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Immunolocalization of hypochlorite-induced, catalase-bound free radical formation in mouse hepatocytes

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Abstract

The establishment of oxidants as mediators of signal transduction has renewed the interest of investigators in oxidant production and metabolism. In particular, H₂O₂ has been demonstrated to play pivotal roles in mediating cell differentiation, proliferation and death. Intracellular concentrations of H₂O₂ are modulated by its rate of production and its rate of decomposition by catalase and peroxidases. In inflammation and infection some of the H₂O₂ is converted to hypochlorous acid, a key mediator of the host immune response against pathogens. *In vivo* HOCl production is mediated by myeloperoxidase, which uses excess H₂O₂ to oxidize Cl⁻. Mashino and Fridovich (1988) observed that a high excess of HOCl over catalase inactivated the enzyme by mechanisms that remain unclear. The potential relevance of this as an alternative mechanism for catalase activity control and its potential impact on H₂O₂-mediated signaling and HOCl-production compelled us to explore in depth the HOCl-mediated catalase inactivation pathways. Here, we demonstrate that HOCl induces formation of catalase protein radicals and carbonyls, which are temporally correlated with catalase aggregation. Hypochlorite-induced catalase aggregation and free radical formation that paralleled the enzyme loss of function *in vitro* were also detected in mouse hepatocytes treated with the oxidant. Interestingly, the novel immunospin-trapping technique was applied to image radical production in the cells. Indeed, in HOCl-treated hepatocytes, catalase and protein-DMPO nitron adducts were colocalized in the cells' peroxisomes. In contrast, when hepatocytes from catalase-knockout mice were treated with hypochlorous acid, there was extensive production of free radicals in the plasma membrane. Because free radicals are short-lived species with fundamental roles in biology, the possibility of their detection and localization to cell compartments is expected to open new and stimulating research venues in the interface of chemistry, biology and medicine.

INTRODUCTION

Catalase is a 247 kDa enzyme composed of four identical monomer polypeptide chains containing one prosthetic heme group and a tightly bound NADPH cofactor per monomer [1–3]. Catalase is very efficient in catalyzing H₂O₂ dismutation and, by influencing its rate of decomposition, is capable of regulating the intracellular hydrogen peroxide steady state, which should impact intracellular redox signaling (see for example [4–6]). Also, the enzyme has been

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considered to be a pivotal cellular defense against reactive oxygen species (ROS) because of its role in the detoxification of H_2O_2 [1,7,8], which is the precursor of highly reactive species such as hydroxyl radical, $\cdot\text{OH}$ [9,10], and hypochlorous acid [11–13].

Hypochlorous acid (HOCl/OCl^- , hereafter referred to as HOCl) production is mediated *in vivo* by myeloperoxidase (MPO) [11], which catalyzes the two-electron oxidation of halides by H_2O_2 . Although catalase and myeloperoxidase compound I intermediates can be produced upon reaction of the enzymes with H_2O_2 at comparable rates, MPO compound I has been shown to be in equilibrium with the resting enzyme and H_2O_2 . For example, 20-fold molar excess of H_2O_2 has been used to completely convert MPO to its compound I state [13–15]. Once produced, MPO compound I has been demonstrated to be reduced back to the resting enzyme with variable bimolecular reaction rate constants which depend on the concentration of Cl^- . At physiological Cl^- concentrations, the value $k = 4.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ [11,12] has been measured. Therefore, it is difficult to understand how MPO can efficiently compete with catalase for available H_2O_2 even during the oxidative burst when high concentrations of the peroxide are likely to accumulate. Nevertheless, it has been demonstrated *in vitro* that at a catalase/MPO heme ratio of 1:6, catalase efficiently inhibits MPO-mediated HOCl production [16].

In 1988, Mashino and Fridovich studied the reaction between catalase and HOCl [17] and showed that a large molar excess of HOCl over catalase provoked the enzyme's inactivation. This seminal paper suggested to us that free radical intermediates could potentially play a role in catalase inactivation. Surprisingly, further studies have not been conducted so far, and the chemical modifications leading to catalase inactivation have not been characterized, which precludes the search for intracellular products formed in the interaction of catalase with HOCl .

Here we demonstrate that the reaction of catalase with HOCl leads to protein oxidation *in vitro* and in cell cultures, which is a primary event leading to catalase aggregation and consequent loss of function. The detailed, in depth initial characterization of catalase products and mechanism of inactivation enabled further studies using cell cultures by providing a suitable background to search for catalase-DMPO-nitrone adducts and aggregates in the cells. Indeed, through immunospin-trapping in conjunction with confocal microscopy, our results demonstrate that for the first time we have localized radical formation to specific organelles within a cell. Biologically, these findings suggest a possible regulatory mechanism by which catalase activity is modulated, which could have a significant impact on redox signaling.

MATERIALS AND METHODS

Chemicals

NaOCl , succinic anhydride, dimethyl sulfoxide, methionine and 3,5-dibromo-sulfanilic acid were purchased from Sigma Chemical Company (St. Louis, MO); DBNBS was synthesized according to [18]. Horseradish peroxidase (HRP) grade I was purchased from Roche (Indianapolis, IN). Isotopically labeled tyrosine was obtained from Cambridge Isotope Laboratories (Andover, MA) and contained 95–99% uniformly labeled ^{13}C carbon atoms. Catalase was from Calbiochem-Novabiochem Corp. (La Jolla, CA). Catalase solutions were prepared in phosphate buffer; their concentrations were determined spectrophotometrically at 405 nm ($\epsilon = 3.97 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$) and expressed as heme concentrations throughout this manuscript. H_2O_2 was obtained from Fisher Scientific (Fair Lawn, NJ). DMPO (high purity, $\geq 99\%$) was purchased from Alexis Biochemicals (San Diego, CA), sublimed twice under vacuum at room temperature and stored at -70°C . All other chemicals were of analytical grade or better. All protein separations were performed on Invitrogen's NuPage 4–12% Bis-Tris Gels.

Catalase inactivation by HOCl

Catalase inactivation by HOCl was assayed by measuring the inhibition of guaiacol (2 mM) oxidation by horseradish peroxidase (HRP) (1.5 nM)/H₂O₂ (0.5 mM) by means of monitoring a guaiacol oxidation product at 470 nm. Catalase (75 µM) was incubated with HOCl (75–1500 µM) at room temperature for 40 min. The reaction was stopped by the addition of methionine (10 mM). Catalase incubations were then diluted 4,000 times and competition assays were performed.

Measurement of hepatocyte-mediated H₂O₂ consumption by electrochemical detection

Hepatocytes were washed twice with PBS (pH 7.4, 0.1M) and plated at 1.25×10^6 cells/ml concentration. Then two pulses of HOCl (0–250 µM) were added with a 30-min interval between them. After a further 30-minute incubation, cells were lysed with RIPA buffer containing protease inhibitors (Roche complete protease inhibitor cocktail). H₂O₂ uptake experiments were performed using a H₂O₂ electrode attached to an Apollo 4000 *Free radical analyzer* unit (World Precision Instruments, WPI, Sarasota, FL). Amperometric detection of H₂O₂ was carried out using a poise voltage of +400mV. The oxidation of H₂O₂ at the surface of the sensor produced an electric current in the nA range. The reaction was constantly stirred throughout the course of the experiment. The amount of catalase added was a function of the total protein content in the sample as measured with Pierce's BCA Protein Assay Reagent Kit.

EPR and EPR spin-trapping studies

The reaction mixtures containing catalase and HOCl in the presence or in the absence of DBNBS (0.25 mM) were transferred to a flat cell, and EPR spectra were obtained with a Bruker EMX spectrometer. The instrument was equipped with an ER 4122 SHQ cavity operating at 9.78 GHz and 100 KHz modulation field at room temperature. For the spin-trapping experiments, DBNBS and taurine were added together immediately after HOCl. Taurine was included to scavenge excess HOCl(13). Digestion of DBNBS/protein radical adducts was performed by incubation with 0.9 mg/ml bovine pancreas trypsin at 37 °C for 10 minutes.

Immunoprecipitation of catalase from mouse hepatocytes

Mouse hepatocytes were collected and washed twice with PBS (pH 7.4, 0.1 M). Subsequently, cells were lysed in lysis buffer (NaCl, 150 mM; Tris-HCl, 10 mM; EGTA/EDTA, 1mM each; PMSF, 0.2 mM; Triton-X, 1%, pH 7.4), and the supernatants collected. Immunoprecipitation of catalase was performed using the Pierce seize X protein G immunoprecipitation kit according to the manufacturer's instructions.

Confocal microscopy

Hepatocytes were plated onto microscope cover glasses (22 × 22 mm, 1 ½ mm thickness) (Erie Scientific Co., Portsmouth, NH). After 20h the cells were washed with PBS twice, and DMPO (40 mM) was added. After fifteen minutes of equilibration, HOCl was added in 3 pulses (one every half hour) of 25–50 µM. Hepatocytes were then washed and fixed with 4% formaldehyde. Excess formaldehyde was washed with PBS, and cold methanol was then added. Cover slips were washed and blocked overnight with a 2 % bovine serum albumin/2% amicase solution. Primary anti-catalase (mouse host) and anti-DMPO (rabbit host) antisera were diluted in wash buffer (0.05% tween 20, 0.1 % bovine serum albumin/0.1 % amicase in TBS, pH 7.4, 0.1 M) and incubated with the cover slips for one hour. After this period, the cover slips were washed, and secondary Alexafluor anti-mouse conjugated with rhodamine and Alexafluor anti-rabbit conjugated with fluorescein were added. Finally, cells were washed and cover slips were placed over microscope slides (gold seal, rite on micro slides, Portsmouth, NH) containing one drop of mounting medium (Shur/Mount, water base, from EMS, Ft. Washington, PA).

Protein carbonyl assay

Protein carbonyls were accessed through the use of an OxyBlot kit purchased from Chemicon (Temecula, CA) according to the manufacturer's directions with minor modifications. To avoid peroxidase/active catalase interference with the assay, the secondary antibody of choice was Pierce's anti-rabbit conjugated to alkaline phosphatase.

SDS-PAGE and Western blot assay

Primary and secondary antibodies used were rabbit anti-DMPO [19] and goat anti-rabbit IgG conjugated with alkaline phosphatase (Pierce Biotechnology Inc., Rockford, IL), respectively.

HPLC/mass spectrometry studies

Catalase (0.8 mM) was treated with HOCl (10–20 mM) in the presence or in the absence of DMPO (200 mM) in phosphate buffer (pH 7.4, 100 mM) for 30 min at 37 °C. Reactions were stopped by passing the incubations through PD-10 columns previously equilibrated with water. PD-10 columns were eluted using ammonium bicarbonate buffer (10 mM, pH 7.8), and the fractions containing catalase were collected and digested overnight with pronase (100 µg/ml). Digested samples were then filtered through 5 kDa mass cut filters and analyzed through HPLC. HPLC separations were performed on an Agilent 1100 apparatus equipped with an Agilent, Zorbax, reverse phase C-18 column (4.6 × 150 mm, 5 µm particle size). Typically, 50 µl aliquots were injected and eluted using a gradient of solvent A (water containing 0.1% v/v trifluoroacetic acid) and solvent B (acetonitrile containing 0.1% v/v trifluoroacetic acid) as follows: 0–20 minutes (0–5% solvent B); 20–30 minutes (5–10% solvent B); 30–40 minutes (10–20% solvent B); 40–50 minutes (20–50% solvent B); 50–70 minutes (50–90% solvent B), followed by 20 minutes re-equilibration with solvent A between samples. Eluting fractions containing 400 nm absorbing compounds were collected and lyophilized overnight. After resuspending the products in water/methanol (50:50 v/v, containing 1% v/v acetic acid), the solutions were analyzed on an ESI Finnigan LCQ ion trap mass spectrometer operating in the positive ion mode. Finnigan's xcalibur software (version 1.2) was used to analyze the results. Sheath gas flow rate was set at 35% (arbitrary units), capillary voltage and temperature were 7 V and 150 °C, respectively, and the spray voltage was 4.5 kV.

Hepatocyte isolation

Wild-type and catalase-knockout mice fed *ad libitum* were used for these experiments. Liver hepatocytes were isolated as previously described [20]. The studies adhered to National Institutes of Health guidelines for the care and handling of experimental animals. Catalase-knockout mice were a generous gift from Jackson Laboratory, and their catalase levels were verified using Western Blot analysis of their hemolysate samples.

RESULTS

Catalase inactivation by HOCl

Catalase activity was assayed through its ability to inhibit guaiacol oxidation mediated by HRP in the presence of H₂O₂ (for details see Materials and Methods). Figure 1, Panel A, shows that purified catalase not exposed to HOCl efficiently competes with HRP for available H₂O₂, inhibiting guaiacol oxidation. Significantly, HOCl pre-incubation hampered the ability of catalase to scavenge H₂O₂ in a dose-dependent manner, leading to higher yields of guaiacol oxidation over time (Figure 1, Panel A). Interestingly, pre-incubation of catalase with equimolar concentrations of HOCl marginally affected the capacity of the enzyme to catalyze H₂O₂ dismutation. However, under conditions of excess HOCl, the rate of guaiacol oxidation increased proportionally to the HOCl concentration used for catalase pre-treatment (Figure 1, Panel A). At a 10:1 HOCl/catalase ratio, the guaiacol oxidation rate was comparable to that

obtained in the absence of catalase, indicating a substantial inactivation of the enzyme by the oxidant under these conditions (Figure 1, Panel A). Changes in the visible spectrum of catalase triggered by HOCl were also observed (Figure 1, Panel B). Briefly, HOCl addition induced a dose-dependent peak at 570 nm and a marked reduction of the absorption peaks at 405 (Soret) and 627 nm. These modifications are in agreement with previously published studies [17] and confirm compound II formation (characteristic absorption peaks at 435, 535 and 570 nm). Addition of DMPO prior to HOCl considerably protected catalase from inactivation even at the highest HOCl concentration used (Figure 1, Panel A). DMPO can not be considered a good substrate for peroxidases since its oxidizing potential ($E^\circ = 1.63$ V) [21] is far higher than that of the peroxidases' compound I. Neither, is DMPO likely to have acted by inhibiting the reaction of HOCl with catalase by scavenging the oxidant [22] since the addition of DMPO at the concentrations studied did not significantly influence compound II formation obtained from catalase incubation with HOCl (Figure 1, Panel B). This result indicates that DMPO does not affect the interaction of the catalase and HOCl but rather inhibits subsequent chemical modifications of the protein. Also, hepatocytes were treated with two pulses of hypochlorite (25–250 μ M, every 30 minutes). Similarly, they also exhibited considerably lower levels of cellular catalase activity even at the lowest dose used, as demonstrated by the reduction of the rate of H_2O_2 decomposition measured with a H_2O_2 electrode as in Materials and Methods (Figure 1, panel B). This result is consistent with the deactivation seen with purified catalase.

HOCl-induced catalase aggregation and protein carbonyl formation

The studies reported above led us to suspect of significant protein chemical modification in this system. In addition to its notable impact upon catalase activity and absorption spectra, HOCl led to catalase monomer polymerization. SDS-PAGE analysis of non-treated catalase showed a single band at approximately 62 kDa corresponding to the catalase monomeric chain (Figure 2, Panel A). However, incubations of catalase (29 μ M) with varying concentrations of HOCl demonstrated a dose-dependent aggregation of the monomeric chain (Figure 2, Panel A). Interestingly, the addition of the spin trap DMPO prevented catalase aggregation. Similar behavior was observed when DMPO was replaced by DNBBS, a nitroso spin trap (data not shown). Based on these findings, we next used the spin-trapping technology combined with the immunochemical detection of protein-DMPO nitron adducts. As shown in Figure 2, Panel B, catalase samples treated with HOCl in the presence of DMPO were positive for protein-DMPO nitron adducts, demonstrating the production of catalase protein radicals. The amount of protein radical adduct was proportional to the HOCl concentration. Indeed, when catalase was exposed to a 10-fold higher HOCl concentration, protein-DMPO adducts were detected at higher molecular weights, which suggests the existence of multiple protein radical sites on catalase when the enzyme is treated with excess HOCl. It is known that hypochlorous acid is a very reactive compound that can lead to multiple types of protein modification [23]. Next, we evaluated the time course of catalase aggregation, protein radical formation and HOCl-mediated protein carbonyl production. All time course studies used methionine (10 mM) added at the times indicated to stop HOCl reactions with catalase. As shown in Figure 3, Panel A, catalase aggregation could easily be detected as early as 90 seconds after initiating the incubation of the protein with HOCl. At 3 minutes, maximal catalase aggregation seemed to have already been achieved. A similar time course for catalase-DMPO nitron adduct formation was obtained with detectable protein-DMPO derivatives formed after 90 seconds of incubation and maximal yield obtained after 3 minutes (Figure 3, Panel B). In addition, we evaluated protein carbonyl formation. As shown in Figure 3, Panels C and D, protein carbonyl formation was dependent on HOCl concentrations (Figure 3, Panel C) and on the incubation times (Figure 3, Panel D). At 90 seconds, significant protein carbonyl could be detected which increased with time, peaking between 5 and 15 minutes of incubation. Interestingly, pretreatment of catalase (80 μ M in heme) with cyanide (140 μ M) considerably inhibited protein carbonyl formation (Figure 3, Panel C) and catalase aggregation (Figure 4).

EPR detection of catalase protein radicals induced by HOCl

To unequivocally demonstrate catalase radical formation mediated by HOCl, EPR studies were performed. EPR techniques generally require high concentrations of reactants because of sensitivity limitations. In the absence of spin trap agents, catalase incubations with HOCl produced a detectable EPR signal whose intensity depended on HOCl concentration (Figure 5 A–C). As expected, EPR signals could not be detected in the absence of catalase or HOCl (data not shown), indicating that radical formation was a consequence of HOCl interaction with the protein. The signal was detectable for several minutes at room temperature using air-equilibrated buffers. This observation, together with the EPR parameters for this spectrum ($g \sim 2.004$, peak to peak line width = 28 G), suggested the detection of a protein tyrosyl radical [24–29].

To provide further insight into protein radical formation, we next performed spin-trapping experiments. Not surprisingly, in the presence of the spin trap DBNBS, a broad triplet previously characterized as anisotropic protein-derived nitroxide adducts [30–32] could be observed (Figure 5 D). Indeed, the broadening of the line width is the result of the spatial hindrance imposed on to the free rotation of the nitroxide group by the protein backbone, which, *per se*, is convincing evidence of macromolecule-derived radical trapping. Digestion of the mixture with trypsin rendered a mobile triplet ($a^N = 13.6$ G) characteristic of DBNBS radical products, with the radical centered on tertiary carbon atoms such as those expected for tyrosyl radical trapping (Figure 5 I) [31,32]. Indeed, as shown in Figure 5, inset, the DBNBS-tyrosyl radical adduct could be produced with HRP/H₂O₂/tyrosine and had identical EPR parameters, thus furnishing additional support for tyrosyl radical trapping on catalase.

As demonstrated in the case of 2-methyl-2-nitrosopropane (MNP)/tyrosyl radical adduct, proton hyperfine couplings were not observed on the EPR spectrum [33]. Because of that, tyrosyl radical trapping by DBNBS was unequivocally confirmed by the use of tyrosine uniformly labeled with ¹³C, which is expected to induce further splittings in the EPR spectra due to the ¹³C nuclear spin ($I = 1/2$). As in Figure 5, inset, replacing tyrosine with the labeled compound yielded a six-line spectrum as anticipated instead of the three-line one. Addition of DBNBS to catalase/HOCl incubations inhibited catalase aggregation, as analyzed by SDS-PAGE, due to protein radical trapping (data not shown); this inhibition was consistent with EPR results.

The correlation of radical spin trapping with inhibition of catalase aggregation indicates that, to some extent, free radical intermediates contribute to enzyme cross-link formation. Intact and digested incubations of catalase with DBNBS in the absence of HOCl rendered much less intense EPR signals, demonstrating that most of the signal obtained in the presence of HOCl was due to its interaction with catalase and not to the well-characterized ene-addition reaction [34](Figure 5 E and J), which consists of nucleophilic attack of lipids and electron-rich residues to the nitroso moiety of the spin traps and leads to hydroxylamine adduct formation. Hydroxylamines that were originally produced by non-radical mechanisms are likely to then be oxidized to EPR-active nitroxides by oxygen, trace metals or oxidants present in the system. Indeed, in the absence of the oxidant, we observed weaker EPR signals that most likely arose from the direct reaction of protein residues with the nitroso group of the spin trap [34].

Interestingly, addition of cyanide (4 mM) to the incubations before HOCl significantly inhibited the formation of trappable protein radicals, indicating an essential role for the heme moiety in mediating protein radical production (Figure 5F). The addition of lysine also inhibited the formation of the protein radical (Figure 5 G, H). Indeed, lysine residues have been shown to react with HOCl to produce lysine chloramines, which decompose to protein radicals and carbonyl compounds [35,36]. Lysine residues have also been shown to influence tyrosine

chlorination in peptides, but no evidence for a radical-based mechanism has been provided [37,38].

Heme chemical modification studied by HPLC/mass spectrometry

The studies presented above clearly demonstrated the central role played by the heme group in mediating catalase oxidation. Thus, to obtain further information about possible HOCl-induced chemical modifications of the heme prosthetic group, we next digested HOCl-treated catalase and analyzed the peptide mixture and the heme derivatives through HPLC. According to Figure 6, treatment of catalase with a 20-fold excess of HOCl greatly modified the chromatographic elution pattern of catalase digests. In fact, digests monitored at 280 nm also revealed that the protein polypeptide chain suffered major chemical modifications (data not shown). To a lesser extent, the chromatographic pattern monitored at 400 nm for the detection of heme group derivatives also presented important changes (Figure 6, left panel). Two new peaks (marked A and B) appeared and were collected for analysis through mass spectrometry. Peak A had a mass spectrum consistent with the oxidized heme group ($m/z = 616 + O^{16}$) (Figure 6, right panel A), which corresponded to one oxygen atom added per heme. The higher molecular mass ($m/z = 664$) present in the spectrum corresponds to the oxygenated heme/methanol complex ($m/z = 632 + 32$) [39]. Peak B could also be characterized and is attributable to the chlorinated heme ($m/z = 616 + Cl^{35}$) (Figure 6, right panel B). In this case, the methanol ($m/z = 32$) complex ($m/z = 682$) [39] could also be detected. Notably, although some heme modification could be detected and characterized at extremely high HOCl: catalase ratios (e.g. < 10:1, respectively), most of the heme remained intact as shown by the HPLC chromatogram, demonstrating that although it is a primary site of interaction, the heme group remains virtually unmodified by HOCl under our experimental conditions.

Catalase radical formation in mouse hepatocytes induced by HOCl, localization of protein radicals in intact cells

Having established the mechanisms by which HOCl oxidizes catalase, we then sought the characterized catalase radical products and aggregates produced in living cells by HOCl. For that, we employed the newly developed immuno-spin-trapping technique coupled with confocal microscopy [19,40]. The data shown in Figure 7 demonstrate that HOCl induced DMPO binding to catalase, which is considered to be a major peroxisomal protein. Indeed, the results demonstrated the colocalization of the anti-DMPO antibody (stained in green with anti-rabbit fluorescein conjugate in Figure 7, panels A, B, E and F) and anti-catalase (stained with anti-mouse conjugated with rhodamine, Figure 5, panels A–D). Thus, for the first time protein radical formation was captured and localized in living cells.

In the absence of HOCl or DMPO, catalase was still easily detectable (Figure 7, Panels C and D, respectively); nevertheless, no green fluorescein staining was observed due to the absence of anti-DMPO antibody binding. These data indicate that protein-radical formation was a consequence of the HOCl-induced protein oxidation to free radicals, which were trapped by DMPO.

Localization of catalase and protein-DMPO adducts rendered a dotted stain pattern and were not evenly distributed throughout the cytosol. This pattern is consistent with the fact that catalase is confined within the peroxisomes and that HOCl diffused through such organelles to oxidize catalase. Also, a higher density of DMPO adduct was observed closer to the plasma membrane, which is consistent with the reduced lifetime of HOCl in the presence of cell components such as thiols, proteins, DNA and lipids.

Comparison with cells obtained from knockout mice, which exhibited considerably lower levels of catalase (Figure 7, panels E and F), rendered very interesting results. In this case,

most of the anti-DMPO staining was observed on the plasma membrane with a much reduced amount in the cytosol. This pattern demonstrated that catalase is indeed a major internal target for HOCl and acts as a sink for the oxidant in the hepatocytes. Interestingly, in catalase-rich cells, a much lower membrane radical production was observed even when the higher dose of HOCl was used.

To further confirm catalase as an important target of HOCl in cells, we immunoprecipitated catalase from mouse hepatocytes exposed to different HOCl concentrations before lysis. The protein that was recovered from the immunoprecipitates was then analyzed through Western blot with anti-DMPO-nitrone antibody. According to Figure 8 panel A, catalase-DMPO-nitrone adduct formation was dependent on HOCl concentration, although some minor nonspecific antibody binding to mouse-catalase could also be detected. Moreover, an increased degree of catalase aggregation could be observed in HOCl-treated cell lysates (Figure 8 B), unequivocally establishing catalase as a target for HOCl in cells.

DISCUSSION

Hypochlorous acid production by neutrophils during infectious and inflammatory conditions is a pivotal event leading to both pathogen killing and host tissue damage [41–46]. Myeloperoxidase (MPO), which is believed to be the main source of HOCl, depends on H_2O_2 to convert chloride ions present in high concentrations in biological fluids to HOCl. Nevertheless, MPO and catalase compete for available H_2O_2 . Besides, MPO complete conversion to its compound I state is challenging because such intermediate can decay regenerating the reactants ($MPO + H_2O_2$) [47]. Under normal conditions H_2O_2 is rapidly scavenged and detoxified by peroxidases and catalase, thus keeping not only H_2O_2 but also HOCl at low levels. Indeed, catalase has been shown to efficiently inhibit HOCl formation [16]. Although confined to particular cellular organelles (e.g. peroxisomes), catalase is clearly exposed to compounds that freely diffuse through membranes such as H_2O_2 , HOCl and DMPO (Scheme 1). Indeed, although the locations of particular reactions are different (for example extracellular for MPO and intraperoxisome in the case of catalase), the diffusion of H_2O_2 to the peroxisomes significantly reduces the amount of the peroxide available for MPO use. Conversely, the HOCl diffusion through cellular membranes (clearly demonstrated by our confocal studies in Figure 7), mediates catalase inactivation. Taken together, these processes demonstrate the possibility of an active cross talk between the two enzymes through their substrates and products. Because of that, catalase inactivation by HOCl may be a primary event necessary to guarantee efficient HOCl production during pathological conditions. Even more importantly, catalase inactivation is expected to result in H_2O_2 accumulation, which is likely to trigger redox-sensitive processes in neighboring cells and tissues.

Here we have confirmed that HOCl's action upon catalase inhibits the enzyme's ability to compete with a model peroxidase for H_2O_2 (Figure 1, Panel A) [17]. Of note is that HOCl extensively provoked catalase aggregation in a concentration-dependent manner (Figure 2, Panels A and B), which correlated with protein radical formation (Figures 3, 4 and 5) and protein carbonyl production.

Of note was the central role played by the heme group in the catalysis of the formation of both the radical and carbonyls. Indeed, pre-treatment of catalase with cyanide inhibited all processes evaluated, which included protein radical formation (studied through EPR and immunospin trapping), protein carbonyl production (accessed through OxyBlot) and protein aggregation. Interestingly, although demonstrated to be a primary reaction site, heme was not significantly modified by HOCl as demonstrated by HPLC/mass spectrometry analysis (Figure 6). Our results thus clearly demonstrate that in the particular case of catalase, heme is a central player, catalyzing the HOCl-mediated oxidation of the protein which leads to enzyme aggregation and

consequent loss of function. This might happen through diverse mechanisms which include HOCl activation by coordination to the porphyrin metal center, the heme catalyzed oxidation of aminoacids or even heme-mediated decomposition of chloramines to radicals and carbonyls, which further supports a physiological role for HOCl-mediated catalase activity control.

Although not particularly accessible in its native state, the catalase active site solvent exposure may be severely influenced by chemical modifications demonstrated to take place in the backbone. Significantly, spin-trapping agents were demonstrated to inhibit both catalase aggregation and loss of function without markedly affecting the catalase reaction with HOCl, which, together with the intermediacy of free radical intermediates, suggests an intimate link between the two processes.

Notably, lysine reaction with HOCl produced low molecular weight radicals which were trapped by DBNBS, probably generated by the decomposition of lysine chloramines (Figure 5 G, H). Previously, Hawkins and Davies trapped nitrogen-centered radicals produced when protein and DNA were exposed to HOCl using DMPO as the spin trap [34,35,48,49]; such nitrogen-centered radicals have been demonstrated to be precursors of protein carbonyls. Nevertheless, under their experimental conditions, significant chloramine decomposition was shown to take several minutes to one hour in contrast to HOCl-mediated catalase aggregation and carbonyl formation, which peaked within a couple of minutes after HOCl addition to catalase.

Another product of catalase interaction with HOCl is the tyrosyl radical (Scheme 1). The identification of this particular intermediate, produced upon catalase interaction with HOCl *in vitro*, was fundamental to direct further studies using immunospin-trapping, free radical imaging in cells. Actually, HOCl interaction with catalase in cell cultures was unequivocally demonstrated through the localization of catalase-derived free radical formation in the hepatocytes. Catalase and protein-DMPO nitron adducts were colocalized in the hepatocytes, demonstrating that the enzyme is a target for HOCl in these cells (Figure 7), a finding that was confirmed by the use of hepatocytes obtained from knockout mice and immunoprecipitation experiments. These experiments demonstrated not only catalase-radical formation but also enzyme aggregation in the cellular milieu (Figure 8, Panels A and B). Even more important was the demonstration of the use of immunospin-trapping as a valid methodology to capture radical formation in time and space in living cells (see scheme 1).

Presently, it is clear that oxidant balance plays a fundamental role in cellular homeostasis and signaling. A better characterization of the biologically relevant catalase reactions is, thus, of concern to the understanding of the subtle equilibrium imposed by oxidative challenge as it applies to health and disease. In addition, this work clearly demonstrates how the newly developed immunospin-trapping approach in conjunction with confocal microscopy can significantly contribute to snap-shot protein radical formation on model proteins in test tubes and intact cells.

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Figure 1.

Panel A. Absorption traces at 470 nm of incubations containing HRP (20 nM)/H₂O₂ (200 μM)/guaiacol (1 mM) in the absence (A) or in the presence of catalase (16 μM, in heme) exposed to different HOCl concentrations for 40 minutes. Trace B (HOCl 160 μM, 10:1 molar ratio with catalase); Trace C (HOCl, 80 μM, 5:1); Trace D (48 μM, 3:1); Trace E, dotted line (HOCl was 160 μM, 10:1, but DMPO 40 mM was present); Trace F (16 μM, 1:1); Trace G, control experiment catalase was not exposed to HOCl. Before assay, methionine (25 mM) was added to catalase incubations containing HOCl. The mixtures were diluted in phosphate buffer (pH 7.4, 0.1M) by a factor of 1000 × to a final catalase concentration of 16 nM. Assays were performed at pH 7.4, 0.1 M, at room temperature. **Panel B.** Absorption spectra of incubations

of catalase (16 μM) with HOCl (160 μM) in the absence and in the presence of DMPO 40 mM recorded immediately after the oxidant addition. Incubations were performed in Pi buffer (pH 7.4, 0.1M) at room temperature. **Panel C.** H_2O_2 (100 μM) decomposition rates induced by the addition of hepatocyte lysates normalized by protein concentration treated with HOCl (2x, 0–250 μM) at 37 °C for 1h, assayed through the use of a H_2O_2 electrode according to materials and methods. Trace A, control cells in the absence of HOCl; trace B, cells were treated with two pulses of HOCl (25 μM) at an interval of 30 minutes; trace (C) same as B, but HOCl was 50 μM ; trace D, same as B, but HOCl was 250 μM . Traces shown in Panels A and C are representative curves obtained from 3 independent experiments.

Figure 2. SDS-PAGE analysis of incubations of catalase (29 μ M in heme) exposed to different HOCl concentrations (29 – 290 μ M) in the absence or in the presence of DMPO (200 mM) at pH 6.9. **Panel A.** Gel was stained for protein using Coomassie Brilliant Blue dye. **Panel B.** Same as panel A, but the auto-radiograph picture of catalase Western blot, using anti-DMPO as the primary antibody, is shown.

Figure 3.

Panel A SDS-PAGE analysis of incubations of catalase (40 μ M in heme) exposed to HOCl concentrations (400 μ M) and incubated for the indicated times in phosphate buffer (pH 7.4, 0.1) at room temperature. Reactions were stopped through methionine addition (10 mM). **Panel B.** Basically, the experiment shown in Panel A was repeated in the presence of DMPO (40 mM), and Western blot analysis was performed using anti-DMPO as the primary antibody. **Panel C.** Catalase (40 μ M, in heme) and heme-blocked catalase (produced by pretreating the enzyme with 140 μ M cyanide) were incubated with HOCl at the indicated concentrations for 30 minutes at room temperature in phosphate buffer (pH = 7.4, 0.1M). After this time, methionine (10 mM) was added to scavenge remaining HOCl. Catalase carbonyl content was

then assayed through OxyBlot. Remaining unreacted cyanide was removed prior to HOCl addition by passing the enzyme through Millipore mass cut (< 5000 KDa) filters. Panel D. Essentially, experiment shown in Panel C was repeated fixing the concentration of HOCl (400 μ M) and varying incubation times as indicated. Reactions were stopped by methionine (10 mM) addition prior to carbonyl derivatization.

Figure 4. SDS-PAGE analysis of incubations of catalase (40 μM in heme) exposed to different HOCl concentrations (80 – 400 μM). The experiment was reproduced with catalase pretreated with cyanide (140 μM). Incubations were performed for 30 minutes at room temperature in phosphate buffer (pH = 7.4, 0.1M) and were stopped by methionine (10 mM) addition.

Figure 5.

(A–C). EPR spectra of catalase (0.8 mM, in heme) incubated with different HOCl concentrations (0–8 mM). (A) HOCl was 0.7 mM; (B) 3.5 mM; (C) 8 mM. All incubations were performed in phosphate buffer (pH 7.4, 0.1M) at room temperature. Instrumental conditions were: power, 20 mW; scan rate, 0.47 G/s; modulation amplitude, 2.5 G; time constant, 163 ms; gain, 6.32×10^4 . Each spectrum is the result of the accumulation of 6 scans. Figure 5 (D–J) EPR spectra of catalase (0.8 mM) incubated with HOCl (2 mM) in the presence of DBNBS (0.25 mM) added together with taurine (40 mM) immediately after HOCl. (D) in the presence of HOCl; (E) in the absence of HOCl; (F) in the presence of KCN (4 mM); (G) in the presence of lysine (5 mM); (H) in the presence of lysine (10 mM); (I) same as D, but samples were digested with 0.9 mg/ml trypsin at 37 °C for 10 minutes; (J) same as E, but samples were digested with 0.9 mg/ml trypsin at 37 °C for 10 minutes. All incubations were performed in phosphate buffer (pH 7.4, 0.1 M) at room temperature. Instrumental conditions

were: power, 20 mW; scan rate, 0.47 G/s; modulation amplitude, 2.5 G; time constant, 163 ms; gain, 6.32×10^4 . Each spectrum is the result of the accumulation of 2 scans. Figure 5, inset, EPR spectra of DBNBS/tyrosyl adducts produced in the incubation of tyrosine (2 mM) with H_2O_2 (0.5 mM) in the presence of HRP (500 U/ml) and DBNBS (5 mM). Spectrum 2, tyrosine was replaced with tyrosine ^{13}C -labeled at all four aromatic ring carbons. Spectrum 3, catalase (200 μM) incubated with HOCl (1 mM). DBNBS was added with taurine immediately after HOCl and the sample was digested with trypsin. Instrumental conditions: power, 20 mW; scan rate, 0.23 G/s; modulation amplitude, 1 G; time constant, 327 ms; gain, 6.32×10^5 . Each spectrum is the result of the accumulation of 4 scans.

Figure 6.

Left Panels. Chromatographic elution patterns of catalase (100 μ M, in heme) (upper panel) and catalase treated with HOCl (2.0 mM) for 1 h and digested overnight with trypsin (0.1% w/v). The elution gradient and solvents are detailed in materials and methods.

Right Panels. Mass spectrometry analysis of peaks A and B collected from HPLC separations of HOCl-treated catalase digests. Peaks were collected and concentrated before analysis. For the analysis, solutions were injected in the mass spectrometer operating as described in materials and methods.

Figure 7.

Representative confocal microscopy images of the colocalization of catalase (red stain) and protein-DMPO adducts (green stain) obtained by treating mouse hepatocytes (2.5×10^6 cells/ml) with HOCl. (A) cells were treated with three pulses of HOCl (20 μ M, 30-minute intervals) in the presence of DMPO; (B) same as A, but HOCl was 50 μ M; (C) same as B, but in the absence of DMPO; (D) same as B, but in the absence of HOCl; (E) and (F) same as A and B, respectively, but cells were obtained from catalase-knockout mice. Clockwise, the quadrants in each picture are laser microscopy showing DAPI (for nuclear stain), anti-DMPO stained with anti rabbit (green/FITC conjugate); overlain picture of laser microscopy obtained from

anti-catalase and anti-DMPO (yellow shade obtained by overlaying red and green); laser microscopy showing anti-catalase stained with anti-mouse conjugated with rhodamine.

Figure 8.

Mouse hepatocytes (2.5×10^6 cells/ml) were treated with HOCl (0–220 μM) for 30 minutes in the presence of DMPO (100 mM). Then, Taurine (50 mM) was added to quench unreacted HOCl. Cells were immediately centrifuged and washed twice with PBS (pH 7.4, 0.1M). After addition of lysis buffer (materials and methods for details), the lysates were frozen at -80°C . Immunoprecipitation of catalase was performed as described in the **Materials and Methods** section and Western blots using anti-DMPO (to detect protein radical formation, Panel A) or anti-catalase (to ensure catalase content, Panel B) as primary antibodies were conducted on the recovered protein. The resulting autoradiographs are presented.

MECHANISM OF HOCl-INDUCED CATALASE RADICAL PRODUCTION

Scheme 1.

Schematic representation of the HOCl-induced radical formation in cells. The protonated form of HOCl permeates cell membranes and has access to peroxisome-confined catalase. Once in the peroxisome, HOCl reacts with catalase producing catalase protein radicals which are trapped by freely diffusing DMPO. The oxidation of the protein-bound nitroxides to nitroxide renders the formation of protein radicals detectable in the cells as DMPO adducts.