The Metabolism and Dechlorination of Chlorotyrosine in Vivo*

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During inflammation, neutrophil- and monocyte-derived myeloperoxidase catalyzes the formation of hypochlorous acid, which can chlorinate tyrosine residues in proteins to form chlorotyrosine. However, little is known of the metabolism and disposition of chlorotyrosine in vivo. Following infusion of deuterium-labeled [D₄]chlorotyrosine into Sprague-Dawley rats, the major urinary metabolites were identified by mass spectrometry. 3-Chloro-4-hydroxyphenylacetic acid was identified as the major chlorinated metabolite of chlorotyrosine and accounted for 3.6 ± 0.3% of infused [D₄]chlorotyrosine. The striking observation was that ~40% (39 ± 1%) of infused [D₄]chlorotyrosine was dechlorinated and excreted in the urine as deuterated 4-hydroxyphenylacetic acid, a major metabolite of tyrosine. 1.1 ± 0.1% of infused [D₄]chlorotyrosine was excreted as [D₄]tyrosine. To determine whether protein-bound chlorotyrosine could undergo dechlorination, chlorinated albumin was incubated with liver homogenate from mutant rats, which did not synthesize albumin. There was ~20% decrease in the chlorotyrosine content over 1 h. This study is the first to describe the dechlorination of chlorotyrosine as the major metabolic pathway to eliminate this modified amino acid in vivo.

Myeloperoxidase (MPO) is a phagocytic enzyme secreted during inflammation, which undergoes transcytosis into vascular endothelium where it can lead to vascular dysfunction (1, 2). Myeloperoxidase catalyzes the formation of hypochlorous acid, a potent chlorinating reagent, from hydrogen peroxide and chloride anion. In turn, hypochlorous acid can react with nitrite to form nitryl chloride (3, 4). Hypochlorous acid and nitryl chloride can react with tyrosyl residues in proteins to form chlorotyrosine and nitrotyrosine, respectively (3, 4). Thus, the measurement of chlorotyrosine has been extensively used to assess the formation of MPO-derived hypochlorous acid in vivo (5, 6). Likewise measurement of nitrotyrosine has been used as a footprint for the formation of peroxynitrite or nitryl chloride in vivo (1, 7–9). However, one of the major disadvantages of measuring chlorotyrosine or nitrotyrosine is that modified proteins may undergo rapid catabolism (10), and the resulting free chlorotyrosine or nitrotyrosine are taken up by cells, metabolized, and excreted. Thus, it is well established that nitrotyrosine is metabolized to 3-nitro-4-hydroxy-phenylacetic acid (nitro-HPA), which is the major urinary metabolite (11, 12), and urinary levels increase during endotoxemia (12).

Tyrosine is iodinated by iodine in a reaction catalyzed by thyroid peroxidase to form iodotyrosine, a key intermediate in thyroid hormone synthesis. It is well recognized that pathways exist for the dehalogenation of iodotyrosine to form tyrosine in vivo (13), and historically this pathway has been assumed to exist for the conservation of iodine. Iodotyrosine dehalogenase, which catalyzes the deiodination of iodotyrosine to tyrosine and iodide, was characterized over 50 years ago (13). This enzyme has recently been cloned (DEHAL1) and is expressed in the thyroid gland and other organs including the liver and kidney (14). It is well known that DEHAL1 has a nitroreductase domain and can catalyze the deiodination of both mono- and diiodotyrosine (15, 16). We postulated that chlorotyrosine released during proteolysis may undergo dechlorination to form tyrosine, either in a reaction catalyzed by DEHAL1 or through an alternative pathway. The aim of this study was to identify the major urinary metabolites of chlorotyrosine and to determine whether chlorotyrosine undergoes dechlorination in vivo.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals were purchased from Sigma-Aldrich unless stated otherwise. [13C₉]Tyrosine, [D₄]tyrosine, [D₄]lactacetic acid, D₂O, and deuterium chloride (2HCl) were purchased from Cambridge Isotope Laboratories (Andover, MA).

Animals—Male Sprague-Dawley rats (270–300 g) were obtained from the Comparative Biology Unit at University College London. Snap frozen liver tissues from male Nagase analbuminemic rats (NAR) were used to study the dechlorination of chlorinated albumin. Systemic inflammation was induced by intraperitoneal injection of lipopolysaccharide (Salmonella typhimurium, 1 mg/kg) and animals sacrificed at 24 h under pentobarbital anesthesia. Animal procedures were in accordance with the Home Office, UK guidelines.

Synthesis of Deuterium-labeled Compounds—[D₄]Chlorotyrosine, [D₄]chloro-HPA, and [D₄]HPA were synthesized by deuterium exchange as described (12, 17) using 50 mg of either chlorotyrosine, chloro-HPA, or HPA as starting material. The resulting products were dissolved in 0.1% (v/v) TFA/water (adjusted to pH 5.0 with ammonia solution) and extracted on an LC18 reverse-phase column, pre-washed with 2 ml of methanol and 5
ml of 0.1% (v/v) TFA/water (pH 5.0). The products were washed with water, and the deuterated products eluted with 4 ml of 30% (v/v) methanol in water. The products were purified further by thin-layer chromatography (12), and their concentrations determined against known amounts of unlabeled standards by GC/MS (see below).

**Synthesis of 13C-labeled Internal Standard for Measurement of Chlorotyrosine, HPA, and Chloro-HPA—**[13C₈]3-chlorotyrosine was synthesized by reaction of [13C₈]tyrosine with sodium hypochlorite (4% solution) at room temperature for 1 h followed by solid phase extraction. The products were purified using high pressure liquid chromatography as described (12). [13C₈]HPA was synthesized following the deamination and decarboxylation of [13C₈]tyrosine using Taiwan cobra venom as described previously (12). [13C₈]Chloro-HPA was synthesized in the same way as chlorotyrosine, and the concentrations of 13C-labeled standards determined against known amounts of unlabeled standards by GC/MS.

**Synthesis of Chlorinated Albumin—**Human serum albumin was dissolved in 0.1 M phosphate buffered saline (PBS) to give a final concentration of 10 mg/ml, and sodium hypochlorite solution was added to give a final concentration of 0.4% and left for 1 h at room temperature. Chlorinated albumin was dialyzed against PBS for 48 h.

**Measurement of Chlorotyrosine and Tyrosine by GC/MS—**13C-labeled internal standard (5 ng) was added to 20 μl of rat urine and diluted to 1 ml of total volume with 0.1% (v/v) TFA/water (pH 5.0). The samples were extracted using LC18 reverse-phase column as described above. Chlorotyrosine and tyrosine were derivatized with ethyl heptafluorobutyrate and silylated with tert-butyldimethylsilyl as described (18, 19). Derivatized samples were dried under nitrogen and redissolved in 20 μl of n-undecane. Samples were applied to a GC equipped with a 15-m DB-1701 (J&W Scientific, Folsom, CA) capillary column (0.25-mm internal diameter, 0.25-mm film thickness) interfaced with a mass spectrometer (Trio 1000; Fisons Instruments, Beverly, MA). The ion source and interface temperatures were set at 200 and 320 °C, respectively. Samples were analyzed in negative-ion chemical ionization mode with ammonia as the reagent gas, using 1 μl of each sample for injection. The initial column temperature was maintained at 150 °C for 1 min increasing to 300 °C at 20 °C/min. Ions were monitored at 489, 493, and 498 mass units for authentic, [D₄] and [13C₈]chloro-HPA, respectively. For measurement of tyrosine, samples were monitored at 407, 411, and 339 m/z for authentic, [D₄] and [13C₈]tyrosine, respectively. Concentrations were calculated by ratio to known 13C₈-labeled internal standards.

**Measurement of Chloro-HPA and HPA by GC/MS—**13C-labeled internal standard (5 ng) was added to 20 μl of rat urine and diluted to 1 ml with 0.1% (v/v) TFA/water (pH 5.0). The samples were extracted using LC18 reverse-phase column as described above. The samples were derivatized to the pentafluorobenzyl ester by the addition of 20 μl of di-isopropyl ethylamine in acetonitrile and 40 ml of 10% (v/v) pentafluorobenzyl bromide in acetonitrile for 1 h at room temperature (21–23 °C), dried under nitrogen, and redissolved in 20 ml of n-undecane for full scan GC/MS analysis.

**Measurement of Chlorotyrosine in Vivo Studies—**A similar method used by Ohshima et al. (11) was used for identification of urinary metabolites of chlorotyrosine. To obtain enough material for GC/MS analysis, male Sprague-Dawley rats (n = 3 in each group) were given drinking water containing either chlorotyrosine or tyrosine at a concentration of 1 mg/ml for 24 h, and urine was collected in metabolic cages for this period. Urine samples were acidified to pH 1.0 with HCl and extracted with an equivalent volume of ethyl acetate. The concentrated extracts were derivatized to the pentafluorobenzyl ester by the addition of 20 μl of di-isopropyl ethylamine in acetonitrile and 40 ml of 10% (v/v) pentafluorobenzyl bromide in acetonitrile for 1 h at room temperature (21–23 °C), dried under nitrogen, and redissolved in 20 ml of n-undecane for full scan GC/MS analysis.

**Dechlorination of Chlorotyrosine in Vivo Studies—**Male Sprague-Dawley rats were divided into four groups (n = 4 in each group), which were given a single intraperitoneal injection of [D₄]chlorotyrosine, [D₄]tyrosine, or [D₅]chloro-HPA or [D₄]HPA (0.50 μmol). The animals then were transferred to standard metabolic cages, and urine samples were collected for 24 h. The levels of deuterated chlorotyrosine, tyrosine, chloro-HPA, and HPA were measured in the collected urine samples as described above. To determine whether the observed dechlorination of chlorotyrosine was dependent on a cytochrome P450, one group of rats (n = 3) were pre-treated with 1-amino-nobenzotriazole (50 mg/kg) to block cytochrome P450 activity at 24 and 4 h before the intraperitoneal injection of 0.50 μmol [D₄]chlorotyrosine (20). Urine was collected over 24 h and analyzed as above.
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**Dechlorination of Chlorinated Albumin by Liver Homogenate in Vitro**—To determine whether protein-bound chlorotyrosine was a substrate for dechlorination on an intact protein, chlorinated albumin was incubated with liver homogenates obtained from mutant rats, which lacked albumin (NAR rats) (22). This allows us to quantitate chlorinated and dechlorinated albumin without contamination from endogenously synthesized albumin. Added albumin was separated from other proteins in liver homogenates by electrophoresis. In brief, snap-frozen liver from analbuminemic rats was homogenized in PBS (1:10, w/v) containing NADPH (100 μM), FAD, and FMN (200 μM final concentration). 50 μl of liver homogenate was incubated with 50 μl of chlorinated albumin (10 mg/ml) in a circulating water bath (37 °C). After 1-h incubation, the reaction was stopped by adding 100 μl of lysis buffer containing SDS (20%), 2-mercaptoethanol (25 μl/ml), and bromphenol blue (10 mM). Mixtures were incubated in triplicate for 60 min at 37 °C, and the reaction was stopped by addition of 0.9 ml of 10% acetic acid on ice. The level of chlorotyrosine was then measured using GC/MS as described above.

**Structural Confirmation of Chlorotyrosine by GC/MS**—For GC/MS analysis of chlorotyrosine and tyrosine the amine group was derivatized with ethyl heptafluorobutyrate, and the hydroxyl and carboxylic groups were silylated with tert-butyldimethylsilyl and the products analyzed by negative-ion chemical ionization mass spectrometry. Full scan mass spectra obtained for these molecules are shown in Fig. 1 (A and B). The major fragment ion for chlorotyrosine has a m/z 489, which corresponds to loss of tert-butyldimethylsilyl and chloride. The major fragment ion of tyrosine is consistent with previously published data and corresponds to loss of O and tert-butyldimethylsilyl, which results in a dominant ion at m/z 407 (18).

**Dechlorination of Chlorotyrosine**

**Interaction of Chlorotyrosine and DEHAL1 in Vitro**—DEHAL1 was obtained from HEK293 cells transfected with DEHAL1 cDNA (14). To determine whether chlorotyrosine inhibited or activated DEHAL1, deiodinase activity was measured by radioimmunoassay based on radioiodide formation from [125I]iodotyrosine. Incubation mixtures contained [125I]iodotyrosine (~100,000 cpm mixed with 0.1 μM unlabeled iodothyrosine), DEHAL1 enzyme, and varying amounts of chlorotyrosine (0.01–100 μM final concentration) in 100 μl of PBS containing NADPH (100 μM), EDTA (2 mM), and diithiothreitol (10 mM). Mixtures were incubated in duplicate for 60 min at 37 °C, and the reactions were stopped by addition of 0.9 ml of 10% acetic acid on ice. The radioiodide in the mixture was fractionated by SDS-PAGE. The gel was then stained with Coomassie Blue, and the albumin band (64 KDa) was cut out and subjected to hydrolysis using 1 ml of NaOH (4 M) for 15 h. To quantify the level of chlorotyrosine and tyrosine, isotopic internal standards, [13C9]chlorotyrosine (100 ng), and [13C9]tyrosine (1 μg) were added to the samples prior to alkaline hydrolysis. Following two steps of solid phase extraction, chlorotyrosine and tyrosine were quantitated as described (18, 19). Data are expressed as chlorotyrosine/tyrosine ratio. Each experiment was repeated four times (n = 4).

**Structural Confirmation of Chloro-HPA and HPA by GC/MS**—The structures of the pentafluorobenzyl ester derivatives of chloro-HPA and HPA are shown in Fig. 1 (D and E). Analysis of the pentafluorobenzyl ester derivatives of chloro-HPA and HPA by full scan mode showed that chloro-HPA had a dominant ion at m/z 493, which was 4 mass units heavier than the authentic chlorotyrosine and corresponded to [D₄]chlorotyrosine.

**Metabolism of Chlorotyrosine**—Analysis of urine extracts from rats drinking water containing chlorotyrosine showed one main additional peak on the GC compared with urine obtained from rats given tyrosine alone (Fig. 2). GC/MS analysis identified this peak as chloro-HPA (Figs. 1E and 2C). Following injection of 0.5 μmol [D₈]chlorotyrosine, 3.6 ± 0.3% of the dose was excreted as [D₈]chloro-HPA, and 0.9 ± 0.5% of infused chlorotyrosine was excreted in the urine unchanged (Fig. 3).

Although chloro-HPA was observed as the major chlorinated metabolite present in urine of rats ingesting chlorotyrosine, compared with tyrosine-ingesting controls, when we followed the pathways of deuterated chlorotyrosine metabolism, we found that the major urinary metabolite observed was deuterated HPA. Because HPA is also a major metabolite of tyrosine metabolism (17), this peak was not evident when urinary samples in the drinking water of rats containing chlorotyrosine or tyrosine were compared. Thus, urinary HPA may be formed by

For statistical analysis, a non-parametric test was used for statistical analysis.
metabolism of either chlorotyrosine or tyrosine. Almost 40% (39 ± 1%) of infused [D₄] chlorotyrosine was excreted in the urine as [D₄]HPA, and 1.1 ± 0.1% excreted as [D₄]tyrosine (Fig. 3). When 0.5 μmol [D₄]tyrosine was injected into rats, 41.5 ± 1% of the dose was excreted as [D₄]HPA, and 0.9 ± 0.7% was excreted in the urine unchanged (i.e. similar to that obtained by injection of deuterated chlorotyrosine). To determine whether HPA undergoes further metabolism, we also injected 0.5 μmol [D₆]HPA, and 94.3 ± 2.1% was recovered unchanged in urine within 24 h, indicative of little, if any, metabolism (Fig. 3B). However, 8.3 ± 0.6% of injected [D₅]chloro-HPA was excreted as its dechlorinated metabolite ([D₅]HPA), and 88.2 ± 1.4% was detected in 24-h urine samples unchanged (Fig. 3B). These data show that chloro-HPA can also serve as a substrate for dechlorination but is a poor substrate compared with chlorotyrosine.

Rats pre-treated with 1-aminobenzotriazole, a potent inhibitor of cytochrome P450, showed the same quantitative pattern of [D₄]HPA and [D₅]chloro-HPA excretion compared with saline pre-treated rats following [D₄]chlorotyrosine injection (data not shown), suggesting that metabolism by a cytochrome P450 enzyme is unlikely to be responsible for the dechlorination pathway.

Dechlorination of Chlorotyrosine by HepG2 Cells—Because the liver is the most likely site of metabolism we incubated chlorotyrosine with cultured HepG2 cells, which contained most of the normal enzymes present in hepatocytes. Detectable amounts of [D₄]tyrosine were observed in the cell fraction obtained from HepG2 cells following incubation with [D₄]chlorotyrosine (Fig. 4). As shown in Table 1, ∼4% (4.3 ± 0.3) of incubated [D₄]chlorotyrosine was incorporated into proteins as protein-bound [D₄]tyrosine. Free [D₄]tyrosine was also detectable in cell extracts (Fig. 4) but not the culture media following incubation with [D₄]chlorotyrosine. Incubation of HepG2 cells with equimolar concentration (10 μM) of [D₄]tyrosine was associated with incorporation of ∼24% of [D₄]tyrosine into cellular proteins. A small fraction (∼0.5%) of incubated tyrosine was detected as free [D₄]tyrosine in cell extracts (Table 1).

Dechlorination of Chlorinated Albumin—GC/MS analysis of the hydrolysate of albumin following its reaction with hypochlorous acid showed that 6.2 ± 0.2% of tyrosine residues of albumin were chlorinated. To determine whether protein-bound chlorotyrosine could be dechlorinated, chlorinated albumin was incubated with liver tissue homogenates obtained from mutant rats, which lacked albumin (NAR) (22). The liver from these rats was used to avoid interference from albumin formed by "normal liver". Murad and colleagues (23, 24) have observed that denitrination of nitrotyrosine is up-regulated following induction of a systemic inflammatory response by injection of endotoxin; therefore we used both liver from analbuminemic rats as well as liver obtained 24 h after injection of endotoxin (lipopolysaccharide, 1 mg/kg). Thus, chlorinated albumin was incubated with either buffer alone, liver homogenate from analbuminemic rats, or liver homogenate from NAR.
rats injected with endotoxin. Fig. 5 shows the levels of protein-bound tyrosine, chlorotyrosine, and the chlorotyrosine to tyrosine ratio after GC/MS analysis of the hydrolyzed albumin band. Following incubation of chlorinated albumin with liver homogenate for 1 h there was a significant decrease in the chlorotyrosine/tyrosine ratio in both control and endotoxemic groups. The reduction in both chlorotyrosine content and the chlorotyrosine/tyrosine ratio was more prominent in the liver homogenate obtained from endotoxemic rats (Fig. 5). There was no statistical difference in tyrosine levels of the hydrolyzed albumin among the groups.

Inhibition of DEHAL1-induced Deiodination by Chlorotyrosine—Chlorotyrosine inhibits iodotyrosine deiodination in vitro in a dose-dependent manner (Fig. 6A). None of the other tested phenolic compounds (chloro-HPA and tyrosine) showed the ability to inhibit deiodination induced by DEHAL1. The Michaelis-Menten plot of the velocity of deiodination reaction in presence of chlorotyrosine is shown in Fig. 6B. At any given chlorotyrosine concentration (1 and 10 μM), enzyme inhibition could be overcome by increasing the substrate (iodotyrosine) concentration, consistent with reversible inhibition of DEHAL1 dehalogenase. We then tested the hypothesis that chlorotyrosine might serve as a substrate for DEHAL1 dehalogenase; however, DEHAL1 was unable to catalyze the dechlorination of chlorotyrosine. Indeed almost 100% of added chlorotyrosine could be recovered following incubation with DEHAL1 in the presence of its cofactors (Fig. 6C).

DISCUSSION

During inflammation, neutrophil and monocyte-derived myeloperoxidase catalyze the formation of hypochlorous acid, which can chlorinate tyrosine residues in proteins to form chlorotyrosine. Chlorination of tyrosine decreases the pKa of...
the phenolic group from 10.1 to 8.3 (25). This can lead to altered protein conformation and function in the same way that nitration of tyrosine can alter enzymatic activity or protein function (26–28). Our understanding on the fate of chlorinated proteins is limited to the assumption that chlorinated proteins undergo proteolysis to release free chlorotyrosine, which undergoes metabolism. Thus, it was attractive for us to identify the urinary metabolite(s) of chlorotyrosine to develop assays for the non-invasive assessment of chlorotyrosine formation in vivo.

We and others have previously shown that the major urinary metabolite of nitrotyrosine is 3-nitro-4-hydroxyphenylacetic acid (11, 12), and it seemed likely that chloro-HPA would be the corresponding major urinary metabolite of chlorotyrosine. Indeed when we first screened the urine for differences in the metabolite profile of rats ingesting chlorotyrosine compared with tyrosine ingesting controls, one peak was markedly prominent and corresponded to chloro-HPA. Subsequent studies confirmed that this was the major chlorinated metabolite of chlorotyrosine, accounting for nearly 4% of infused chlorotyrosine, but this simple screening approach failed to identify the dominant non-chlorinated metabolites which hid behind those formed endogenously from tyrosine metabolism.

Further studies using infused deuterated chlorotyrosine enabled us to follow the deuterium, knowing that it could only have been derived from infused chlorotyrosine. We observed that almost 40% of infused [D$_4$]chlorotyrosine was excreted in the urine as the dechlorinated derivative, [D$_4$]HPA, which was comparable with the metabolism of injected [D$_4$]tyrosine in rats (Fig. 3). These data demonstrate that either chlorotyrosine or chloro-HPA undergoes dechlorination in vivo. However, whereas chloro-HPA can be dechlorinated in vivo, less than 10% of infused [D$_5$]chloro-HPA is excreted in the urine as the dechlorinated derivative. This data suggests that the major substrate for dechlorination is chlorotyrosine. We further showed that human hepatoblastoma cells (HepG2) in culture could catalyze dechlorination of chlorotyrosine in vitro (Table 1). To date, various microbial and protistal (e.g. Dictyostelium) dechlorinases have been isolated and characterized (29–34). However, this is the first report to demonstrate

![FIGURE 4. Chromatogram of cell lysate after incubation of HepG2 cells with [D$_4$]chlorotyrosine (10 μM).](image)

![FIGURE 5. The levels of protein-bound tyrosine, chlorotyrosine as well as the chlorotyrosine/tyrosine ratio in chlorinated albumin before (buffer) and after incubation with liver homogenates from control or endotoxemic lipopolysaccharide (LPS) analbuminemic rats.](image)

**TABLE 1**

| Incubated amino acid | Medium | Free amino acid in cell fraction | Protein-bound amino acid in cell fraction |
|----------------------|--------|---------------------------------|----------------------------------------|
|                      | [D$_4$]Cl-tyrosine | [D$_4$]tyrosine | [D$_5$]Cl-tyrosine | [D$_5$]tyrosine | [D$_4$]Cl-tyrosine | [D$_4$]tyrosine |
|                      | μmol   | nmol              | μmol              | nmol   | μmol              | nmol   |
| [D$_4$]Chlorotyrosine| 8.2 ± 0.4 (82.5 ± 4.5%) | 0 ± 0 (0 ± 0%)   | 0.9 ± 0.4 (0.3 ± 0.1%) | 1.1 ± 0.1 (0.4 ± 0.1%) | 0 ± 0 (0 ± 0%)  | 13 ± 4 (4.3 ± 0.4%) |
| [D$_4$]Tyrosine      | 0 ± 0 (0 ± 0%)     | 7.1 ± 0.8 (71.4 ± 8.4%) | 0 ± 0 (0 ± 0%)     | 2.2 ± 0.7 (0.5 ± 0.2%) | 0 ± 0 (0 ± 0%)  | 111 ± 12 (23.6 ± 2.5%) |
that chlorotyrosine undergoes a dehalogenation reaction in mammals (Fig. 7).

Most of the tyrosine present in a cell is protein-bound, and chlorination of tyrosine residues site specifically affects the functions of the proteins (27). We therefore investigated whether protein-bound chlorotyrosine could undergo dechlorination in vitro. Chlorinated albumin was incubated with liver homogenates obtained from analbuminemic rats. The main advantage of using these rats is that incubated albumin could then be separated from other proteins by SDS-PAGE because analbuminemic rats do not express albumin. Incubation of chlorinated albumin with liver homogenates led to a decrease of chlorotyrosine levels as well as the chlorotyrosine/tyrosine ratio in both control and endotoxemic rats. The decrease in chlorotyrosine levels or ratio was highest when chlorinated albumin was incubated with liver tissue obtained from endotoxemic rats suggesting that this process was inducible. These data are in line with the existence of a mechanism responsible for either dechlorination or proteolysis of chlorinated albumin in our model system. We excluded simple proteolysis by co-incubation with a mixture of protease inhibitors to the lysis buffer before tissue homogenization. Furthermore there was no statistical difference in tyrosine content of the hydrolyzed albumin among the groups, whereas chlorotyrosine content showed a significant reduction following incubation with tissue homogenate (Fig. 5). These data suggest that intact proteins can undergo dechlorination in vivo, whereas current thinking views protein chlorination as an irreversible event. A similar mechanism has been suggested for nitration reaction by Murad and colleagues (23, 24) who reported an activity in rat tissues that denitrates nitrotyrosine-containing proteins.

Although the responsible mechanisms for dehalogenation of chlorotyrosine have not yet been identified, we tested two important enzymatic pathways which might be involved. We initially tested the effect of a nonspecific cytochrome P450 inhibitor, 1-aminobenzotriazole, on metabolism of intraperitoneally injected [D4]chlorotyrosine in rats. Rats pre-treated with 1-aminobenzotriazole showed the same pattern of [D4]HPA and [D4]chloro-HPA excretion compared with saline-treated rats. Furthermore, HepG2 monolayers, which do not adequately express cytochrome P450 isozymes (35), also catalyze the dechlorination of [D4]chlorotyrosine in vitro.

In mammals, iodotyrosines account for two-thirds of the iodine in thyroglobulin and serve as precursors in the formation of the thyroid hormones. Secretion of thyroid hormones requires the proteolysis of thyroglobulin, in the course of which, the free amino acids iodotyrosine and di-iodotyrosine are released from peptide linkages. The free iodotyrosine and

![Figure 6](image-url)

**FIGURE 6.** A, the effect of chlorotyrosine, chloro-HPA, iodotyrosine, and tyrosine on DEHAL1-induced [125I]iodotyrosine deiodination. Chlorotyrosine clearly inhibits the deiodination of iodotyrosine in vitro. B, Michaelis-Menten plot of the velocity of deiodination reaction in the presence of chlorotyrosine (1 and 10 μM). C, the level of chlorotyrosine after 1-h incubation of chlorotyrosine (1, 10, and 100 μM) with DEHAL1 in the presence of NADPH.

![Figure 7](image-url)

**FIGURE 7.** The metabolism of chlorotyrosine in Vivo. Both chlorotyrosine and tyrosine can be deaminated and decarboxylated to form chloro-HPA and HPA, respectively. However, the major metabolic pathway for chlorotyrosine is dechlorination. Both chlorotyrosine and chloro-HPA can undergo dechlorination to form a dechlorinated product.
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di-iodotyrosine cannot be reutilized as such for thyroglobulin synthesis but are enzymatically deiodinated. This enzymatic deiodination is a reductive process that leads to the formation of iodide and tyrosine, both of which can then be reused for thyroglobulin synthesis (16). One of the first mammalian dehalogenase (DEHAL1) activity was characterized 50 years ago, which catalyzes the deiodination of iodotyrosine to tyrosine and iodide (13). Thus, we postulated that chlorotyrosine might undergo dechlorination to form tyrosine in a reaction catalyzed by DEHAL1. Our data showed that although chlorotyrosine reversibly inhibits DEHAL1 deiodinase activity, it is not a substrate for this enzyme.

This is the first description of a dechlorination pathway for chlorotyrosine in mammals and demonstrates that this is the major metabolic route to eliminate this modified amino acid formed during inflammation. Although the mechanism of this reaction is not identified in the present study, it is likely that a specific enzyme is responsible for this newly identified metabolic pathway. Identification of enzyme(s) responsible for this reaction, its mechanism of action, and regulation will increase our understanding of the control of inflammation and repair.

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REFERENCES

1. Baldus, S., Eiserich, J. P., Mani, A., Castro, L., Figueroa, M., Chumley, P., Ma, W., Tousson, A., White, C. R., Bullard, D. C., Brennan, M. L., Luis, A. J., Moore, K. P., and Freeman, B. A. (2001) J. Clin. Investig. 108, 1759–1770
2. Eiserich, J. P., Baldus, S., Brennan, M. L., Ma, W., Zhang, C., Tousson, A., Castro, L., Luis, A. J., Nauseef, W. M., White, C. R., and Freeman, B. A. (2002) Science 296, 2391–2394
3. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Castro, L., Lusis, A. J., Nauseef, W. M., White, C. R., and Freeman, B. A. (2002) J. Biol. Chem. 277, 17415–17427
4. Brennan, M. L., Wu, W., Fu, X., Shen, Z., Song, W., Fos, T. H., Vadsseth, C., Narine, L., Lenkiewicz, E., Borchers, M. T., Lusis, A. J., Lee, J. J., Lee, N. A., Abu-Soud, H. M., Ischiropoulos, H., and Hazen, S. L. (2002) J. Biol. Chem. 277, 17415–17427
5. Hazen, S. L., and Heinecke, J. W. (1997) J. Clin. Investig. 99, 2075–2081
6. Brennan, M. L., Anderson, M. M., Shih, D. M., Qu, X. D., Wang, X., Mehta, A. C., Lim, L. L., Shi, W., Hazen, S. L., Jacob, J. S., Crowley, J. R., Heinecke, J. W., and Luis, A. J. (2001) J. Clin. Investig. 107, 419–430
7. MacMillan-Crow, L. A., Crow, J. P., Kerby, J. D., Beckman, J. S., and Thompson, J. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11853–11858
8. Aslan, M., Ryan, T. M., Townes, T. M., Coward, L., Kirk, M. C., Barnes, S., Alexander, C. B., Rosenfeld, S. S., and Freeman, B. A. (2003) J. Biol. Chem. 278, 4194–4204
9. Domigan, N. M., Charlton, T. S., Duncan, M. W., Winterbourn, C. C., and Kettle, A. J. (1995) J. Biol. Chem. 270, 16542–16548
10. Souza, J. M., Choi, I., Chen, Q., Weisse, M., Daikkin, E., Yudkoff, M., Obin, M., Ara, J., Horwitz, J., and Ischiropoulos, H. (2000) Arch. Biochem. Biophys. 380, 360–366
11. Ohshima, H., Friesen, M., Brouet, I., and Bartsch, H. (1990) Food Chem. Toxicol. 28, 647–652
12. Mani, A. R., Pannala, A. S., Orié, N. N., Olloosson, R., Harry, D., Rice-Evans, C. A., and Moore, K. P. (2003) Biochem. J. 374, 521–527
13. Roche, J., Michel, R., Michel, O., and Lissitzky, S. (1952) Biochim. Biophys. Acta 9, 161–169
14. Moreno, J. C., van der Hout, C., Klootwijk, W., and Visser, T. J. (2006) Horm. Res. (Basel) 65, 31
15. Moreno, J. C. (2003) Horm. Res. (Basel) 60, 96–102
16. Gnidehou, S., Caillou, B., Talbot, M., Ohayon, R., Kaniewski, J., Noel-Hudson, M. S., Morand, S., Aignangdi, D., Sezan, A., Courtin, F., Virion, A., and Dupuy, C. (2004) FASEB J. 18, 1574–1576
17. Fell, V., Hoskins, J. A., and Pollitt, R. J. (1978) Clin. Chim. Acta 83, 259–269
18. Frost, M. T., Halliwell, B., and Moore, K. P. (2000) Biochem. J. 345, 453–458
19. Gaut, J. P., Byun, J., Tran, H. D., and Heinecke, J. W. (2002) Anal. Biochem. 300, 252–259
20. Garner, C. E., Sumner, S. C., Davis, J. G., Burgess, J. P., Yueh, Y., Remeter, J., Zhan, Q., Valentine, J., Jeffcoat, A. R., Burka, L. T., and Mathews, J. M. (2006) Toxicol. Appl. Pharmacol. 215, 23–36
21. Mavri-Damelin, D., Eaton, S., Damelin, L. H., Rees, M., Hodgson, H. J., and Selden, C. (2007) Int. J. Biochem. Cell Biol. 39, 555–564
22. Nagase, S., Shimamune, K., and Shimiya, S. (1979) Science 205, 590–591
23. Kamisaki, Y., Wada, K., Bian, K., Balabanli, B., Davis, K., Martin, E., Behbod, F., Lee, Y. C., and Murad, F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11584–11589
24. Irie, Y., Saeki, M., Kamisaki, Y., Martin, E., and Murad, F. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5634–5639
25. Faleev, N. G., Aksenova, O. V., Demidkina, T. V., and Phillips, R. S. (2003) Biochim. Biophys. Acta 1647, 260–265
26. Zheng, L., Nukuna, B., Brennan, M. L., Sun, M., Goormastic, M., Settle, M., Schnitt, D., Fu, X., Thomson, L., Fox, P. L., Ischiropoulos, H., Smith, J. D., Kinter, M., and Hazen, S. L. (2004) J. Clin. Investig. 114, 529–541
27. Shao, B., Bergt, C., Fu, X., Green, P., Voss, J. C., Oda, M. N., Oram, J. F., and Heinecke, J. W. (2005) J. Biol. Chem. 280, 5983–5993
28. Shao, B., Oda, M. N., Bergt, C., Fu, X., Green, P. S., Brot, N., Oram, J. F., and Heinecke, J. W. (2006) J. Biol. Chem. 281, 9001–9004
29. Sun, B., Griffin, B. M., Ayala-del-Rio, H. L., Hashsham, S. A., and Tiedje, J. M. (2002) Science 298, 1023–1025
30. Quensen, J. F., 3rd, Mueller, S. A., Jain, M. K., and Tiedje, J. M. (1998) Science 282, 722–724
31. Cole, J. R., Cascarelli, A. L., Mohn, W. W., and Tiedje, J. M. (1994) Appl. Environ. Microbiol. 60, 3536–3542
32. Valverde, C., Orozco, A., Becerra, A., Jezierski, M. C., Villalobos, P., and Solis, J. C. (2004) Int. Rev. Cytol. 234, 143–199
33. Kay, R. R., Large, S., Traynor, D., and Nayler, O. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 887–891
34. Nayler, O., Insall, R., and Kay, R. R. (1992) Eur. J. Biochem. 208, 531–536
35. Wilkening, S., Stahl, F., and Bader, A. (2003) Drug Metab. Dispos. 31, 1035–1042