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Original Contribution

The metabolism and de-bromination of bromotyrosine *in vivo*

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ABSTRACT

During inflammation, leukocyte-derived eosinophil peroxidase catalyses the formation of hypobromous acid, which can brominate tyrosine residues in proteins to form bromotyrosine. Since eosinophils are involved in the pathogenesis of allergic reactions, such as asthma, urinary bromotyrosine level has been used for the assessment of children with asthma. However, little is known about the metabolism and disposition of bromotyrosine *in vivo*. The aim of this study was to identify the major urinary metabolites formed during bromotyrosine metabolism and to develop mass spectrometric methods for their quantitation. Deuterium-labeled bromotyrosine was synthesized by deuterium exchange. [D_3]bromotyrosine (500 nmole) was injected intraperitoneally into Sprague-Dawley rats and urine was collected for 24 h in a metabolic cage. ^{13}C-labeled derivatives of bromotyrosine and its major urinary metabolite were synthesized and used as internal standards for quantitation. Following solid phase extraction, urine samples were derivatized to the pentafluorobenzyl ester, and analyzed using isotope dilution gas chromatography and negative-ion chemical ionization mass spectrometry. A novel brominated metabolite, 3-bromo-4-hydroxyphenylacetic acid (bromo-HPA), was identified as the major brominated metabolite of bromotyrosine. Bromo-HPA only accounted for 0.43 ± 0.04% of infused [D_3]bromotyrosine and 0.12 ± 0.02% of infused [D_3]bromotyrosine was excreted in the urine unchanged. However, ~ 1.3% (6.66 ± 1.33 nmole) of infused [D_3]bromotyrosine was excreted in the urine as the de-brominated metabolite, [D_3]4-hydroxyphenylacetic acid, which is also a urinary metabolite of tyrosine in mammals. We also tested whether or not iodotyrosine dehalogenase can catalyse de-bromination of bromotyrosine and showed that iodotyrosine dehalogenase is able to de-brominate free bromotyrosine *in vitro*. We identified bromo-HPA as the main brominated urinary metabolite of bromotyrosine in rats. However, de-halogenation of bromotyrosine is the major metabolic pathway to eliminate free brominated tyrosine *in vivo*.

1. Introduction

Eosinophils play an important role in the mammalian immune system during allergic reactions, as well as in immune responses against extracellular parasites. Following activation, eosinophils release effectors, such as cationic proteins and eosinophil peroxidase, that lead to host defense and/or tissue damage [1]. Eosinophil peroxidase is a halide peroxidase that preferentially uses bromide to generate hypobromous acid, even at physiological halide concentrations where chloride is almost 1000-fold greater than bromide concentrations [1]. Hypobromous acid is a brominating agent that can brominate nucleophilic aromatic compounds, such as tyrosine [2,3]. Wu et al., reported that 3-bromotyrosine and 3,5-di-bromotyrosine are detectable following eosinophil peroxidase-induced protein oxidation [2]. Likewise, neutrophil-derived myeloperoxidase uses chloride, bromide and nitrite ions to generate halogenating and nitrating agents that lead to the formation of 3-chlorotyrosine, 3-bromotyrosine and 3-nitrotyrosine [4–6]. Therefore, halogenated tyrosine residues are commonly used to quantify leukocyte-mediated damage in diseased tissues [7,8]. However, one of the major disadvantages of measuring bromotyrosine or chlorotyrosine is that oxidized proteins undergo proteolysis, and the resulting free halogenated amino acids are metabolized and excreted in the urine [9].

Since eosinophils play an important role in the pathogenesis of allergic asthma, quantitation of urinary bromotyrosine levels has been used as a non-invasive biomarker for the monitoring of children with asthma [10–12]. Thus, Wedes et al. demonstrated that urinary bromotyrosine levels predict the risk of future asthma exacerbations in children [11]. Moreover, Cowan et al., have

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recently reported that urinary bromotyrosine levels can be used to predict the responsiveness to inhaled corticosteroid therapy in asthmatic patients [12]. Although urinary bromotyrosine measurements have been used for the assessment of eosinophil activation, very little is known about the metabolism and disposition of bromotyrosine *in vivo*. This is surprising, given the fact that major urinary metabolites of chlorotyrosine and nitrotyrosine have been identified [9,13,14] and their measurement has been employed for assessment of systemic inflammation *in vivo* [15,16]. Thus, it is known that chlorotyrosine is metabolized to 3-chloro-4-hydroxyphenylacetic acid (chloro-HPA) and 4-hydroxyphenylacetic acid (HPA), the major urinary metabolites of chlorotyrosine *in vivo* [9]. We previously found that, following infusion of deuterium-labeled chlorotyrosine, the urinary excretion rate of deuterium-labeled chloro-HPA and HPA was 3.3- and 35.6-fold greater than the un-metabolized chlorotyrosine, respectively [9]. Likewise, quantitation of the urinary metabolites of bromotyrosine may provide a better, non-invasive assessment of bromination reactions *in vivo* than the urinary concentration of un-metabolized bromotyrosine. In the present study, we used mass spectrometry in order to identify the major urinary metabolites of bromotyrosine and di-bromotyrosine.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich, unless stated otherwise. [$^{13}C_9$]Tyrosine, [D_4]acetic acid, D_2O, and deuterium chloride (37% in D_2O) were purchased from Cambridge Isotope Laboratories (Andover, MA).

2.2. Animals

Male Sprague-Dawley rats (270–300 g) were obtained from the Comparative Biology Unit at University College London. Animal procedures were in accordance with the Home Office, UK guidelines.

2.3. Synthesis of deuterium-labeled compounds

[D_3]Bromotyrosine was synthesized by deuterium exchange as described [9,14], using authentic bromotyrosine as starting material. In brief, 50 mg of bromotyrosine were dissolved in a mixture of [D_4]acetic acid and D_2O, and the solvent was evaporated in a stream of nitrogen at 90 °C. This procedure was repeated twice to remove active protons. The resulting residue was dissolved in a mixture of [D_4]acetic acid, D_2O and deuterium chloride. The solution was sealed in an acid-digestion bomb and heated in an autoclave at 190 °C for 8 h [9,14]. The resulting product was dissolved in 0.1% (v/v) trifluoroacetic acid (TFA)/water (adjusted to pH 5.0 with ammonia solution) and extracted on a 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 [14], and their concentrations determined against known amounts of unlabeled standards by GC/MS (see below).

2.4. Synthesis of ^{13}C-labeled internal standard for the measurement of bromotyrosine, 4-hydroxyphenylacetic acid (HPA) and 3-bromo-4-hydroxyphenylacetic acid (bromo-HPA)

[$^{13}C_9$]Bromotyrosine was synthesized by the reaction of [$^{13}C_9$]tyrosine with hypobromous acid (HOBr) at room temperature for 1 h. HOBr was prepared freshly by adding HOCl to NaBr as described [17]. The products were purified using LC18 solid phase extraction, followed by HPLC. [$^{13}C_8$]4-hydroxyphenylacetic acid (HPA) was synthesized following deamination and decarboxylation of [$^{13}C_9$]tyrosine, using Taiwan cobra venom as described previously [14]. [$^{13}C_8$]3-Bromo-4-hydroxyphenylacetic acid (bromo-HPA) was synthesized and purified by bromination of [$^{13}C_8$]HPA in the same way as bromotyrosine, and the concentrations of ^{13}C-labeled standards determined against known amounts of unlabeled standards by GC/MS.

2.5. Measurement of bromotyrosine and tyrosine by GC/MS

^{13}C-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. Bromotyrosine and tyrosine were derivatized with ethyl heptafluorobutyrate and silylated with tert-butyldimethylsilyl, as described [9,17,18]. 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, 492, and 498 mass units for authentic, [D_3] and [$^{13}C_9$]bromotyrosine, respectively (Table 1). For measurement of tyrosine, samples were monitored at 407, 410 and 415 m/z for authentic, [D_3]tyrosine and [$^{13}C_9$]tyrosine, respectively. Concentrations were calculated by ratio to known $^{13}C_9$-labeled internal standards.

Table 1
The mass balance of the derivatized authentic, ^{13}C and deuterium-labeled compounds that were used for single-ion monitoring during mass spectrometry.

Compound	Authentic	^{13}C-labeled	Deuterium-labeled
Tyrosine	Tyrosine 407	[$^{13}C_9$]Tyrosine 415	[D_3]Tyrosine 410
3-Bromotyrosine	Bromotyrosine 489	[$^{13}C_9$]Bromotyrosine 498	[D_3]Bromotyrosine 492
4-Hydroxyphenylacetic acid (HPA)	HPA 331	[$^{13}C_8$]HPA 339	[D_3]HPA 334
3-Bromo-HPA	Bromo-HPA 409	[$^{13}C_8$]Bromo-HPA 417	[D_3]Bromo-HPA 412
3,5-Di-bromo-HPA	Di-bromo-HPA 489	[$^{13}C_8$]Di-bromo-HPA 497	Not used in this study