases. Our laboratory has recently found that low doses of HOCl can cause selective phosphorylation of components of the MAP kinase cascade, with activation of extracellular signal regulated protein kinase (ERK) and p38 kinase, dependent on the amount of oxidant added (R. Midwinter, C. C. Winterbourn, M. C. M. Vissers, unpublished observations).

CONCLUSIONS AND FUTURE DIRECTIONS

The tendency to refer to oxidants generically as ROS (reactive oxygen species) or RONS (reactive oxygen and nitrogen species) encourages the concept that these oxidants are similar and that they react similarly. Based on known differences in chemical reactivity, this is unlikely to be true (*82*). Other oxidant species, particularly H_2O_2 and peroxynitrite, are known to affect cell viability and functions. However, there are notable differences between the various oxidative systems. In particular, the amount of HOCl required for toxicity is markedly lower than the concentration of H_2O_2. This may reflect the absence of an enzymatic scavenger system for HOCl, or alternatively, its greater ability to damage specific cell targets. Another important consideration is that HOCl causes irreversible loss of intracellular GSH, which can presumably be replaced only by resynthesis. The same may be true for protein thiols, in which case, repair mechanisms will be less effective than for oxidants such as H_2O_2. These effects would have major consequences for the maintenance of the cell's redox state.

There is currently much interest in the role of oxidants in cell signaling. H_2O_2 and peroxynitrite have been shown to activate kinase pathways and to cause transcription factor activation, and there is evidence for the generation of undefined oxidants in many receptor-mediated signaling events. The findings that some signaling processes can also be initiated by low doses of HOCl, together with the profile of targets for this oxidant, may help in the search for the mechanism of oxidant-induced signaling. It could be noted that the growth arrest and apoptosis initiated in response to low and intermediate doses of HOCl are stress responses, and there is currently no evidence that HOCl

causes modulation of function, as has been seen with H_2O_2. This will become of interest as the differences between oxidants and the role they play in inflammation are better defined.

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