Hypochlorous acid (HOCl) is a highly reactive product generated by the myeloperoxidase reaction during the oxidative burst of activated neutrophils, which is implicated in many bactericidal and cytotoxic responses. Recent evidence suggests that HOCl may also play a role in the modulation of redox sensitive signaling pathways. The short half-life of HOCl and the requirement for a continuous presence of H₂O₂ as a substrate for its myeloperoxidase-catalyzed generation make the study of HOCl-mediated responses very difficult. We describe here an enzymatic model consisting of glucose/glucose oxidase, catalase, and myeloperoxidase (GOX/CAT/MPO) that allows the controlled generation of both HOCl and H₂O₂ and thus, mimics the oxidative burst of activated neutrophils. By employing this model we show that HOCl prevents the H₂O₂-mediated activation of iron regulatory protein 1 (IRP1), a central post-transcriptional regulator of mammalian iron metabolism. Activated IRP1 binds to “iron-responsive elements” (IREs) within the mRNAs encoding proteins of iron metabolism and thereby controls their translation or stability. The inhibitory effect of HOCl is not a result of a direct modification of IRP1 by this oxidant. Kinetic experiments provide evidence that HOCl intervenes with the signaling cascade, which results in the activation of IRP1. We further demonstrate that HOCl antagonizes the H₂O₂-mediated increase in the levels of transferrin receptor, which is a downstream target of IRP1. Our findings suggest that HOCl can modulate signaling pathways in a concerted action with H₂O₂. The GOX/CAT/MPO system provides a valuable tool for studying the regulatory function of HOCl.

Phagocytic cells, including neutrophils and macrophages, have an important function in the inflammatory response. Upon stimulation, they undergo an "oxidative burst" resulting in the generation of large amounts of superoxide by a membrane associated NADPH oxidase, which is further metabolized to H₂O₂ by either spontaneously or by superoxide dismutases (1). Hydrogen peroxide is mostly utilized by the heme enzyme myeloperoxidase (MPO)¹ that is released from azurophilic granules upon neutrophil activation. Being activated, myeloperoxidase is able to oxidize Cl⁻ to hypochlorous acid (HOCl). Approximately up to 70% of H₂O₂ is converted by myeloperoxidase (2) to hypochlorous acid (HOCl), a highly reactive species with potent microbicidal and cytotoxic properties (1). On the other hand, activated MPO oxidizes a wide variety of different substrates by abstracting only one electron under formation of radical products. These substrates include tyrosine, tryptophan, sulfhydryls, phenol, and indole derivatives, nitrite, hydrogen peroxide, xenobiotics, and others (3–5). Thus, myeloperoxidase produces several reactive oxidants by utilizing hydrogen peroxide.

While the generation of HOCl is of fundamental significance for combating infection, sustained levels of this oxidant are associated with side effects of inflammation, such as cell injury and tissue damage (6–8). Recent studies demonstrate the presence of HOCl-modified proteins in inflammatory bowel disease (9) and in atherosclerotic plaques (10). HOCl is cell permeable and reacts readily with thiols, thioethers and amino groups (11, 12). Exposure of red cells or endothelial cells to HOCl results in an early decrease of the glutathione pool (13, 14). This suggests that HOCl could modulate cell processes in a manner similar to that seen with H₂O₂ or peroxynitrite. Most studies on cellular responses to HOCl have focused on the toxic nature of this oxidant (6, 7). However, regulatory functions of MPO and MPO-derived products have recently been identified. Cell-bound MPO rapidly transcytoses the intact endothelium and localizes at the basolateral site of the endothelium closely associated with interstitial matrix proteins such as fibronectin (15). In inflammatory models, the immunoreactivity of MPO strongly colocalizes with the formation of nitrotyrosine in subendothelial and epithelial tissue regions (16). Moreover, MPO impairs the NO-dependent blood vessel relaxation (17). MPO and especially HOCl are apparently involved in apoptosis induction (18, 19). While HOCl prevents the expression of endothelial adhesion molecules at sublethal concentrations (20), it has also been demonstrated to activate important regulatory molecules as the tumor suppressor protein p53 (21) and members of the MAP kinase pathway (22).

The lack of appropriate models for HOCl generation poses an

¹ The abbreviations used are: MPO, myeloperoxidase; GOX, glucose/glucose oxidase; CAT, catalase; IRE, iron responsive element; IRP, iron regulatory protein; EMSA, electrophoretic mobility shift assay; PMA, phorbol myristate acetate; TR, transferrin receptor

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obvious limitation for studying and understanding the signaling functions of this oxidant. The high reactivity of HOCl toward functional protein groups makes impossible to sustain steady-state nontoxic concentrations of the oxidant in cell culture media. In addition, the employment of myeloperoxidase for a physiologically relevant continuous release of HOCl requires sustained nontoxic levels of substrate (\(\text{H}_2\text{O}_2\)), which is extremely labile and gets rapidly metabolized by catalases and/or glutathione peroxidases. \(\text{H}_2\text{O}_2\) itself has signaling functions and is involved in redox-sensitive regulatory pathways (23).

Exposure of mammalian cells or tissues to \(\text{H}_2\text{O}_2\) results in activation of iron regulatory protein 1 (IRP1) (24–27), a central posttranscriptional regulator of cellular iron metabolism (28, 29). In iron-replete cells, IRP1 assembles a cubic iron-sulfur cluster and functions as a cytosolic aconitase. In iron-starved cells, or cells exposed to nitric oxide (NO) or \(\text{H}_2\text{O}_2\), the iron-sulfur cluster is removed and IRP1 is activated for binding to mRNA “iron-responsive elements” (IREs). IRE/IRP interactions in the 3′ untranslated region (UTR) stabilize transferrin receptor (TfR) mRNA against degradation. Conversely, IRE/IRP interactions in the 5′ UTR inhibit translation of ferritin (H- and L-) mRNAs. Thus, the IRE/IRP system accounts for the coordinate regulation of TfR and ferritin expression, key proteins involved in cellular iron uptake and storage, respectively, but also of other proteins of iron and energy metabolism (24–29).

While iron chelators promote a slow (>4 h) response, \(\text{H}_2\text{O}_2\) activates IRP1 within 30 min. The mechanism does not involve a direct attack of the iron-sulfur cluster by \(\text{H}_2\text{O}_2\), and a mere increase in intracellular \(\text{H}_2\text{O}_2\) levels is not sufficient to activate IRP1 (27). It appears that extracellular \(\text{H}_2\text{O}_2\), but not \(\text{H}_2\text{O}_2\) released from the mitochondrial or peroxisomal compartments, elicits a signaling cascade, which ultimately leads to IRP1 activation. The oxidative stress-mediated activation of IRP1 is associated with an increase in iron uptake via the TfR (30). The well-established role of iron and \(\text{H}_2\text{O}_2\) in tissue injury (31), based on Fenton chemistry, e.g., the iron-catalyzed decomposition of \(\text{H}_2\text{O}_2\) to aggressive hydroxyl radicals, suggests that this response may have important pathophysiological implications. This is particularly relevant in inflammation, where cytotoxic immune cells release large amounts of reactive oxygen species. As extracellular \(\text{H}_2\text{O}_2\) poses an efficient and rapid activating signal for IRP1, much attention has been drawn to inflammatory cells like neutrophils, which represent a major source of extracellular oxidative stress during their respiratory burst (32). In stimulated neutrophils the release of \(\text{H}_2\text{O}_2\) can increase by a factor of 10 and reach micromolar concentrations (26).

Our recent findings on IRP1 activation by \(\text{H}_2\text{O}_2\), the importance of both \(\text{H}_2\text{O}_2\) and HOCl in the context of inflammation and the potential of both molecules to be involved in cytotoxic and signaling activities, prompted us to establish a model for quantitative HOCl generation and study the role of respiratory burst products in iron homeostasis.

**MATERIALS AND METHODS**

**Reagents and Solutions**—Luminol, phosphate-buffered saline, \(\text{H}_2\text{O}_2\), catalase, sodium hypochlorite, glucose oxidase, and sodium azide were from Sigma. Purified human myeloperoxidase (MPO) was a gift from Christine C. Winterbourn. Stock solutions of luminol were prepared in 10 mM phosphate-buffered saline and adjusted to pH 7.4. Stock solutions of NaOCl and \(\text{H}_2\text{O}_2\) were prepared in water. Their concentrations were determined spectrophotometrically (\(\epsilon_{\text{max}} = 350\) liter mol \(^{-1}\) cm \(^{-1}\) at pH 12 (33)) and \(\epsilon_{\text{max}} = 74\) liter mol \(^{-1}\) cm \(^{-1}\) (34) for NaOCl and \(\text{H}_2\text{O}_2\), respectively. For cell experiments with catalase, luminol, and NaCl, a Dianorm buffer (HBSs) was used: pH 7.4, 137 mM NaCl, 5 mM KCl, 5 mM glucose, 2 mM Na\(\text{H}_2\text{PO}_4\), 2 mM KH\(\text{PO}_4\), 1.47 mM Mg\(\text{Cl}_2\), 0.9 mM Ca\(\text{Cl}_2\). Cell Culture—Murine B6 fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM glutamate, 100 units/ml penicillin, 0.1 ng/ml streptomycin, and 10% fetal calf serum.

**Exposure of Cells to HOCl**—NaOCl was diluted in HBSS immediately before addition to the cells. NaOCl solutions contain about equimolar concentrations of HOCl and OCI\(^-\) (\(\rho_{\text{Ka}} = 7.5\)) at pH 7.4 and can be applied as sources of HOCl. Unless otherwise indicated, all cell experiments were performed in 10-cm culture dishes (Fa. Greiner, Germany) with a cell density of \(8 \times 10^5\) cells/fish containing 900 μg of protein (confluency ~80%); prediluted HOCl was then added in a total volume of 8 ml HBSS. HOCl was rapidly consumed on addition to the cells with a half-time of less than 1 min, as reported earlier (35). In addition, HBSS was demonstrated to modulate TIR expression when exposed longer than 1 h and longer incubation of cells with HBSS mimicked cell viability (60% at 8 h). Exposure of cells to HBSS were kept below 60 min. In 24-hour experiments to study TIR expression, HBSS was replaced by cell culture medium after the 60-min incubation period. In the pulse-chase experiments, cells were only exposed for 5 min to HBSS and NaOCl at varying time points as indicated. Cell lysates were treated with HOCl in a total volume of 60 μl. The addition of HOCl was at room temperature; subsequent incubations were at 37 °C.

**Determination of Catalase and GOX Activity**—Actual activities of GOX, catalase, and MPO were determined at very low \(\text{H}_2\text{O}_2\) concentrations prior to the experiment using a sensitive chemiluminescence technique (36, 37). Appropriate amounts of both enzymes were mixed in HBSS containing 5 mM glucose to low \(\text{H}_2\text{O}_2\) concentrations. Continuous measurements on culture supernatants confirmed the maintenance of steady-state concentrations of \(\text{H}_2\text{O}_2\) during the experiments (38).

**Determination of MPO Activity**—The enzymatic activity of MPO was determined by measuring the \(\text{H}_2\text{O}_2\) degradation as previously described for catalase activity (36, 38), with some modifications. Briefly, 450 μl of HBSS containing known concentrations of \(\text{H}_2\text{O}_2\) were mixed with luminol. After addition of 50 μl of MPO, MPO-mediated \(\text{H}_2\text{O}_2\) decay was measured by addition of NaOCl in intervals of 10 s. Prior to the NaOCl injection, the so-called luminol-dependent chemiluminescence by luminol, and MPO was measured and subtracted. The zero order decay of \(\text{H}_2\text{O}_2\) at high \(\text{H}_2\text{O}_2\) concentrations, an exponential decay of \(\text{H}_2\text{O}_2\) was expected herein as \(k_{\text{MPO}}\) and found to be 31 s\(^{-1}\) (stock solution). The rate constants \(k_{\text{MPO}}\) and \(k_{\text{MPO}}\) for \(\text{H}_2\text{O}_2\) decomposition by MPO were determined by using linear fit software (Microcal Origin 6.0, Microcal Software Inc.). To better compare our results with those reported in the literature, MPO activity was also determined by means of the conventional guaiacol assay (39, 40). The standard molar activity of MPO was defined reaction mix of 10 μM luminol, 88.7 mM guaiacol, and 10 mM MPO in 10 mM phosphate buffer, pH 7.4. The oxidation of guaiacol was followed spectrophotometrically at 470 nm. An activity \(k_{\text{MPO}}\) of \(15 \pm 0.8\) s\(^{-1}\) was obtained for the MPO stock solution that corresponded very well with 18 μM s\(^{-1}\) as determined with the luminol/NaOCl technique.

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSAs were performed as described recently using a radiolabeled human ferritin H-chain IRE probe (45). RNA-protein complex formation was quantified by densitometric scanning of the depicted autoradiographs.

**Western Blotting**—Cells were lysed directly in radioimmune precipitation assay (RIPA) lysis buffer and lysates immediately boiled for 10 min. Equal aliquots were resolved by SDS-PAGE on 8% gels and proteins were transferred onto nitrocellulose filters. The blots were saturated with 5% nonfat milk in phosphate-buffered saline and probed with TIR (Zymed Laboratories Inc., San Francisco, CA) or β-actin (Sigma) antibodies. Dilutions for antibodies were 1:400 (TfR), 1:500 (β-actin), and 0.05% (v/v) Tween 20. Blots with TIR monoclonal antibodies were further incubated with rabbit anti-mouse IgG (1:6000 dilution). The blots with β-actin antibodies were incubated with goat anti-rabbit IgG (1:10,000 dilution). Detection of the peroxidase-coupled secondary antibodies was performed with the ECL® method (Amersham Biosciences). The blots were quantified by densitometric scanning using the TotalLab software version 1.11 (Nonlinear Dynamics Inc.).

**Cytotoxicity Studies**—Cell viability was determined with the MTT assay. Briefly, the tetrazolium salt (MTT) is converted into a blue formazan product that is detected using a 96-well plate reader at 570
The kit was obtained from Roche Applied Science and used according to the manufacturer’s recommendations.

RESULTS

A Titrated Mixture of GOX/CAT/MPO Allows the Continuous Generation of HOCl at Physiologically Relevant Concentrations—A treatment of cultured cells with a single bolus of HOCl does not mimic in vivo conditions of HOCl release by polymorphonuclear (PMN) cells. On the other hand, the employment of MPO for generation of HOCl requires the continuous presence of H2O2 that may itself regulate redox-sensitive pathways. No system allowing the controlled and independent generation of HOCl and H2O2 has been described thus far.

Fig. 1. H2O2 kinetics by (a) glucose oxidase, (b) catalase, and (c) myeloperoxidase at very low H2O2 concentrations. Linear (left panel) and semilogarithmic plots (right panel) are depicted to visualize the characteristic kinetic behavior. While catalase is not saturated by its substrate H2O2, MPO becomes saturated at concentrations higher than 0.5 μM H2O2. For stable H2O2 steady-state generation, the H2O2 degrading enzyme needs to work at non-saturating conditions. All experiments were carried out in HBSS, at pH 7.4 and 37 °C, and with 5 mM glucose. Final enzyme activities were for panel A, glucose oxidase (1:100 000) with k_{GOX} = 3.4 × 10^{-8} M s^{-1}; for panel B, catalase (1:50,000) with k_{CAT} = 0.019 s^{-1}; and for panel C, 2.3 nM myeloperoxidase (1:1000) with k_{1MPO} = 1.8 × 10^{-8} M s^{-1}; and k_{2MPO} = 0.031 s^{-1}.

We have recently shown that a mixture of glucose/glucose oxidase titrated with catalase (GOX/CAT) generates stable H2O2 steady-state concentrations over hours (38, 46). Fig. 1 shows real-time H2O2 measurements at very low H2O2 concentrations for GOX (a), CAT (b), and MPO (c). The specific kinetic patterns are visualized using linear as well as semilogarithmic plots. In the GOX/CAT system, the stability of the equilibrium is based on the specific kinetics of these enzymes: While GOX generates H2O2 under saturating conditions at pseudo zero order rate (Fig. 1a), catalase is not saturated by its substrate H2O2 up to molar concentrations, and H2O2 decay follows an exponential pattern (Fig. 1b). As a consequence, at a given GOX
concentration, H₂O₂ is formed at a constant rate. In the presence of catalase, the concentration of H₂O₂ reaches steady-state levels when the rate of its degradation by catalase equals the rate of its production by GOX.

GOX has already earlier been used as H₂O₂ source for MPO (18–22). However, in these reports the concentration and generation rate of H₂O₂ was not characterized. Aimed at developing a system for the controlled generation of H₂O₂ and HOCl, we first set up experiments to study the removal of H₂O₂ by MPO. Direct and real-time measurement of MPO-mediated H₂O₂ degradation reveal two different phases (Fig. 1c). At H₂O₂ concentrations above 5 μM, the enzyme is saturated and degradation rate is independent of substrate, following a zero order decay. At H₂O₂ concentrations below 0.5 μM, and similar to catalase, the degradation rate linearly depends on substrate. In conclusion, no stable H₂O₂ steady state can be formed by simply mixing MPO and GOX when substrate concentrations exceed 0.5 μM.

To establish controlled conditions for H₂O₂ generation and degradation, in order to reach steady-state concentrations, the addition of catalase is necessary. In such a triple enzyme system (GOX/CAT/MPO) glucose/glucose oxidase and catalase are mainly responsible for maintaining stable H₂O₂ concentrations in a wide concentration range, while myeloperoxidase controls the rate of HOCl formation.

The formation of the H₂O₂ equilibrium for the GOX/CAT system in the presence or absence of myeloperoxidase is illustrated in Fig. 2a. The steady state is reached within 10 min and can be maintained over several hours, regardless of the presence of myeloperoxidase. Fig. 2b demonstrates the real-time generation of MPO-derived oxidants (mainly HOCl) by the GOX/CAT/MPO system using the unspecific luminol-dependent chemiluminescence (41). Since the amount of GOX remains constant, H₂O₂ generation rate and oxygen consumption are similar in the presence or absence of myeloperoxidase. Consequently, the GOX/CAT/MPO system represents a first experimental model that mimics the release of HOCl and H₂O₂ by myeloperoxidase and GOX. Identical steady-state H₂O₂ concentrations at 10 μM were formed in both systems allowing studies on MPO-derived HOCl functions independent of H₂O₂. MPO-derived oxidants were unspecifically detected in real-time using the luminol-dependent chemiluminescence technique (41, 42). Conditions: HBSS, pH 7.4, 5 mM glucose, 37°C, GOX/CAT/MPO system: kGOX = 3.4 × 10⁻⁴ M s⁻¹, kCAT = 0.0031 s⁻¹; kMPO = 1.8 × 10⁻¹ M s⁻¹; kGOX/CAT = 0.0031 s⁻¹; GOX/CAT/MPO system: kGOX = 3.4 × 10⁻⁴ M s⁻¹, kCAT = 0.0034 s⁻¹.

**Establishment of Nontoxic Conditions for HOCl and H₂O₂ Release by the GOX/CAT/MPO System**—The primary focus of this study is to investigate the regulatory functions of HOCl. This requires to establish conditions where HOCl is continuously generated at relatively low, nontoxic and physiologically relevant concentrations. Due to the high reactivity of HOCl, the GOX/CAT/MPO system in the presence or absence of myeloperoxidase (Fig. 2a) demonstrates the real-time generation of MPO-derived oxidants (b) by a GOX/CAT system w/o myeloperoxidase. Identical steady-state H₂O₂ concentrations at 10 μM and turnover rates of 0.34 μM s⁻¹ are formed in both systems allowing studies on MPO-derived HOCl functions independent of H₂O₂. MPO-derived oxidants were unspecifically detected in real-time using the luminol-dependent chemiluminescence technique (41, 42). Conditions: HBSS, pH 7.4, 5 mM glucose, 37°C, GOX/CAT/MPO system: kGOX = 3.4 × 10⁻⁴ M s⁻¹, kCAT = 0.0031 s⁻¹; kMPO = 1.8 × 10⁻¹ M s⁻¹; kGOX/CAT = 0.0031 s⁻¹; GOX/CAT/MPO system: kGOX = 3.4 × 10⁻⁴ M s⁻¹, kCAT = 0.0034 s⁻¹.

**Table I**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>H₂O₂ LD₅₀</th>
<th>NaOCl LD₅₀</th>
</tr>
</thead>
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<tr>
<td>24 h DMEM</td>
<td>300 μM</td>
<td>2000 μM</td>
</tr>
<tr>
<td>1 h HBSS – 23 h DMEM</td>
<td>150 μM</td>
<td>400 μM</td>
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</table>

**Fig. 2.** Generation of steady-state H₂O₂ concentration (a) and MPO-derived oxidants (b) by a GOX/CAT system w/o myeloperoxidase. Identical steady-state H₂O₂ concentrations at 10 μM and turnover rates of 0.34 μM s⁻¹ are formed in both systems allowing studies on MPO-derived HOCl functions independent of H₂O₂. MPO-derived oxidants were unspecifically detected in real-time using the luminol-dependent chemiluminescence technique (41, 42). Conditions: HBSS, pH 7.4, 5 mM glucose, 37°C, GOX/CAT/MPO system: kGOX = 3.4 × 10⁻⁴ M s⁻¹, kCAT = 0.0031 s⁻¹; kMPO = 1.8 × 10⁻¹ M s⁻¹; kGOX/CAT = 0.0031 s⁻¹; GOX/CAT/MPO system: kGOX = 3.4 × 10⁻⁴ M s⁻¹, kCAT = 0.0034 s⁻¹.
is also nontoxic and far below the rate found during inflammation.

As expected (27), treatment of B6 cells with a bolus of 100 μM H₂O₂ or 10 μM steady-state H₂O₂ results in IRP1 activation (Fig. 3a, lanes 1–3). However, the presence of myeloperoxidase in the H₂O₂-generating mix clearly prevents IRP1 activation (lane 4), suggesting that HOCl modulates the IRE binding activity. This activity can be completely recovered upon treatment of the cell extracts with the reducing agent 2-mercaptoethanol, suggesting that the effect of HOCl on IRP1 is reversible.

We addressed whether the effect of HOCl on IRP1 could be a result of catalase inactivation (47), or a possible direct interaction of HOCl with H₂O₂. However, no inhibition in the activity of exogenously added or endogenous cellular catalase could be observed under these experimental conditions (data not shown). Furthermore, real-time measurements confirmed identical H₂O₂ concentrations in both the GOX/CAT and the GOX/CAT/MPO samples (Fig. 3b), thus excluding a possible interference of HOCl with H₂O₂ metabolism. The H₂O₂ generation rates were identical in all experiments. In conclusion, HOCl seems to prevent H₂O₂-mediated IRP1 activation, without affecting the concentration of H₂O₂.

Effects of HOCl on IRP1 in Cell Extracts—It is well established that H₂O₂ fails to activate IRP1 in cytoplasmic cell extracts (48). Nevertheless, considering that HOCl readily reacts with amino- and sulfhydryl groups of amino acids and thus, has the potential to modify and inactivate proteins (11, 12), experiments with cell extracts could provide some insights on possible direct effects of HOCl on IRP1. To this end, cytoplasmic lysates were incubated with various HOCl concentrations (bolus or steady state) and IRE binding activity was analyzed by EMSA (Fig. 4). Nontoxic (<15 μM) concentrations of HOCl, either administered as a single bolus (lanes 2 and 3), or generated by the GOX/CAT/MPO system (lane 6) do not affect IRP1 activity. However, treatment of the lysates with higher concentrations of the oxidant results in a clear inhibition of IRP1 activity (lanes 4, 5, and 7). Latent IRE binding activity can be readily recovered by 2-mercaptoethanol in extracts treated with up to 110 μM HOCl (bottom panel), but this is not the case in extracts exposed to 500 μM HOCl (lane 5, bottom panel). We conclude that only high and toxic doses of HOCl irreversibly inactivate IRP1, while low, nontoxic concentrations of the oxidant leave IRP1 unaffected.

Evidence That HOCl Interferes with the Signaling Pathway for H₂O₂-mediated Activation of IRP1—The data shown in Fig. 4 exclude a direct modification of IRP1 in the presence of nontoxic HOCl concentrations, and argue against this plausible scenario as a mechanistic basis for the inhibitory effects of HOCl on IRP1 activation by H₂O₂ (Fig. 3a). Thus, the possibility remains that HOCl may interfere with the signaling pathway that leads to IRP1 activation by H₂O₂. To address this, the inhibitory function of HOCl in H₂O₂-mediated activation of

### Table II

<table>
<thead>
<tr>
<th>H₂O₂ as HOCI flux</th>
<th>Cumulative HOCI</th>
<th>GOX catal.</th>
<th>CAT catal.</th>
<th>MPO catal.</th>
<th>Survival</th>
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<tr>
<td>H₂O₂ μM</td>
<td>nmol s⁻¹</td>
<td>μM</td>
<td>%</td>
<td></td>
<td></td>
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<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>1</td>
<td>25</td>
<td>100</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

| α | GOX (+) = k_{GOX} 3.4 × 10⁻⁵ M s⁻¹. |
| β | GOX (+) = k_{GOX} 3.4 × 10⁻⁷ M s⁻¹. |
| γ | CAT (+) = k_{CAT} 0.0034 s⁻¹. |
| δ | CAT (+) = k_{CAT} 0.034. |
| ε | MPO (+) = k_{MPO} 1.8 × 10⁻⁹ M s⁻¹. |
| ζ | MPO (+) = k_{MPO} 3.4 × 10⁻⁸ M s⁻¹. |

Fig. 3. MPO prevents H₂O₂-mediated IRP-1 activation. a, fibroblasts (10⁷ B6) were treated with a pulse of H₂O₂ (lane 2) or H₂O₂ steady-state system without (lane 3) or with (lane 4) myeloperoxidase for 1 h at 37 °C. The samples were centrifuged, supernatants were chilled on ice for up to 1 h, and 10 μl (2.5 μg/μl) were analyzed by EMSA with 25,000 cpm of ³²P-labeled IRE probe in the absence (top panel) or presence of 2% 2-mercaptoethanol (2-ME) (bottom panel). The position of the IRE/IRP complexes and excess free IRE probe is indicated by arrows. Lane 1, untreated control; lane 2, treatment with 100 μM bolus H₂O₂ for 60 min; lane 3, treatment with GOX 1:20,000 = 170 ns s⁻¹ and catalase 1: 23,500 k₅₀ = 0.017 s⁻¹, lane 4, treatment with GOX 1:20,000 = 170 ns s⁻¹ and catalase 1: 25,300 k₅₀ = 0.0168 s⁻¹, MPO 1:10,000, k_{MPO} = 1.8 nm s⁻¹, 6.5 μM cumulative HOCl, resulting steady-state concentrations of H₂O₂: 10 μM. The depicted experiment is representative of three independent measurements. b, correct H₂O₂ steady-state concentrations at 10 μM were measured during the experiment by taking 500 μM from the culture media every 10 min. The figure shows the average of six such measurements over 60 min. No significant differences are observed under conditions w/o MPO and w/o cells.
tants were chilled on ice for up to 1 h, and 10 and 6 treated with a pulse of NaOCl (lanes) or a flux of GOX/MPO (lanes 2–5). By contrast, NaOCl added 15 min, and to a lesser extent 1–3 MPO at nontoxic conditions. The experiment shown in Fig. 6 indicates that H2O2 increases IRP1 activation by H2O2. Previous work has shown a treatment of cells with a single bolus of 100 μM H2O2 results in IRP1 activation within 30–60 min following biphasic kinetics with a characteristic “activation” and an “execution” phase (27, 49). The former requires the cumulative HOCl), lane 7, treatment with GOX (kGOX = 340 nM s−1), MPO + kMPO = 180 nM s−1, 324 μM cumulative HOCI). The depicted experiment is representative of three independent measurements.

IRP1 was evaluated in a time course experiment. Previous work has shown that a treatment of cells with a single bolus of 100 μM H2O2 results in IRP1 activation within 30–60 min following biphasic kinetics with a characteristic “activation” and an “execution” phase (27, 49). The former requires the cumulative HOCl), lane 7, treatment with GOX (kGOX = 340 nM s−1), MPO + kMPO = 180 nM s−1, 324 μM cumulative HOCI). The depicted experiment is representative of three independent measurements.

FIG. 4. IRP-1 binding activity is not directly affected by HOCl/ MPO at nontoxic conditions. Lysates of B6 fibroblasts (107 B6) were treated with a pulse of NaOCl (lanes 2–5) or a flux of GOX/MPO (lanes 6 and 7) for 30 min at 37 °C. The samples were centrifuged, supernatants were chilled on ice for up to 1 h, and 10 μl (2.5 μg/ml) were analyzed by EMSA with 25,000 cpm of 32P-labeled IRE probe in the absence (top panel) or presence of 2% 2-mercaptoethanol (2-ME) (bottom panel). The position of the IRE/IRP complexes and excess-free IRE probe is indicated by arrows. Lane 1, untreated control; lanes 2–5, treatment with the indicated NaOCl concentrations; lane 6, treatment with GOX (kGOX = 34 nM s−1), MPO + kMPO = 34 nM s−1), MPO + kMPO = 340 nM s−1), MPO + kMPO = 180 nM s−1, 324 μM cumulative HOCI). The depicted experiment is representative of three independent measurements.

NaOCl completely reacts with cultured cells within 2 min. The bolus additions thus allowed us in a pulse-chase manner to determine the critical time interval of the HOCl-cell interaction that finally blocks IRP-1 activation. NaOCl added 15 min or even 5 min before H2O2 did not prevent IRP1 activation (lanes 1–3). By contrast, NaOCl added 15 min, and to a lesser extent 25 min after H2O2 incubation, inhibited IRP1 activation (lanes 5 and 6). Considering that NaOCl reacts within 2 min with cellular compounds (35), these results strongly suggest that HOCl intervenes with the early steps in the “execution” phase of IRP1 activation by H2O2.

Effects of HOCl on regulation of TfR by H2O2—The TfR is a major downstream target of IRP1. Previous work has shown that the H2O2-mediated activation of IRP1 is associated with an increase in the steady-state levels of TfR (30). We thus investigated whether HOCl could modulate this response. B6 cells were exposed to 100 μM NaOCl at 15 min following addition of 100 μM H2O2 and TfR expression was analyzed by Western blotting after 24 h. Following a bolus of 100 μM H2O2, TfR expression increased by a factor of three within the 24 h. The experiment shown in Fig. 6 indicates that H2O2 increases TfR expression 2.6-fold (lane 2) and NaOCl added before H2O2 does not affect TfR expression (lane 3). However, NaOCl added after H2O2 impairs the H2O2-mediated increase in TfR expression (lane 4). Continuous presence of the iron chelator desferal fully activates TfR (lane 5).

DISCUSSION

We present here a novel enzymatic system to study HOCl-mediated functions independently of the H2O2 concentration,
experiments with human blood and assuming a normal concentration of (26) The value is obtained by virtually replacing the HBSS used in all phils. This is also evident in quantitative terms. In the healthy CAT/MPO system truly mimics the oxygen burst of neutrophils can yield micromolar concentrations of H2O2 (26, 38). With respect to H2O2 and HOCl production, the GOX/ appropriate HOCl-negative control, as established recently co-release of HOCl. Glucose oxidase/catalase alone serve as concentrations and production rates are generated independently of a characteristics of this system, stable H2O2 steady-state concen- trations and production rates are generated independently of a co-release of HOCI. Glucose oxidase/catalase alone serve as appropriate HOCI-negative control, as established recently (38). With respect to H2O2 and HOCI production, the GOX/ CAT/MPO system truly mimics the oxygen burst of neutrophils. This is also evident in quantitative terms. In the healthy human, leukocytes are able to generate H2O2 at a maximum rate of about 0.2 μM s⁻¹ (50). Isolated suspensions of neutrophils can yield micromolar concentrations of H2O2 (26, 38). Reports in the literature widely agree that up to 70% of the consumed oxygen is converted into HOCI (51–54), correspond- ing to a maximum flux rate of ca. 0.1 μM HOCI per second. As demonstrated in Figs. 2 and 3, these rates can be faithfully obtained with the GOX/CAT/MPO system.

The data in Table 3 illustrate a clear difference in cytotoxicity when HOCI is administered as a single bolus or generated continuously by the GOX/CAT/MPO system. We find that ten times lower amounts of HOCI in the flux mode are sufficient to elicit similar toxicity as a single bolus of the oxidant. This is probably related to following issues: First, the real exposure time with HOCI in a bolus treatment is restricted to less than 5 min even in protein-free culture media. By contrast, we find that in serum-containing media comparable effects can only be observed by employing 20 times higher HOCI concentrations (not shown). In addition, the compartmentation of intact cells by the membranes seems to restrict the access of HOCI to proteins within intracellular compartment. When expressed as mol HOCI per cellular protein, our data nicely correspond to recent findings by Pullar et al. (35) using endothelial cells. In this report, HOCI was sublethal at <25 μM HOCI corresponding to less than 630 μmol of HOCI per g of protein. In B6 fibroblasts, less than 1000 μmol of HOCI per g of protein is nontoxic.

By applying the GOX/CAT/MPO system to address the effects of oxidative burst products on cellular iron metabolism, we demonstrate that low, nontoxic concentrations of HOCI prevent IRP1 activation by H2O2. Previous studies (24, 26, 27, 55) have established that exposure of cultured cells, or intact organs, to H2O2 is associated with a rapid activation of IRP1. An IRP1-mediated activation of TIR expression is expected to promote increased cellular uptake of transferrin-bound iron. This response may be related to the decline of serum transferrin-bound iron (hypoferraemia) as a result of shift to cellular compartments, which is characteristic in chronic inflammation. The data presented here show that HOCI can antagonize the H2O2-mediated activation of IRP1. Importantly, this occurs under nontoxic conditions, with HOCI flux rates more than 20 times lower than established in vivo flux rates (2.8 nM s⁻¹ versus 100 nM s⁻¹). Consequently, we propose that the balance between HOCI and H2O2 concentrations reached during the respiratory burst is important with regard to the IRP1 status. While the presence of two opposing signals may, at first glance, appear counterintuitive, “balance” principles as determining factors to drive the direction of biochemical pathways also operate in complex cytokine signaling networks.

The data shown in Figs. 2 and 3b exclude plausible scenarios for an inhibitory effect of HOCI on H2O2 generation by the GOX/CAT/MPO system. In addition, the experiment in Fig. 4 clearly demonstrates that the impairment of H2O2-mediated activation of IRP1 by low, nontoxic concentrations of HOCI cannot be attributed to a direct oxidant inactivation of the protein. Importantly, these inhibitory effects are observed at HOCI levels, which do not suffice to reduce the intracellular GSH pool (35). We provide here evidence that HOCI interferes with the early phase of the signaling pathway, which is trig- gered by H2O2 and leads to IRP1 activation. As a pretreatment of cells with HOCI fails to prevent IRP1 activation by H2O2, we propose that HOCI does not “mask” a putative H2O2-sensor activity, but rather intervenes with downstream signals. We also show that HOCI modulates the expression of TIR, a down- stream IRP1 target.

Generally, the mode of HOCI action in biochemical pathways is poorly understood. It is conceivable that HOCI reacts imme- diately with thiol groups and modulates the activity of thiol- containing proteins (11, 12, 35). Our findings suggest that HOCI should not be viewed as a mere cytotoxic and damaging species. At low, nontoxic levels, HOCI can be clearly involved in signaling functions, as reported earlier in the context of the tumor suppressor protein p53 (21), the MAP kinase pathway (22), or apoptosis (18).

On a final note, although HOCI is regarded as the major product of MPO activity, it should also be emphasized that MPO can catalyze the generation of a wide variety of additional oxidants. Many different substrates are known to be amenable to one-electron oxidation reactions by compound I (and also at smaller rates by compound II), giving rise to radical products. The above substrates include tyrosine, tryptophan, sulphydral, phenol, and indole derivatives, nitrite, hydrogen peroxide, xeno- biotics, and others (3–5). In fact, one-electron oxidations by other peroxidases have been well studied (43, 44, 56). The data presented herein clearly demonstrate that MPO-derived HOCI is able to modulate IRP1 on its own. Putative effects of addi- tional MPO-dependent oxidants on IRP1 activity and on other cell regulatory functions may add different levels of complexity and will be investigated in future studies.

In summary, our results suggest a regulatory interplay be- tween the small oxygen burst molecules HOCI and H2O2 in the regulation of iron metabolism. In addition, the GOX/CAT/MPO system defines an experimental model for the accurate and specific study of additional HOCI-dependent cellular responses.

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<table>
<thead>
<tr>
<th>Biochemical/cellular response</th>
<th>μmol HOCI/g protein</th>
<th>Bolus (NaOCl)</th>
<th>flux (MPO)</th>
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<tbody>
<tr>
<td>Cell lysates</td>
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<tr>
<td>IRP-1 degradation</td>
<td>500</td>
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<tr>
<td>Reversible inhibition of IRP-1</td>
<td>50</td>
<td>11</td>
<td></td>
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<tr>
<td>mRNA binding activity</td>
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<tr>
<td>No change on IRP-1 activity</td>
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<td>11</td>
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<td>Intact cells</td>
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<tr>
<td>Cytotoxicity</td>
<td>15% survival</td>
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<tr>
<td></td>
<td>90% survival</td>
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<td>2800</td>
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<tr>
<td>IRP-1 activity</td>
<td>Reversible inhibition of H2O2-induced IRP-1 activity</td>
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<td>Other cellular responses</td>
<td>No intracellular glutathione loss (35)</td>
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<td></td>
<td>HOCl production of fully stimulated PMN</td>
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</table>

* Treatment for 60 min.
* SD, less than 10%.
REFERENCES

Additions and Corrections


Myeloperoxidase-derived hypochlorous acid antagonizes the oxidative stress-mediated activation of iron regulatory protein 1.

Sabine Mütze, Ulrike Hebling, Wolfgang Stremmel, Jian Wang, Jürgen Arnhold, Kostas Pantopoulos, and Sebastian Mueller

The name of the first author was stated incorrectly. The corrected version is shown above.


Functional characterization of rat brain-specific organic anion transporter (Oatp14) at the blood-brain barrier. High affinity transporter for thyroxine.

Daisuke Sugiyama, Hiroyuki Kusuhara, Hirokazu Taniguchi, Shumpei Ishikawa, Yoshitane Nozaki, Hiroyuki Aburatani, and Yuichi Sugiyama

Page 43493, lines 5 and 15: The abbreviation TCA was written out incorrectly. It should be taurocholate, not trichloroacetic acid.
Myeloperoxidase-derived Hypochlorous Acid Antagonizes the Oxidative Stress-mediated Activation of Iron Regulatory Protein 1
Sebastian Mütze, Ulrike Hebling, Wolfgang Stremmel, Jian Wang, Jürgen Arnhold, Kostas Pantopoulos and Sebastian Mueller

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