Nitration of endogenous *para*-hydroxyphenylacetic acid and the metabolism of nitrotyrosine

Ali R. MANI*, Ananth S. PANNALA†, Nelson N. ORIE*, Richard OLLOSSON*, David HARRY*, Catherine A. RICE-EVANS† and Kevin P. MOORE*¹

*Centre for Hepatology, Department of Medicine, Royal Free and University College Medical School, University College London, Rowland Hill Street, London NW3 2PF, U.K., and †Antioxidant Research Group, Wolfson Centre for Age-Related Diseases, GKT School of Biomedical Sciences, King's College London, St Thomas's Street, London SE1 9RT, U.K.

Reactive nitrogen species, such as peroxynitrite, can nitrate tyrosine in proteins to form nitrotyrosine. Nitrotyrosine is metabolized to 3-nitro-4-hydroxyphenylacetic acid (NHPA), which is excreted in the urine. This has led to the notion that measurement of urinary NHPA may provide a time-integrated index of nitrotyrosine formation *in vivo*. However, it is not known whether NHPA is derived exclusively from metabolism of nitrotyrosine, or whether it can be formed by nitration of circulating *para*-hydroxyphenylacetic acid (PHPA), a metabolite of tyrosine. In the present study, we have developed a gas chromatography MS assay for NHPA and PHPA to determine whether or not NHPA can be formed directly by nitration of PHPA. Following the injection of nitrotyrosine, $0.5 \pm 0.16\%$ of injected dose was recovered unchanged as nitrotyrosine, and $4.3 \pm 0.2\%$ as NHPA in the urine. To determine whether or not NHPA could

be formed by the nitration of PHPA, deuterium-labelled PHPA ($[^2\text{H}_6]\text{PHPA}$) was injected, and the formation of deuterated NHPA ($[^2\text{H}_5]\text{NHPA}$) was measured. Of the infused $[^2\text{H}_6]\text{PHPA}$, $78 \pm 2\%$ was recovered in the urine unchanged, and approx. 0.23% was recovered as $[^2\text{H}_5]\text{NHPA}$. Since the plasma concentration of PHPA is markedly higher than free nitrotyrosine (approx. 400-fold), the nitration of high-circulating endogenous PHPA to form NHPA becomes very significant and accounts for the majority of NHPA excreted in urine. This is the first study to demonstrate that NHPA can be formed by nitration of PHPA *in vivo*, and that this is the major route for its formation.

Key words: *para*-hydroxyphenylacetic acid, 3-nitro-4-hydroxyphenylacetic acid, nitrotyrosine, reactive nitrogen species.

INTRODUCTION

The pathological actions of reactive nitrogen species (RNS) are rooted in their ability to modify cell function through nitration, nitrosation or oxidation of cellular components, such as proteins, lipids or DNA [1]. In proteins, most nitration reactions occur on a tyrosine residue to form 3-nitrotyrosine [2,3], which is chemically stable. However, nitration of tyrosine can alter the protein's function, because nitration of tyrosine lowers the pK_a of the phenolic group and thus affect the maintenance of protein conformation [4–6]. However, little is known about the fate of nitrated proteins *in vivo*. It has been postulated that there is increased proteolytic degradation of nitrated proteins [7]. For example, Souza et al. [7] have shown that nitration of tyrosine residue(s) in proteins is sufficient to induce an accelerated degradation of the modified proteins by the proteasome to release free amino acids as well as free nitrotyrosine. The urinary elimination of free nitrotyrosine is minimal, but its secondary metabolites can potentially serve as biomarkers of overall nitrotyrosine formation *in vivo* [8]. Since the pioneering work of Ohshima et al. [9], it is known that nitrotyrosine is metabolized to 3-nitro-4-hydroxyphenylacetic acid (NHPA), which is excreted in the urine as the major urinary metabolite. This suggests that measurement of urinary NHPA could be used as a marker of systemic nitrotyrosine formation *in vivo*. It is not known, however, whether urinary NHPA is derived solely from metabolism

of nitrotyrosine or whether it can also be formed by nitration of endogenous *para*-hydroxyphenylacetic acid (PHPA), a metabolite of tyrosine [10] and various phenolic compounds [11]. PHPA readily undergoes nitration *in vitro* [12], and has been used as a chemical probe to monitor nitration reactions *in vitro* [13,14]. However, it is not known whether or not this reaction occurs endogenously, and whether or not measurement of the formation of NHPA can be used as an index of nitration reactions *in vivo*. The aims of the present study were thus to determine whether or not NHPA can be formed endogenously from nitration of circulating PHPA, and whether or not measurement of urinary NHPA can be used to monitor systemic nitration reactions *in vivo*.

EXPERIMENTAL

Chemicals

All chemicals were purchased from Sigma Aldrich (Poole, Dorset, U.K.) unless stated otherwise. $[^{13}\text{C}_9]\text{Tyrosine}$ ($[1,2,3,1',2',3',4',5',6'-^{13}\text{C}]\text{tyrosine}$), $[^2\text{H}_4]\text{ethanoic acid}$, $^2\text{H}_2\text{O}$ and ^2HCl were purchased from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Methanol, ethanol, ethyl ethanoate, acetonitrile and chloroform were from BDH (Lutterworth, Leicestershire, U.K.).

Abbreviations used: GC, gas chromatography; LPS, lipopolysaccharide; NHPA, 3-nitro-4-hydroxyphenylacetic acid; $[^{13}\text{C}_8]\text{NHPA}$, $[1,2,1',2',3',4',5',6'-^{13}\text{C}]\text{NHPA}$; $[^2\text{H}_5]\text{NHPA}$, $[2,2,2',5',6'-^2\text{H}]\text{NHPA}$; NICI, negative-ion chemical ionization; PHPA, *para*-hydroxyphenylacetic acid; $[^{13}\text{C}_8]\text{PHPA}$, $[1,2,1',2',3',4',5',6'-^{13}\text{C}]\text{PHPA}$; $[^2\text{H}_6]\text{PHPA}$, $[2,2,2',3',5',6'-^2\text{H}]\text{PHPA}$; RNS, reactive nitrogen species; TFA, trifluoroacetic acid; $[^{13}\text{C}_9]\text{tyrosine}$, $[1,2,3,1',2',3',4',5',6'-^{13}\text{C}]\text{tyrosine}$.

¹ To whom correspondence should be addressed (e-mail kmoore@rfc.ucl.ac.uk).

Synthesis of internal standards, [$^{13}\text{C}_8$]PHPA ([1,2,1',2',3',4',5',6'- ^{13}C]PHPA) and [$^{13}\text{C}_8$]NHPA ([1,2,1',2',3',4',5',6'- ^{13}C]NHPA)

[$^{13}\text{C}_8$]PHPA was synthesized following the de-amination and decarboxylation of [$^{13}\text{C}_9$]tyrosine using Taiwan cobra (*Naja naja atra*) venom as a catalysing enzyme [15]. In brief, [$^{13}\text{C}_9$]tyrosine was added to 50 mM ammonium formate buffer (pH 7.4) at a concentration of 0.2 mg/ml, and venom was added at a concentration of 1 mg/ml, before incubation at 37 °C for approx. 2 h. [$^{13}\text{C}_8$]PHPA was extracted into ethyl ethanoate, dried under nitrogen, and was then further purified by HPLC on a Techsphere C₁₈ column (25 mM $\times$ 4.6 mM). This employs a gradient of water containing 0.1 % (v/v) TFA (trifluoroacetic acid) (solution A) and 0.1 % (v/v) TFA/acetonitrile (solution B). Initial conditions were 100 % solution A, changing to 9:1 solution A/solution B over 15 min, then to 1:1 solution A/solution B from 15 to 30 min. The fractions containing [$^{13}\text{C}_8$]PHPA were identified by their retention times and characteristic UV spectra with a photodiode array system. Fractions containing [$^{13}\text{C}_8$]PHPA were pooled and freeze-dried under vacuum. [$^{13}\text{C}_8$]NHPA was synthesized after exposure of [$^{13}\text{C}_8$]PHPA to acidified nitrite (1 M hydrochloric acid and sodium nitrite, 200 mM) followed by ethyl ethanoate extraction, and drying under nitrogen.

Synthesis of deuterium-labelled PHPA

[$^2\text{H}_6$]PHPA ([2,2,2',3',5',6'- ^2H]PHPA) was synthesized by deuterium exchange [16]. In brief, 50 mg of PHPA was dissolved in a mixture of [$^2\text{H}_4$]ethanoic acid (0.4 ml) and $^2\text{H}_2\text{O}$ (0.5 ml) and the solvent was evaporated in a stream of nitrogen at 90 °C. This procedure was repeated twice to remove active protons as completely as possible. The resulting residue was dissolved in a mixture of [$^2\text{H}_4$]ethanoic acid (0.3 ml), $^2\text{H}_2\text{O}$ (0.3 ml) and ^2HCl (37 % in $^2\text{H}_2\text{O}$, 0.8 ml). The solution was sealed in an acid-digestion bomb and heated in an autoclave at 190 °C for 8 h. The product was extracted with ethyl ethanoate and washed with water. After drying of the organic phase, the resulting materials were dissolved in 0.1 % (v/v) TFA/water (adjusted to pH 5.0 with ammonia solution) and were then applied to an LC₁₈ reverse-phase columns (Supelclean SPE tubes; Sigma) that had been pre-washed with 2 ml of methanol and 5 ml of 0.1 % (v/v) TFA/water (pH 5.0). The column was washed with 2 ml of water and [$^2\text{H}_6$]PHPA was eluted with 4 ml of 25 % (v/v) methanol in water.

The concentrations of ^{13}C -labelled and deuterated standards were determined against known amounts of unlabelled standards using the method described below.

Sample purification and derivatization for urinary NHPA and PHPA

[$^{13}\text{C}_8$]NHPA (10 ng) or [$^{13}\text{C}_8$]PHPA (100 ng) was added to 100 μl of human urine, or 20 μl of rat urine, and was diluted to 1 ml with de-ionized water. Following the addition of 1 ml of ethyl ethanoate and vortex mixing, the organic phase was removed, and the sample was evaporated under a stream of nitrogen. The residue was reconstituted in 30 μl of acetonitrile, applied to a silica TLC plate (LK6 60 Å, 250 μm layer thickness, 5 cm $\times$ 20 cm; Whatman, Clifton, NJ, U.S.A.), and was run to the top of the plate in chloroform/methanol (80:20, v/v). Compounds migrating in the region of $R_f \sim 0.22$ for NHPA and at 0.20 for PHPA, and 1 cm above and below, were scraped from the plate and extracted in 1 ml of ethyl ethanoate/ethanol (50:50, v/v), dried under nitrogen, and derivatized to the pentafluorobenzyl ester, by the addition of

20 μl of 10 % (v/v) di-isopropyl ethylamine in acetonitrile and 40 μl of 10 % (v/v) pentafluorobenzyl bromide in acetonitrile for 1 h at room temperature (21–23 °C), dried under nitrogen and re-dissolved in 20 μl of *n*-undecane for analysis of NHPA or 50 μl of *n*-undecane for analysis of PHPA.

Measurement of plasma concentrations of NHPA and PHPA

To measure plasma levels of NHPA and PHPA, blood was taken from three male Sprague–Dawley rats into tubes containing EDTA, which were centrifuged at 2300 *g* for 30 min at 4 °C. The internal standards (10 ng of [$^{13}\text{C}_8$]NHPA or 100 ng of [$^{13}\text{C}_8$]PHPA) were added to 1 ml of plasma, which was immediately filtered by centrifugation at 9000 *g* in a microfuge through a 30 kDa molecular-mass cut-off centrifugal membrane (Ultrafree; Millipore, Bedford, MA, U.S.A.) to remove high-molecular-mass proteins. The filtrate was applied to an LC₁₈ reverse-phase column that had been pre-washed with 2 ml of methanol and 5 ml of 0.1 % (v/v) TFA/water (pH 5.0). The column was washed with 2 ml of water. Then NHPA and PHPA were eluted with 4 ml of 25 % (v/v) methanol in water and dried under vacuum before TLC extraction as described above. The samples were derivatized to the pentafluorobenzyl ester by the addition of 20 μl of 10 % (v/v) di-isopropyl ethylamine in acetonitrile and 40 μl of 10 % (v/v) pentafluorobenzyl bromide in acetonitrile for 1 h at room temperature, dried under nitrogen and re-dissolved in 20 μl of *n*-undecane before GC (gas chromatography)/MS analysis.

GC/MS analysis of derivatized samples

Samples were analysed on a GC equipped with a 15 m DB-1701 (J&W Scientific, Folsom, CA, U.S.A.) capillary column (0.25 mm internal diameter, 0.25 μm film thickness) interfaced with a mass spectrometer (Trio 1000; Fisons Instruments, Beverly, MA, U.S.A.). The ion source and interface temperature were set at 200 °C and 300 °C respectively. Samples were analysed in negative-ion chemical ionization (NICI) 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. Samples were quantified by isotope-dilution GC/MS and ions were monitored at 376 and 384 mass units for NHPA and at 311 and 319 mass units for PHPA with single-ion monitoring. The concentrations were calculated from the known $^{13}\text{C}_8$ -labelled internal standards, which are 8 mass units heavier than the authentic NHPA and PHPA.

Exclusion of artifactual nitration of PHPA during analysis

Initial studies using buffers at pH 4.0 showed that there was significant artifactual nitration of PHPA during the preparation of samples (results not shown). Therefore, to ensure that there was no artifactual nitration of PHPA during the preparation of urine samples, the formation of [$^2\text{H}_5$]NHPA ([2,2,2',5',6'- ^2H]NHPA) from 100 ng of [$^2\text{H}_6$]PHPA added to 100 μl of human urine was monitored in a separate series of experiments.

Standard curves for evaluation of the assays

To determine the accuracy of the assay, known amounts (0–20 ng of NHPA or 0–10 μg of PHPA) were added to 100 μl of human urine and the samples were analysed as unknowns. For measurement of intra-assay variability, six aliquots from a urine

sample of one volunteer were analysed as described above and the intra-assay variability was calculated as percentage coefficients of variation ($100 \times \text{S.D.}/\text{mean}$).

Animal studies

Male Sprague Dawley rats (body mass 270–300 g) were obtained from the Comparative Biology Unit at the Royal Free and University College Medical School (Royal Free Campus, UCL, London, U.K.). All animal procedures were in accordance with Home Office recommendations. The animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). The jugular vein and the urinary bladder were cannulated with polyethylene cannulae. Physiological saline solution was infused into the animals at 25 ml/kg per h through the jugular vein and urine samples were collected every 30 min as described below.

Metabolism of nitrotyrosine to NHPA

To determine the metabolism of nitrotyrosine to NHPA, 250 nmol of nitrotyrosine was dissolved in 1 ml of sterile PBS and was injected intravenously into three rats as a bolus. Urine samples were collected every 30 min for 5 h, with a single 30 min collection (basal) immediately before injection of nitrotyrosine. The urinary concentration of NHPA and PHPA was determined as described above. Urinary content of un-metabolized nitrotyrosine was also measured in each sample using GC with NICI MS, as previously described [17].

Nitration of PHPA *in vivo*

[$^2\text{H}_6$]PHPA was injected intravenously as a bolus (250 nmol, dissolved in 1 ml of PBS) after the initial basal urine collection ($t = 30$ min) into three rats, and urine samples were collected every 30 min for 4.5 h. Nitration of [$^2\text{H}_6$]PHPA produces [$^2\text{H}_5$]NHPA as a result of replacement of a single deuterium of the aromatic ring by the NO_2 group.

Effect of endotoxaemia on the formation of NHPA

LPS (lipopolysaccharide) from *Salmonella typhimurium* was injected intravenously into three rats as a bolus (5 mg/kg in 1 ml of PBS). Urine samples were collected every 30 min for 4.5 h, with a single 30 min collection (basal) immediately before the injection of LPS. The urinary content of NHPA, PHPA and nitrotyrosine was determined as above. In a further study, 250 nmol of [$^2\text{H}_6$]PHPA was injected intravenously into three rats 2 h after administration of LPS (5 mg/kg). The urinary content of NHPA, PHPA, [$^2\text{H}_5$]NHPA and [$^2\text{H}_6$]PHPA were then quantified by GC/MS using $^{13}\text{C}_8$ -labelled internal standards. For measurement of the deuterium-labelled compounds, ion masses (m/z) of 381 and 337 were selected in selective-ion monitoring, which are 5 and 6 mass units heavier respectively than the authentic NHPA and PHPA.

Statistical analysis

All data are presented as means $\pm$ S.E.M. Statistical evaluation of the data was carried out with the analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparisons, and $P < 0.05$ was considered statistically significant.

Figure 1 Structures and spectra from NICI scans of the pentafluorobenzyl derivative of NHPA (upper panel) and PHPA (lower panel)

The molecular mass of derivatized NHPA is 557 Da, which is 45 mass units heavier than derivatized PHPA (512 Da). The de-protonated molecular ion for the NHPA derivative is evident at m/z 556 [$M-H$] $^-$ and for PHPA at 511 [$M-H$] $^-$. NHPA has a dominant ion at m/z 376 [$M-181$], loss of one pentafluorobenzyl ester group). PHPA has a dominant ion at m/z 331 [$M-181$], which is also compatible with the loss of a pentafluorobenzyl ester group.

RESULTS

To investigate the formation of NHPA *in vivo*, it was necessary to develop a method that can accurately measure this metabolite, and avoid the problems that are encountered during the assay of other nitrated products, such as nitrotyrosine [3,17]. Thus the method needs to be reproducible with biological samples and free of artifactual nitration of its precursor (PHPA).

Characterization of the derivatives of NHPA and PHPA

The structures of the pentafluorobenzyl ester derivatives of NHPA and PHPA are shown in Figure 1. The molecular mass of derivatized NHPA is 557 Da, which is 45 mass units heavier than derivatized PHPA (512 Da). The de-protonated molecular ion for the NHPA derivative is evident at m/z 556 [$M-H$] $^-$ and for PHPA at 511 [$M-H$] $^-$, which are visible on the NICI spectra shown in Figure 1. Analysis of the pentafluorobenzyl ester derivatives of NHPA and PHPA by full-scan mode (Figure 1) showed that NHPA has a dominant ion at m/z 376 [$M-181$], loss of one pentafluorobenzyl ester group). PHPA has a dominant ion at m/z 331 [$M-181$], which is also compatible with the loss of a pentafluorobenzyl ester group. On the basis of these results, all further work was focused on developing the analytical method for NHPA and PHPA as the pentafluorobenzyl ester derivative with selective-ion monitoring at 376 and 331 mass units for NHPA and PHPA respectively. The detection limit was approx. 1 pg on column injection (for both NHPA and PHPA). After GC/MS analysis of urine samples, a single peak was eluted with the respective ^{13}C -labelled internal standards, which were 8 mass units heavier than the authentic NHPA and PHPA (Figure 2).

Standard curves of NHPA and PHPA

There was a good correlation between the added and measured amounts of NHPA ($r^2 = 0.998$) and PHPA ($r^2 = 0.995$) in urine,

Figure 2 Typical chromatogram showing peak shape and resolution for both NHPA (A) and PHPA (B) in a urine sample

The $^{13}\text{C}_8$ -labelled NHPA and PHPA resulted in derivatives that were 8 mass units heavier than the parent compound. NHPA (A) was quantified by monitoring ions of m/z 376 and 384, and PHPA (B) by monitoring ions of m/z 331 and 339. The x-axis represents the retention time (rt).

with a linear standard curve superimposed on the basal concentrations of these analytes (Figure 3). The intra-assay variation on a single urine sample was 7.4 % and 6.1 % for NHPA and PHPA respectively ($n = 6$).

Nitration of PHPA as an artifact

Artificial nitration of PHPA during sample preparation was investigated after the addition of $[\text{H}_6]\text{PHPA}$ to 100 μl of urine. Based on the evaluation of m/z 381 and retention time, no $[\text{H}_5]\text{NHPA}$ was detected after extraction and derivatization of samples using the method described. However, if buffers of pH 4.0 or below were used, there was significant artifactual nitration (results not shown).

Effect of nitrotyrosine infusion on urinary NHPA and nitrotyrosine levels

The basal excretion rate of urinary NHPA in this group of rats was 8.1 ± 0.5 pmol/min (Figure 4). Following the bolus injection of nitrotyrosine (250 nmol), the urinary excretion rate of NHPA increased to a peak of 84.8 ± 6.6 pmol/min within 30 min of injection. The urinary excretion of NHPA remained at 81.7 ± 12.2 pmol/min until 60 min, and thereafter decreased back towards baseline levels of 9.1 ± 1.5 pmol/min by 5 h (Figure 4). These results indicate that only $4.3 \pm 0.2\%$ of the injected nitrotyrosine is excreted as urinary NHPA. Free nitrotyrosine was undetectable in the urine under basal conditions. However,

Figure 3 Standard curves for NHPA (upper panel) and PHPA (lower panel) in human urine

Known amounts of NHPA and PHPA were added to urine. Following analysis as unknowns, the expected amount of added NHPA or PHPA was recovered with a good correlation when analysed by GC/MS.

following the intravenous injection of 250 nmol of nitrotyrosine, urinary nitrotyrosine became detectable with an excretion rate of peaking at 16.8 ± 5.3 pmol/min at 30 min post-injection, and was undetectable by 4 h (Figure 4). Only $0.49 \pm 0.16\%$ of the injected nitrotyrosine was excreted as urinary free nitrotyrosine.

Effect of LPS administration on urinary NHPA and nitrotyrosine levels

Injection of LPS into rats decreased the urine output from 26.5 ± 5.2 to 16.6 ± 7.1 $\mu\text{l}/\text{min}$. As shown in Figure 5, there was initially no change in the urinary excretion of NHPA, but 2 h after the injection of LPS, there was a sustained increase in the urinary excretion of NHPA from 7.61 ± 1.3 to 15.7 ± 2.1 pmol/min ($P < 0.01$). Urinary nitrotyrosine was undetectable until 90 min after bolus injection of LPS, but, thereafter, small amounts of free nitrotyrosine were detectable ($P < 0.01$) (Figure 5).

Nitration of infused $[\text{H}_6]\text{PHPA}$

In order to determine the extent of endogenous nitration of circulating PHPA *in vivo*, the formation of $[\text{H}_5]\text{NHPA}$ was

Figure 4 Urinary excretion of NHPA after intravenous injection of nitrotyrosine

Urinary excretion rate of NHPA and free nitrotyrosine increased by 30 min following the intravenous bolus injection of 250 nmol of nitrotyrosine into anaesthetized rats. Results are means $\pm$ S.E.M. ($n = 3$).

Figure 5 Urinary excretion of NHPA after intravenous injection of LPS

Urinary excretion rate of NHPA and free nitrotyrosine both increased following the injection of LPS (5 mg/kg) into anaesthetized rats at 30 min. Results are means $\pm$ S.E.M. ($n = 3$). ANOVA shows a significant increase in NHPA and PHPA excretion after LPS administration ($P < 0.01$).

investigated in rats injected with [$^2\text{H}_6$]PHPA. A GC/MS chromatogram revealed a peak with an m/z of 381, consistent with the nitration of [$^2\text{H}_6$]PHPA to [$^2\text{H}_5$]NHPA (Figure 6). The urinary excretion rate of [$^2\text{H}_5$]NHPA was 12.6 ± 3.0 and 5.1 ± 1.8 pmol/min at 30 and 60 min after the bolus injection of [$^2\text{H}_6$]PHPA respectively (Figure 7). The urinary excretion of unmetabolized [$^2\text{H}_6$]PHPA peaked within 30 min after the bolus injection, and total recovery of [$^2\text{H}_6$]PHPA was $78 \pm 2\%$ within the initial 4 h of urine collection. This suggests that there is relatively little metabolism of PHPA *in vivo*. At 60 min after [$^2\text{H}_6$]PHPA injection, the urinary excretion rate of [$^2\text{H}_6$]PHPA was very close to the normal range of authentic PHPA excretion (2.3 ± 0.7 nmol/min) (Figure 7), and this time interval was selected for comparison of the endogenous NHPA/PHPA ratio with the [$^2\text{H}_5$]NHPA/[$^2\text{H}_6$]PHPA ratio. At 60 min, the ratio of

Figure 6 Chromatogram of a urine sample obtained from a rat injected with [$^2\text{H}_6$]PHPA

In vivo formation of [$^2\text{H}_5$]NHPA, represented as a distinguished peak, was detected with m/z 381. The values of m/z 376 and m/z 384 represent authentic NHPA and $^{13}\text{C}_8$ -labelled internal standard respectively.

Figure 7 Urinary excretion of [$^2\text{H}_5$]NHPA and [$^2\text{H}_6$]PHPA after intravenous injection of [$^2\text{H}_6$]PHPA

The urinary excretion rate of [$^2\text{H}_5$]NHPA (upper panel) and [$^2\text{H}_6$]PHPA (lower panel) both increased following the intravenous bolus injection of [$^2\text{H}_6$]PHPA (250 nmol) into anaesthetized rats. As shown in the upper panel, there was a marked increase in the formation of [$^2\text{H}_5$]NHPA, which can only arise from nitration of infused [$^2\text{H}_6$]PHPA. The total recovery of injected [$^2\text{H}_6$]PHPA was $78 \pm 2\%$ during the first 4 h of urine collection. Results are means $\pm$ S.E.M. ($n = 3$). The mean urinary excretion rate of endogenous PHPA was 2.3 ± 0.7 nmol/min, and the range of excretion rate observed in normal rats is shown by the dotted lines (a).

[$^2\text{H}_5$]NHPA/[$^2\text{H}_6$]PHPA was 6.1 ± 1.0 (pmol/nmol), and the ratio of endogenous NHPA/PHPA was 7.0 ± 0.9 (pmol/nmol). Assuming that endogenous PHPA is handled in the same way

Figure 8 NHPA/PHPA ratio as an index of RNS formation

Under basal conditions, the ratio of NHPA/PHPA gives an index of the rate of nitration of PHPA, and the metabolism of nitrotyrosine. $[^2\text{H}_6]\text{PHPA}$ was used as a probe to assess the nitration of PHPA to NHPA *in vivo*. Injection of LPS increases the ratio of endogenous NHPA/PHPA as well as that of $[^2\text{H}_5]\text{NHPA}/[^2\text{H}_6]\text{PHPA}$, suggesting that much of the NHPA that is excreted in urine during endotoxaemia is derived from the nitration of circulating PHPA. Results are means $\pm$ S.E.M. * $P < 0.05$ compared with the saline-treated group.

as infused PHPA, there is approx. 0.9 pmol/nmol of NHPA unaccounted for, and which is assumed to be derived from metabolism of nitrotyrosine. Injection of LPS resulted in a significant increase ($P < 0.05$) in the NHPA/PHPA ratio as well as the $[^2\text{H}_5]\text{NHPA}/[^2\text{H}_6]\text{PHPA}$ ratio (Figure 8). Meanwhile, the total recovery of $[^2\text{H}_6]\text{PHPA}$ was significantly decreased after LPS administration ($39.2 \pm 4\%$ compared with $78 \pm 2\%$; $P < 0.05$) during 4 h of urine collection. We attribute this to the marked decrease in urine volume post-LPS.

Plasma concentrations of PHPA and NHPA

The plasma concentration of PHPA was $2.7 \pm 1.1 \mu\text{mol/l}$ ($410 \pm 171 \text{ ng/ml}$) in rats. In contrast, the plasma concentrations of NHPA were undetectable.

DISCUSSION

One of the major disadvantages of measuring nitrotyrosine in tissues or biological fluids is that nitrated proteins are broken down at variable rates, and nitrotyrosine is metabolized and excreted [7,9]. Thus the concept that measurement of NHPA, as the major urinary metabolite of nitrotyrosine, is attractive, since it can provide a time-integrated index of nitrotyrosine formation *in vivo*. However, it is important to know whether this metabolite is formed exclusively from nitrotyrosine, or whether it can be formed via nitration of PHPA, which is present at high concentrations in plasma.

Since the identification of NHPA as the major nitrotyrosine metabolite, there have been no reliable assays for the measurement of NHPA in biological fluids. For instance, an electrochemical-based HPLC method has been described for the measurement of NHPA [18], but the specificity and reproducibility of this method have not been evaluated so far. Beal et al. [19] have also reported an assay for measurement of NHPA in spinal tissue, but their method has the disadvantage that extractions are performed in extremely acidic conditions in which nitrite, always present in biological samples, can cause artifactual nitration of PHPA [12]. In the present study, we have described a novel assay for the

Scheme 1 Two identified pathways, which can produce NHPA *in vivo*

NHPA can be formed by either nitration of PHPA or catabolism of free nitrotyrosine. Tyrosine decarboxylase and tyramine oxidase are two identified metabolic enzymes that are responsible for conversion of tyrosine and nitrotyrosine into PHPA and NHPA.

measurement of urinary NHPA, which has the advantage of being highly sensitive, specific and reproducible.

The major finding of this study is that urinary NHPA is not derived solely from nitrotyrosine metabolism and at least one other pathway is responsible for its formation *in vivo* (Scheme 1). The nitration of $[^2\text{H}_6]\text{PHPA}$ to $[^2\text{H}_5]\text{NHPA}$ provides unequivocal proof that nitration of circulating PHPA can occur. Indeed, based on our animal studies, we estimate that most of the urinary NHPA (approx. 85%) is formed by nitration of PHPA *in vivo* in the rat, despite the fact that only a relatively small fraction of the infused PHPA is nitrated *in vivo*. This occurs because PHPA is present in plasma at a concentration of approx. $2.7 \mu\text{mol/l}$, whereas free nitrotyrosine is only present at approx. 4.2 nmol/l [3].

Although the major source of urinary NHPA is formed by nitration of endogenous PHPA, we also demonstrate that NHPA excretion increased after intravenous nitrotyrosine injection. Less than 5% of injected nitrotyrosine was excreted in the urine as NHPA over 5 h, which is consistent with the observations of Tabrizi-Fard et al. [8], who also observed that only 5–6% of infused nitrotyrosine was metabolized to NHPA. The large volume of distribution of free nitrotyrosine [8] and the absence of detectable nitrotyrosine in normal urine suggest that there is cellular uptake as well as tubular re-absorption of free nitrotyrosine by various amino-acid transporters in the cell membrane. In contrast, $78 \pm 2\%$ of injected $[^2\text{H}_6]\text{PHPA}$ was rapidly excreted into the urine unchanged, demonstrating that there is little metabolism of this compound. Based on a plasma concentration of PHPA of $2.7 \pm 1.1 \mu\text{mol/l}$, and a urinary excretion rate of PHPA at $2.3 \pm 0.7 \text{ nmol/min}$ in rats, the renal clearance of PHPA is estimated to be approx. 0.85 ml/min . This value is very close to the glomerular filtration rate of our laboratory rats ($1.02 \pm 0.1 \text{ ml/min}$) [20], and suggests that PHPA is simply filtered and not re-absorbed by the kidneys.

We have also shown that measurement of $[^2\text{H}_5]\text{NHPA}$ after infusing $[^2\text{H}_6]\text{PHPA}$ may be used as an index of RNS formation *in vivo*. Our observation that endotoxaemia causes a significant

increase in [$^2\text{H}_5$]NHPA excretion (Figure 8) supports the validity of this novel approach. Moreover our observation that the ratio of nitration of [$^2\text{H}_5$]NHPA from infused [$^2\text{H}_6$]PHPA was almost identical with that of endogenous NHPA to PHPA suggests that measurement of the NHPA/PHPA ratio may be useful to assess nitration reactions *in vivo* (Figure 8).

The present study has shown for the first time that endogenous PHPA can be nitrated to NHPA *in vivo*, which, until the present study, has been regarded as the major metabolite of nitrotyrosine. On a practical level, we have also shown that measurement of urinary NHPA can be used as a measure of nitration reactions *in vivo*. We conclude that the measurement of urinary NHPA excretion provides a time-integrated index of RNS formation *in vivo*, although it is not exclusively related to nitrotyrosine metabolism.

This research was supported by grants from the Wellcome Trust, U.K., and the Food Standards Agency, U.K.

REFERENCES

- 1 Droge, W. (2002) Free radicals in the physiological control of cell function. *Physiol. Rev.* **82**, 47–95
- 2 Ischiropoulos, H. (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* **356**, 1–11
- 3 Moore, K. P. and Mani, A. R. (2002) Measurement of protein nitration and S-nitrosothiol formation in biology and medicine. *Methods Enzymol.* **359**, 256–268
- 4 Sokolovsky, M., Riordan, J. F. and Vallee, B. L. (1967) Conversion of 3-nitrotyrosine to 3-aminotyrosine in peptides and proteins. *Biochem. Biophys. Res. Commun.* **24**, 20–25
- 5 Eiserich, J. P., Estevez, A. G., Bamberg, T. V., Ye, Y. Z., Chumley, P. H., Beckman, J. S. and Freeman, B. A. (1999) Microtubule dysfunction by posttranslational nitrotyrosination of α -tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6365–6370
- 6 Tarpey, M. M. and Fridovich, I. (2001) Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. *Circ. Res.* **89**, 224–236
- 7 Souza, J. M., Choi, I., Chen, Q., Weisse, M., Daikhin, E., Yudkoff, M., Obin, M., Ara, J., Horwitz, J. and Ischiropoulos, H. (2000) Proteolytic degradation of tyrosine nitrated proteins. *Arch. Biochem. Biophys.* **380**, 360–366
- 8 Tabrizi-Fard, M. A., Maurer, T. S. and Fung, H. L. (1999) In vivo disposition of 3-nitro-L-tyrosine in rats: implications on tracking systemic peroxynitrite exposure. *Drug Metab. Dispos.* **27**, 429–431
- 9 Ohshima, H., Friesen, M., Brouet, I. and Bartsch, H. (1990) Nitrotyrosine as a new marker for endogenous nitrosation and nitration of proteins. *Food Chem. Toxicol.* **28**, 647–652
- 10 Fell, V., Hoskins, J. A. and Pollitt, R. J. (1978) The labelling of urinary acids after oral doses of deuterated L-phenylalanine and L-tyrosine in normal subjects: quantitative studies with implications for the deuterated phenylalanine load test in phenylketonuria. *Clin. Chim. Acta* **83**, 259–269
- 11 Baker, G. B., Urchuk, L. J., McKenna, K. F. and Kennedy, S. H. (1999) Metabolism of monoamine oxidase inhibitors. *Cell. Mol. Neurobiol.* **19**, 411–426
- 12 Takahama, U., Oniki, T. and Murata, H. (2002) The presence of 4-hydroxyphenylacetic acid in human saliva and the possibility of its nitration by salivary nitrite in the stomach. *FEBS Lett.* **518**, 116–118
- 13 van der Vliet, A., Eiserich, J. P., Halliwell, B. and Cross, C. E. (1997) Formation of reactive nitrogen species during peroxidase-catalysed oxidation of nitrite: a potential additional mechanism of nitric oxide-dependent toxicity. *J. Biol. Chem.* **272**, 7617–7625
- 14 Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B. and van der Vliet, A. (1998) Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature (London)* **391**, 393–397
- 15 Nucaro, E., Jodra, M., Russell, E., Anderson, L., Dennison, P. and Dufton, M. (1998) Conversion of tyrosine to phenolic derivatives by Taiwan cobra venom. *Toxicon* **36**, 1173–1187
- 16 Shimamura, M., Kamada, S., Hayashi, T. and Naruse, H. (1986) Sensitive determination of tyrosine metabolites, *p*-hydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenyl-acetic acid and 4-hydroxy-3-methoxymandelic acid, by gas chromatography-negative-ion chemical-ionization mass spectrometry: application to a stable isotope-labelled tracer experiment to investigate their metabolism in man. *J. Chromatogr.* **374**, 17–26
- 17 Frost, M. T., Halliwell, B. and Moore, K. P. (2000) Analysis of free and protein-bound nitrotyrosine in human plasma by a gas chromatography/mass spectrometry method that avoids nitration artifacts. *Biochem. J.* **345**, 453–458
- 18 Crow, J. P. (1999) Measurement and significance of free and protein-bound 3-nitrotyrosine, 3-chlorotyrosine, and free 3-nitro-4-hydroxyphenylacetic acid in biologic samples: a high-performance liquid chromatography method using electrochemical detection. *Methods Enzymol.* **301**, 151–160
- 19 Beal, M. F., Ferrante, R. J., Browne, S. E., Matthews, R. T., Kowall, N. W. and Brown, R. H. (1997) Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. *Ann. Neurol.* **42**, 644–654
- 20 Anand, R., Harry, D., Holt, S., Milner, P., Dashwood, M., Goodier, D., Jarmulowicz, M. and Moore, K. (2002) Endothelin is an important determinant of renal function in a rat model of acute liver and renal failure. *Gut* **50**, 111–117

Received 7 May 2003; accepted 11 June 2003

Published as BJ Immediate Publication 11 June 2003, DOI 10.1042/BJ20030670