Elevated levels of reactive nitrogen species (RNS) such as peroxynitrite have been implicated in over 50 diverse human diseases as measured by the formation of the RNS biomarker 3-nitrotyrosine. Recently, an additional RNS was postulated to contribute to 3-nitrotyrosine formation in vivo; nitryl chloride formed from the reaction of nitrite and neutrophil myeloperoxidase-derived hypochlorous acid (HOCl). Whether nitryl chloride nitrates intracellular protein is unknown. Therefore, we exposed intact human HepG2 and SW1353 cells or cell lysates to HOCl and nitrite and examined each for 3-nitrotyrosine formation by: 1) Western blotting, 2) using a commercial 3-nitrotyrosine enzyme-linked immunosorbent assay kit, 3) flow cytometric analysis, and 4) confocal microscopic analysis. With each approach, no significant 3-nitrotyrosine formation was observed in either whole cells or cell lysates. However, substantial 3-nitrotyrosine was observed when peroxynitrite (100 μM) was added to cells or cell lysates. These data suggest that nitryl chloride formed from the reaction of nitrite with HOCl does not contribute to the elevated levels of 3-nitrotyrosine observed in human diseases.

There is considerable interest in the role of reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite (ONOO⁻) in human disease (reviewed in Refs. 1 and 2). Numerous cell types are capable of producing high micromolar concentrations of nitric oxide (NO) through the activation of inducible nitric-oxide synthase (reviewed in Refs. 3 and 4). In vivo, NO is readily oxidized via heme proteins to nitrite (NO₂⁻) and nitrate (NO₃⁻). Thus, evidence for an elevated production of NO comes from the measurement of NO₂⁻ and NO₃⁻ in human body fluids such as plasma, cerebrospinal fluid, synovial fluid, respiratory tract lining fluid, saliva, and sputum. This has implicated NO in a large number and diverse range of human diseases (reviewed in Ref. 4). Typically, levels of NO₂⁻ found in plasma taken from healthy human volunteers range between 0.5 and 21.0 μM (5, 6), and levels are significantly elevated during inflammation, e.g. up to 36 μM in patients with human immunodeficiency virus infection (7). Serum NO₂⁻ levels in patients with rheumatoid arthritis (8), systemic sclerosis (9), and systemic lupus erythematosus (10) are reported to be in the millimolar range, whereas in the synovial fluid of patients with rheumatoid arthritis NO₂⁻ levels are reported to range from 0.3 to 15 μM (11–13). Nitrite has been extensively used for decades in the food industry as a preservative and for curbing meat. Approximately 5% of ingested nitrate is reduced to nitrite by oral microflora where it enters the gastrointestinal tract and protonates to form nitrous acid (pKₐ ~ 3.4) (reviewed in Refs. 14 and 15). Furthermore, dietary NO₂⁻ has been proposed as an oral and gut anti-microbial agent (14, 15) where salivary levels of NO₂⁻ of up to 96 μM have been reported (16), and near millimolar concentrations are reported to be reached in the saliva of patients with systemic sclerosis (17).

Over 50 human disease conditions have elevated levels of 3-nitrotyrosine, a biomarker for RNS traditionally attributed to ONOO⁻ formation in vivo. These include neurodegenerative, chronic inflammatory, gastrointestinal tract, and cardiovascular disorders as well as viral and bacterial infections (reviewed in Refs. 1 and 2). Recent research has shown that 3-nitrotyrosine formation is not solely a ONOO⁻-mediated phenomenon. It is also observed with peroxidases such as eosinophil peroxidase (18), myeloperoxidase (released by activated neutrophils at sites of inflammation) (19), and other peroxidases (20) in the presence of NO₂⁻. In addition, hemoglobin and other heme proteins such as catalase may also serve as a mechanism for nitrating tyrosine residues in proteins using NO₂⁻ as a substrate (21).

Recently, a further mechanism for 3-nitrotyrosine formation was proposed (22, 23); the formation of nitryl chloride (NO₂Cl) by reaction of myeloperoxidase-derived hypochlorous acid (HOCl) with NO₂⁻ (Reaction 1) (24).

\[ \text{HOCI} + \text{NO}_2^- + \text{H}^+ \rightarrow \text{NO}_2\text{Cl} + \text{H}_2\text{O} \]

\( k = 7.4 \pm 1.3 \times 10^6 \text{M}^{-1}\text{s}^{-1} \)

pH 7.2, 25 °C

\[ \text{Reaction 1} \]

It has been estimated that up to 80% of the H₂O₂ generated by activated neutrophils during the respiratory burst is used to form HOCI (25). Consequently, NO₂Cl formation from activated human neutrophils and nitrination of extracellular phenolics in the presence of added NO₂⁻ have been demonstrated (23). Although NO₂⁻, NO₃⁻, and HOCl are formed in substantial amounts during inflammation and the former two are normally present in saliva and are present in the gut at high concentrations, whether NO₂Cl plays any part in the tyrosine nitration observed in vivo is unclear. Therefore, in this report we investigated whether HOCI in the presence of NO₂⁻ could nitrate...
intracellular protein using HepG2 hepatoma and SW1353 chondrosarcoma cells as models of human liver (26) and cartilage cells (27, 28) exposed to RNS. We used four analytical approaches: immunochromography with monoclonal and polyclonal antibodies from two commercial sources using confocal microscopy, flow cytometry, and Western blotting as well as a commercial ELISA kit.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin (BSA), oxidized glutathione (GSSG), sodium nitrite (NaNO₂), sodium nitrate (NaNO₃), sodium hy- perchlorite, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO). HOCI concentration was quantified immediately from spectrophotometrically at 290 nm (pH 12, e = 350 M⁻¹ cm⁻¹) (29). Hydrogen peroxide-free peroxinitrite was synthesized as described previously (30) and quantified in 1 N NaOH at 302 nm (e = 1670 M⁻¹ cm⁻¹). Rabbit polyclonal anti-nitrotyrosine antibodies were then added and incubated in PBS containing 1% (v/v) fetal bovine serum for 1 h at room temperature. After washing, fluorescently labeled secondary antibodies (rhodamine or AlexaFluor 488) were then added for 1 h. Cells were then analyzed by flow cytometry using a Epics Elite flow cytometer (ESP, Coulter, Miami, FL) within 5 h of initial treatment. Data were analyzed from 20,000 cells using WinMDI 2.7 software (Scripps Institute, La Jolla, CA), and the percentage of nitrotyrosine-stained cells was determined from histogram analysis.

RESULTS

Assessment of Tyrosine Nitration by Commercial ELISA—The addition of HOCI to SW1353 and HepG2 cells for 5 min resulted in negligible loss of cell viability as measured using MTT. For example, the addition of 125 μM HOCI for 5 min resulted in a 8.8 ± 2.4% and 5.6 ± 4.8% reduction in SW1353 and HepG2 cell viability, respectively. The addition of HOCI, NO₂⁻, or ONOO⁻ did not significantly alter the pH of the reaction mixture.

Using a commercially available nitrotyrosine ELISA kit, extensive tyrosine nitration was observed after human HepG2 cells or SW1353 cells (Fig. 1A) seeded overnight in glass bottom Petri dishes (WillCo-dish, Willco Wells, Amsterdam, The Netherlands) were washed three times in warm PBS and further incubated for 10 min with PBS containing increasing concentrations of NaNO₂ (10 μM to 1 mM). After this time, HOCI was added to give final concentrations between 7 and 125 μM. Cells were then incubated at 37 °C for 5 min. In parallel experiments, cell lysates were obtained by freeze-thawing in 0.5 ml of PBS and sonication at 4 °C for 10 min before addition of HOCI/NO₂⁻ or ONOO⁻ for 5 min and the addition of protease inhibitors (1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) as described (31). Mouse monoclonal anti-nitrotyrosine antibodies were obtained from Calbiochem (La Jolla, CA, #847923) or Alexis (#804-204). Peroxidase-conjugated secondary antibodies for Western blotting were purchased from Promega. Fluorescently labeled rhodamine anti-mouse IgG (#12-329) was obtained from Calbiochem, and AlexaFluor 488 anti-rabbit IgG (#A11008) was obtained from Molecular Probes (Eugene, OR). A commercial ELISA kit (GSSG) was purchased from Cambridge Biosciences (Cambridge, England, #HK501).

The same results were obtained with monoclonal and polyclonal anti-nitrotyrosine antibodies. Fig. 3A–D shows representative Western blots obtained using an Upstate Biotechnology rabbit polyclonal antibody (Fig. 2A) and the Calbiochem mouse monoclonal antibody (Fig. 2B) on whole cell extracts and cell lysates from SW1353 cells. It can be seen that, even after lengthy exposure exposure (20 min) in ECL reagent, tyrosine nitration was only detected in the positive control (100 μM ONOO⁻). The increase in tyrosine nitration was then examined with a variety of the HOCI (7–125 μM) and NO₂⁻ (10 μM to 1 mM) concentrations used. Fig. 1A shows the results obtained with 125 μM HOCI and is representative of all the HOCI concentrations used. Similarly, the addition of HOCI and NO₂⁻ to freshly prepared cell lysates did not show any observable tyrosine nitration (Fig. 1B).

Assessment of Tyrosine Nitration by Western Blotting—Western blotting using monoclonal and polyclonal anti-nitrotyrosine antibodies from several commercial sources was also performed. Treating cells or freshly prepared cell lysates with ONOO⁻ (100 μM, positive control) resulted in extensive tyrosine nitration in cell lysates. However, exposure of the cells or cell lysates to HOCI or HOCl and NO₂⁻ did not result in any detectable tyrosine nitration with any of the commercial antibodies used. Fig. 2 (A and B) shows representative Western blots obtained using an Upstate Biotechnology rabbit polyclonal antibody (Fig. 2A) and the Calbiochem mouse monoclonal antibody (Fig. 2B) on whole cell extracts and cell lysates from SW1353 cells. It can be seen that, even after lengthy exposure exposure (20 min) in ECL reagent, tyrosine nitration was only detected in the positive control (100 μM ONOO⁻) using either monoclonal or polyclonal antibodies. The same results were obtained with HepG2 cells and cell lysates (data not shown).

Assessment of Intracellular Tyrosine Nitration by Flow Cytometry—Flow cytometric analysis of whole cells exposed to ONOO⁻, HOCI, and HOCl with NO₂⁻ was also performed with monoclonal and polyclonal anti-nitrotyrosine antibodies. Fig. 3 is representative of results obtained using monoclonal (Fig. 3, A–D) and monoclonal antibodies (Fig. 3, E–H) against nitrotyrosine.
Lack of Tyrosine Nitration by HOCl and Nitrite

The formation of 3-nitrotyrosine has been observed in over 50 human disease conditions (reviewed in Refs. 1 and 2). The formation of this biomarker has been attributed to an overproduction of NO and subsequent formation of highly reactive nitrogen species (RNS) usually attributed as ONOO⁻. However, an overproduction of NO also results in the accumulation of NO₂, which has been reported to reach millimolar concentrations in certain disease conditions (8–10). Nitrite also serves as a substrate for peroxidases (20) such as myeloperoxidase (20, 23) and eosinophil peroxidase (18) as well as heme proteins (21) to generate tyrosine-nitrating species. At sites of chronic inflammation, neutrophils produce the oxidant HOCl, which in

anti-mouse IgG (Fig. 4, D and E). Substantial positive 3-nitrotyrosine immunostaining was observed in HepG2 cells and SW1353 cells exposed to non-lethal concentrations of ONOO⁻ (100 μM) using either polyclonal (Fig. 4B) or monoclonal (Fig. 4E) anti-nitrotyrosine antibodies. In contrast, treating the cells with PBS alone (Fig. 4A and D) or incubating cells with NO₂ followed by subsequent addition of HOCl (Fig. 4C and F) did not result in any detectable nitrotyrosine formation at any of the NO₂ concentrations (10 μM to 1 mM) or HOCl concentrations (7–125 μM) used.

**FIG. 2. Analysis of tyrosine nitration by Western blotting.** SW1353 cells or fresh cell lysates were incubated with NO₂ at the concentrations stated, and HOCl (125 μM) was added for 5 min or treated with 100 μM ONOO⁻ for 5 min. Lanes: (1) PBS, (2) whole cells exposed to 100 μM ONOO⁻, (3) cell lysates exposed to 100 μM ONOO⁻, (4) 100 μM ONOO⁻, (5) HOCl + 125 μM NO₂, (6) HOCl + 250 μM NO₂, and (7) HOCl + 500 μM NO₂. Lanes 8–10: SW1353 cell lysates exposed to (8) HOCl + 125 μM NO₂, (9) HOCl + 250 μM NO₂, and (10) HOCl + 500 μM NO₂. Lane 11: BSA treated with 1 mM ONOO⁻. Residual HOCl was quenched by the addition of GSSG, and the formation of 3-nitrotyrosine was analyzed by Western blotting as described under “Experimental Procedures.” Data are representative of four separate experiments.

**FIG. 1. Analysis of tyrosine nitration by commercial ELISA.** Cells (A) or fresh cell lysates (B) were incubated with NO₂ at the concentrations stated and HOCl (125 μM) added for 5 min or treated with 100 μM ONOO⁻ for 5 min. Residual HOCl was quenched by the addition of GSSG, and the formation of 3-nitrotyrosine was analyzed by ELISA as described under “Experimental Procedures.” Data are expressed as mean ± S.D. of six or more separate experiments.

**Assessment of Intracellular Tyrosine Nitration by Confocal Microscopy—**Laser scanning confocal microscopy was also used to assess intracellular tyrosine nitration. Fig. 4 is representative of data obtained when cells were exposed to either ONOO⁻ or HOCl in the presence of NO₂. HepG2 cells were immunostained with polyclonal anti-nitrotyrosine antibodies coupled to AlexaFluor 488-conjugated secondary anti-rabbit IgG (Fig. 4, A–C), and SW1353 cells were immunostained with monoclonal anti-nitrotyrosine antibodies coupled to rhodamine-conjugated anti-mouse IgG (Fig. 4, D and E). Substantial positive 3-nitrotyrosine immunostaining was observed in HepG2cells and SW1353 cells exposed to non-lethal concentrations of ONOO⁻ (100 μM) using either polyclonal (Fig. 4B) or monoclonal (Fig. 4E) anti-nitrotyrosine antibodies. In contrast, treating the cells with PBS alone (Fig. 4A and D) or incubating cells with NO₂ followed by subsequent addition of HOCl (Fig. 4C and F) did not result in any detectable nitrotyrosine formation at any of the NO₂ concentrations (10 μM to 1 mM) or HOCl concentrations (7–125 μM) used.

**DISCUSSION**

The formation of 3-nitrotyrosine has been observed in over 50 human disease conditions (reviewed in Refs. 1 and 2). The formation of this biomarker has been attributed to an overproduction of NO and subsequent formation of highly reactive nitrogen species (RNS) usually attributed as ONOO⁻. However, an overproduction of NO also results in the accumulation of NO₂, which has been reported to reach millimolar concentrations in certain disease conditions (8–10). Nitrite also serves as a substrate for peroxidases (20) such as myeloperoxidase (20, 23) and eosinophil peroxidase (18) as well as heme proteins (21) to generate tyrosine-nitrating species. At sites of chronic inflammation, neutrophils produce the oxidant HOCl, which in
the presence of NO$_2$, forms an additional tyrosine-nitrating species, nitryl chloride (NO$_2$Cl) (22, 23). Although there is a wealth of information on RNS-mediated processes, limited information is available on the consequences of HOCl and NO$_2$ accumulation and resulting NO$_2$Cl formation generated from this reaction (28, 32). The relatively fast second order rate constant of reaction of NO$_2$ with HOCl (pH 7.2, 25°C, 7.4±1.3 × 10$^3$ M$^{-1}$ s$^{-1}$) (24) and the high concentrations of HOCl and accumulation of NO$_2$ at sites of chronic inflammation or in the gut after a meal (14, 15), suggest this reaction is plausible in vivo. Recently, Panasenko et al. (24) demonstrated protein modification, low density lipoprotein oxidation, and β-carotene and α-tocopherol depletion by NO$_2$ and HOCl mixtures. NO$_2$ also enhanced formation of some DNA base damage products in HOCl-treated isolated calf thymus DNA (35) as well as DNA isolated from human bronchial epithelial cells exposed to HOCl (32). The majority of reports thus far have focused on the potentiation of oxidative and chlorinative reactions of HOCl by NO$_2$, and the data on nitration of phenolics have been conducted on cell media or buffer (23, 24) rather than the effects on cells themselves. The extent to which NO$_2$Cl penetrates the cell membrane and reacts with intracellular tyrosine residues to contribute to the tyrosine nitration observed in the diverse and large number of human diseases is unknown. The relatively

Fig. 3. Analysis of tyrosine nitration by immunocytochemistry and flow cytometry. HepG2 cells were incubated with NO$_2$ at the concentrations stated, and HOCl (125 µM) was added for 5 min or treated with 100 µM ONOO$^-$ for 5 min. Residual HOCl was quenched by the addition of GSSG, and the formation of 3-nitrotyrosine was analyzed by flow cytometry using polyclonal (A–D) or monoclonal (E–H) anti-nitrotyrosine antibodies. In further control experiments, antibody binding was blocked with 10 mM nitrotyrosine (B and F). Experiments were conducted as described under “Experimental Procedures.” Data are representative of four separate experiments.

Fig. 4. Analysis of tyrosine nitration by immunocytochemistry and confocal microscopy. HepG2 cells (A–C) or SW1353 cells (D and E) were incubated with PBS (A and D), 100 µM ONOO$^-$ (B and E), or NO$_2$ and 125 µM HOCl (C and F) for 5 min. Residual HOCl was quenched by the addition of GSSG, and the formation of 3-nitrotyrosine was analyzed by confocal microscopy using polyclonal (A–C) or monoclonal (D and E) anti-nitrotyrosine antibodies performed as described under “Experimental Procedures.” Data are representative of four separate experiments.
Lack of Tyrosine Nitrlation by HOCl and Nitrite

fast rate of reaction and high concentrations of NO\textsubscript{2} in vivo also confers HOCl-scavenging abilities on NO\textsubscript{2}, such as inhibition of HOCl-mediated anti-microbial activity (36–38) and cell toxicity (28). Therefore, using two human cell lines as models of human cells exposed to RNS in vivo, the extent of tyrosine nitrillation induced by HOCl/NO\textsubscript{2} was investigated using several established analytical techniques.

Using several monoclonal and polyclonal commercial antibodies, substantial nitrotyrosine formation was observed only when cells or cells lysates were exposed to ONOO\textsuperscript{−} added at sublethal concentrations (100 μM). No formation of 3-nitrotyrosine was observed by Western blot with enhanced chemiluminencescence detection (Fig. 2) in either cells or cell lysates exposed to HOCl/NO\textsubscript{2}. Similarly, tyrosine nitrillation was only detected with ONOO\textsuperscript{−} -treated cells using these antibodies with flow cytometric or confocal microscopic analysis. In support, Sampson et al. (20) also failed to detect tyrosine nitrillation by Western blot in homogenates of horse hearts exposed to HOCl/NO\textsubscript{2}, but substantial nitrotyrosine formation was observed when the homogenates were exposed to ONOO\textsuperscript{−}.

It is unlikely that residual HOCl degraded any protein bound nitrotyrosine formed, as we recently reported in vitro (42) for the following reasons: 1) NO\textsubscript{2} reacts with HOCl rapidly (pH 7.2, 25 °C, 7.4 ± 1.3 × 10\textsuperscript{3} M\textsuperscript{−1} s\textsuperscript{−1}) (24); 2) analysis of buffers after experimentation showed NO\textsubscript{2} to be oxidized to NO\textsubscript{3}\textsuperscript{−} (this reaction is stoichiometric (1 mol of NO\textsubscript{2} consumed by 1 mol of HOCl to give 1 mol of NO\textsubscript{3}\textsuperscript{−}) (24, 28); and 3) the time course of exposure used was short (5 min). HOCl-mediated loss of 3-nitrotyrosine formed, as we recently reported (52) (this was investigated using several laboratory.

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Lack of Tyrosine Nitration by Hypochlorous Acid in the Presence of Physiological Concentrations of Nitrite: IMPLICATIONS FOR THE ROLE OF NITRYL CHLORIDE IN TYROSINE NITRATION IN VIVO
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