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Disposition of Protein-bound 3-nitrotyrosine in Rat Plasma Analysed by a Novel Protocol for HPLC–ECD

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3-Nitrotyrosine (NTyr) is considered as a biomarker of the generation of reactive nitrogen species (RNS). However, it is still difficult to determine its concentration in biological samples. To develop a reliable and high-throughput method, we optimized the conditions for high performance liquid chromatography and electrochemical detection (HPLC–ECD). The best separation of NTyr was achieved using a highly acidic mobile phase (pH 2.5). The concentration of protein-bound NTyr in plasma protein was 593.6 ± 53.8 fmol/mg in rats treated with lipopolysaccharide (LPS) and 114.4 ± 27.6 fmol/mg in control. After intravenous administration of *in vitro*-nitrated plasma protein, NTyr concentration decreased; the half-life was 63.4 ± 16.8 h. Consistently, protein-bound NTyr concentration in plasma after LPS treatment declined gradually, but was detectable for 1 week. Our protocol is reproducible and suitable for analysing multiple clinical samples to study RNS production *in vivo*.

Key words: electrochemical detection, HPLC, lipopolysaccharide, nitric oxide, 3-nitrotyrosine.

Abbreviations: ECD, electrochemical detection; GS/MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; LPS, lipopolysaccharide; NO, nitric oxide; NTyr, 3-nitrotyrosine; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; RNS; reactive nitrogen species; TBST, Tris-buffered saline with Tween 20.

Nitric oxide (NO) is a ubiquitous molecule that plays a central role in homeostatic regulation of the cardiovascular, nervous and immune systems (*1*). Despite these important physiological functions, the metabolites of NO can be toxic. For example, NO reacts with superoxide anion to form the reactive peroxynitrite anion ($ONOO^-$), which damages DNA, inhibits a variety of enzymes and initiates lipid peroxidation (*2*, *3*). Excess production of NO and reactive nitrogen species (RNS) is thought to promote a number of inflammatory diseases, including sepsis, ischaemic heart diseases, atherosclerosis and diabetes mellitus (*4–7*).

Nitration of protein, caused by RNS such as $ONOO^-$ produces 3-nitrotyrosine (NTyr) in high yield, and a smaller amount of 3,3′-dityrosine, 3,4-dihydroxyphenylalanine and the corresponding quinine (*8*, *9*). The level of NTyr is regarded as a biomarker of the generation of RNS *in vivo* (*10*). Protein nitration is associated with more than 60 human disorders (*11*). Most studies have attempted to quantify the concentration of free NTyr in plasma, or protein-bound NTyr after extensive hydrolysis of protein samples (*12–18*). However, the reported NTyr levels vary considerably, partly because of the difficulty in quantifying the minute amount of NTyr in highly complex biological samples. It is also unknown how long protein-bound NTyr persists *in vivo*. Such information is indispensable to evaluate the biological significance of NTyr.

Various methods have been used to determine NTyr concentration, including high performance liquid chromatography (HPLC) coupled with electrochemical detector (ECD) (*19–24*) and mass spectrometry coupled with liquid or gas chromatography (LC/MS or GS/MS) (*12*, *18*, *25*, *26*). Although these systems are sensitive enough to detect NTyr in simple aqueous solution, determining the concentration of NTyr in biological samples, which may be as low as 10^{-6} to 10^{-7} relative to tyrosine concentration, is cumbersome (*19*, *25*, *27*). For example, mass spectrometric approaches require expensive and specialized equipment, extensive sample preparation and isotopically labelled internal standards (*15*, *28*). To elucidate the pathophysiological significance of RNS and NTyr formation, it is necessary to establish a

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simple and high-throughput analytical method for accurate quantification of protein-bound NTyr.

To quantify NTyr concentration in biological samples, we have used an HPLC-dual-mode ECD, comprising two serial EC cells in the reduction and oxidation/detection modes (*23*, *24*). This method is highly sensitive and maintains linearity across a wide range of NTyr concentrations (*21*, *23*, *24*). Moreover, ECD offers an intrinsic selectivity function, because electrochemically inert substances are invisible to the ECD. Therefore, the concentration of the reactive components may be determined in a complex matrix without the need for extensive sample purification (*29*).

In this study, we optimized the protocol for NTyr quantification using HPLC–ECD, and examined the persistence of protein-bound NTyr after intravenous administration of *in vitro*-nitrated plasma proteins or after intraperitoneal administration of lipopolysaccharide (LPS).

MATERIALS AND METHODS

Animals—Eight-week-old male Sprague-Dawley (SD) rats were purchased from SLC (Shizuoka, Japan) and housed in a pathogen-free animal facility under a standard 12 h light/dark cycle. All experiments were performed under institutional guidelines approved by the Animal Committee of Kanazawa University.

LPS (*Escherichia coli* 0111:B4, Sigma, St Louis, MO) dissolved in phosphate buffered saline (PBS) was administered intraperitoneally to the rats. At the appropriate time points, whole blood was collected from the inferior vena cava or the tail vein into a heparinized syringe, while the rat was under anaesthesia. The plasma was separated by centrifugation. Control plasma samples were prepared from rats that had been administered PBS only. The concentration of protein was determined using the BCA protein quantification kit (Pierce, Wobum, MA).

In vitro Nitration of Rat Plasma Protein—Rat plasma was dialysed against four exchanges of 100 volumes of PBS at 4°C. The sample was cleared by centrifugation at $2{,}500 \times g$ for 20 min. Plasma proteins were nitrated *in vitro* by adding 5 mM sodium nitrite and adjusting the pH to 3.0 with HCl. After 30-min incubation at room temperature, the reaction mixture was neutralized with 1 M Tris–HCl (pH 8.0) and dialysed against four exchanges of 100 volumes of PBS at 4°C. The nitration of plasma protein was verified by western analysis using an anti-nitrotyrosine antibody, as described later.

The nitrated plasma proteins were infused into rats through the tail vein, to achieve a concentration of 500 pmol NTyr/mg plasma protein 1 h after the infusion. Plasma samples (70 μl) were obtained before; 1, 3, 8 and 24 h after; and 3, 4, 5 and 7 d after the infusion.

Sample Preparation—Rat plasma (95–110 μl, equivalent to 5 mg plasma protein) was diluted to 500 μl with 0.1 M acetate buffer (pH 7.4), precipitated by adding 800 μl of ice-cold acetonitrile and centrifuged at $2800 \times g$ for 10 min. The pellet was washed with acetonitrile/0.1 M acetate buffer (8:5 v/v) to remove nitrite and nitrate present in plasma, and resuspended in 0.1 M acetate buffer (pH 7.4) by two 10-s cycles of sonication. The resuspended protein precipitate was digested with 1 mg pronase (Roche, Basel, Switzerland), which was dialysed against nitrite- and nitrate-free 0.1 M acetate buffer (pH 7.4), for 14 h at 50°C with continuous rotation. The digested product was centrifuged, and the supernatant was ultrafiltrated using Ultra free MC (molecular cutoff 10,000, Millipore, Billerica, MA).

Validity of sample preparation was assessed by measuring the concentration of free tyrosine in the filtrated sample, using an HPLC-ultraviolet detection method. Tyrosine (Sigma, St Louis, MO) was dissolved in 0.1 M acetate buffer (pH 7.4) at the concentration 0.1–10 mM as the standard. A coefficient of variation of the concentration of tyrosine in the sample was <5%.

HPLC–ECD—Fifty microlitres of the filtrated sample was fractionated with an SC-50ODS column (3 mm × 150 mm, 5 μm, Eicom, Kyoto, Japan) and 200 mM phosphate buffer containing 5 μg/ml EDTA and 2% acetonitrile, using the HPLC–ECD system (PEC-510/HTEC-500, Eicom). The flow rate was 500 μl/min. The pH, ionic strength and the concentration of organic solvent in the mobile phase were varied, and their effects on the retention time and resolution of a NTyr peak were examined. 3-Nitrotyrosine (Sigma) dissolved in 0.1 M acetate buffer was used as the standard.

The sensitivity and linearity of the HPLC–ECD system to NTyr were verified by analysing serial dilutions of the NTyr standard, which ranged from 10 pM to 1 μM. Applied potentials for the dual-mode ECD were adjusted to achieve the highest signal-to-noise ratio between NTyr and other components in the digested plasma proteins. The applied potentials were from −900 to −500 mV for the reduction cell and from 50 to 300 mV for the oxidation/detection cell. The concentration of NTyr was determined by using the NTyr standard (Sigma) dissolved in 0.1 M acetate buffer (pH 7.4). The amount of NTyr was expressed as micromol/mg plasma protein as determined using the BCA protein quantification kit.

Western Analysis—Protein samples (5 μg) were fractionated on two 10% polyacrylamide gels in parallel. One gel was stained with Coomassie brilliant blue (CBB) to confirm the protein loading, and the other gel was blotted onto a PVDF membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline with 0.5% Tween 20 (TBST), and incubated with a monoclonal anti-nitrotyrosine antibody diluted 1:2000 (Upstate, Charlottesville, VA) (*30*). After an extensive wash with TBST, the membrane was incubated with anti-mouse antibody conjugated with horseradish peroxidase (Amersham, Piscataway, NJ). Nitrated protein was detected using a Southern lightning system (PerkinElmer, Wellesley, MA).

Statistics—Student's *t*-test was used for statistical analysis using Statview for Windows version 5.0 (SAS Institute, Cary, NJ). $P < 0.05$ was regarded as significant.

RESULTS

Conventional method of NTyr analysis by HPLC–ECD have used phosphate or sodium acetate buffer at pH 4.3 to 7.5 and an organic solvent at a concentration of

several percent for the mobile phase (*19*, *21–23*). We used 200 mM phosphate buffer (pH 4.3) and 2% acetonitrile. Our method has high sensitivity and linearity across the standard NTyr range of 50 fmol to 1 μmol (*23*, *24*, *31*). However, we also observed insurmountable interference with the peak of NTyr caused by various plasma components (Fig. 1). This affected the reproducibility and reliability of NTyr measurement (see Fig. 4).

Improved NTyr Separation—To optimize the separation of NTyr from other plasma components, we examined the effect of a mobile phase on NTyr separation. The retention time of NTyr was highly dependent on the pH of the mobile phase (Fig. 2A) and the concentration of acetonitrile (Fig. 2B), whereas the ionic strength had a minor effect (Fig. 2A). The retention time was prolonged by a strongly acidic mobile phase: 17 min at pH 4.3, 20 min at pH 3.0 and 27 min at pH 2.5.

Because prolongation of the retention time might improve the separation of the NTyr peak from other components in plasma, we tested the effects of pH and the concentration of acetonitrile in the mobile phase on the resolution of the NTyr peak in plasma samples from LPS-treated and control rats. As anticipated, decreasing the pH of the mobile phase improved the resolution of the NTyr peak in the LPS-treated rat plasma. The best separation was achieved with a mobile phase at pH 2.5 and with 2.0% acetonitrile (Fig. 3A). Under this condition, the peak area of NTyr present in the control rat plasma was close to the lower detection limit, and less than one-tenth of that at pH 4.3 (*e.g.* Fig. 3A compare with Fig. 1). To eliminate a possibility to produce artificial NTyr with acidic mobile phase, 1 mM tyrosine standard spiked with or without 10 nM NTyr was analysed with HPLC with acidic mobile phase (ranging from pH 2.1 to 4.5). No artificial NTyr production was observed, probably because we removed nitrite/nitrate from plasma protein, and the separation of tyrosine with nitrite, if present, might start soon after sample injection within the separation column.

Optimizing Applied Potentials—To increase the specificity for NTyr detection further, we optimized the applied potentials in the reduction and oxidation/detection cells of the dual-mode ECD. Our former protocol at pH 4.3 used −900 and 300 mV for the reduction and oxidation/detection cells, respectively. Decreasing the applied potential in the reduction cell from −900 to −600 mV markedly decreased the background current and peak areas of various components in plasma, whereas the peak area of NTyr was well preserved (data not shown).

Further improvement of the signal-to-noise ratio was achieved by decreasing the oxidation/detection potential. The peak of NTyr was maintained by decreasing the oxidation/detection potential from 200 to 75 mV (Fig. 3B). In contrast, most peaks of the other components decreased markedly and some disappeared completely from the chromatogram (Fig. 3C). Accordingly, the optimal applied potentials for NTyr analysis at pH 2.5 were −600 mV for the reduction cell and 75 mV for the oxidation/detection cells.

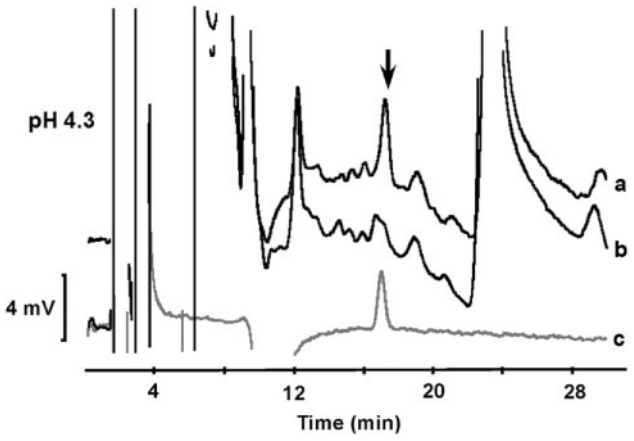

Fig. 1. **Analysis of NTyr in rat plasma samples at pH 4.3.** Rats were treated with or without LPS (5 mg/kg), and plasma samples were prepared 24 h later. Following pronase digestion and ultrafiltration of plasma protein, the sample was analysed with HPLC–ECD, using a mobile phase at pH 4.3. The potentials applied to the reduction and oxidation/detection cells were −900 and 300 mV, respectively. The resolution of the NTyr peak was affected by adjacent peaks at pH 4.3. (a) LPS-treated rat plasma sample; (b) control rat plasma and (c) 5 nM NTyr standard.

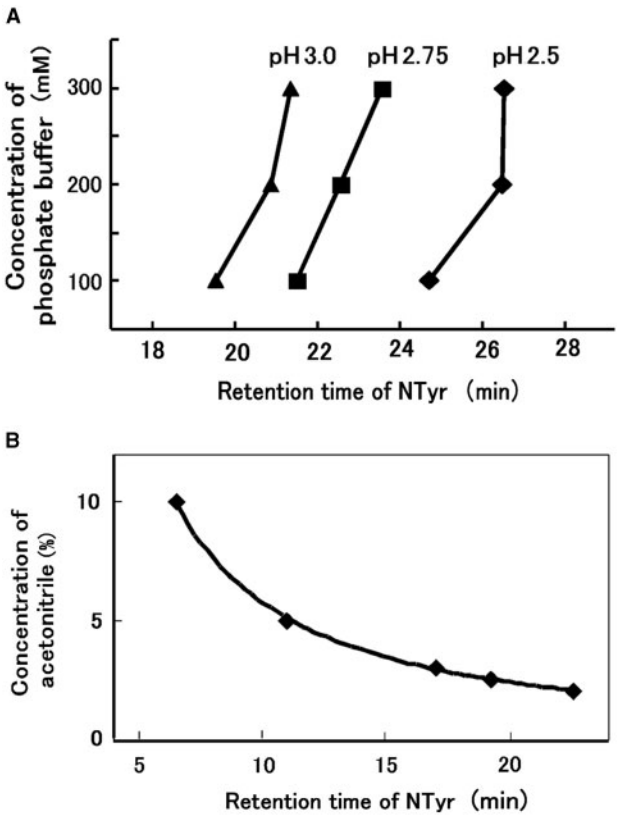

Fig. 2. **The effect of HPLC conditions on the retention time of NTyr.** (A) Relationship between pH, ionic strength of the mobile phase and the retention time of NTyr. The NTyr standard sample was analysed with HPLC–ECD using various mobile phases. (B) The effect of acetonitrile concentration in the mobile phase on the retention time of NTyr. The concentration of acetonitrile was varied from 2 to 10% in a highly acidic mobile phase (200 mM phosphate buffer at pH 2.5 and 5 μg/ml EDTA).

Reproducibility of NTyr Measurement—To assess the reproducibility of the new protocol at pH 2.5, we prepared a batch of sample from pooled rat plasma and analysed it repeatedly using both protocols at pH 2.5 and pH 4.3. The reproducibility of NTyr measurement was apparently improved at pH 2.5 (Fig. 4). Moreover, the shape and resolution of NTyr peak were uniform and reproducible at pH 2.5 (Fig. 3A and B). The concentration of NTyr measured at pH 2.5 was 593.6 ± 53.8 fmol/mg plasma protein from LPS-treated rats and 114.4 ± 27.6 fmol/mg in control rats ($P < 0.001$). The respective values at pH 4.3 were 1434.0 ± 456.0 and 865.2 ± 290.8 fmol/mg in plasma protein from LPS-treated and control rats.

Disposition of Nitrated Plasma Protein in the Rat Circulation—To examine the reliability of the protocol for measuring NTyr concentration, we prepared nitrated rat plasma protein *in vitro* and infused it into four rats to achieve 10^3 times the amount of protein-bound NTyr found in normal rat plasma immediately after the infusion. The amount of NTyr declined rapidly in the rat plasma during the first 24 h. This rapid-elimination phase was followed by a more prolonged elimination phase (Fig. 5A and B). The average half-life of the latter phase for protein-bound NTyr was 63.4 ± 16.8 h ($n = 4$). In addition, the high concentration of nitrated protein in rat plasma allowed us to detect protein-bound NTyr by western analysis, using an anti-NTyr antibody. As shown in Fig. 5B, the amount of all nitrated plasma proteins decreased and that of nitrated albumin detected by western blot analysis correlated with the peak area of NTyr on the HPLC–ECD chromatograms.

Protein-bound NTyr in Rat Plasma after LPS Administration—Finally, we examined the amount of protein-bound NTyr in rat plasma after administration of 0.5, 5 and 10 mg/kg LPS. A series of typical chromatograms is shown in Fig. 6A. The amount of NTyr reached a maximum 24 h after administration of 5 mg/kg LPS. It was 10 times higher than that in control rat plasma (Fig. 6A and B). Thereafter, the amount of NTyr gradually decreased over 1 week (Fig. 6B). The highest dose of LPS (10 mg/kg) elevated NTyr concentration further to a level more than 1.5 times higher than that produced by 5 mg/kg LPS at 12 h (Fig. 6B). The lowest dose of LPS (0.5 mg/kg) produced a 2-fold increase in NTyr concentration at 12 h, compared with control rat plasma, after which it decreased to normal levels within 36 h after the LPS administration (Fig. 6B).

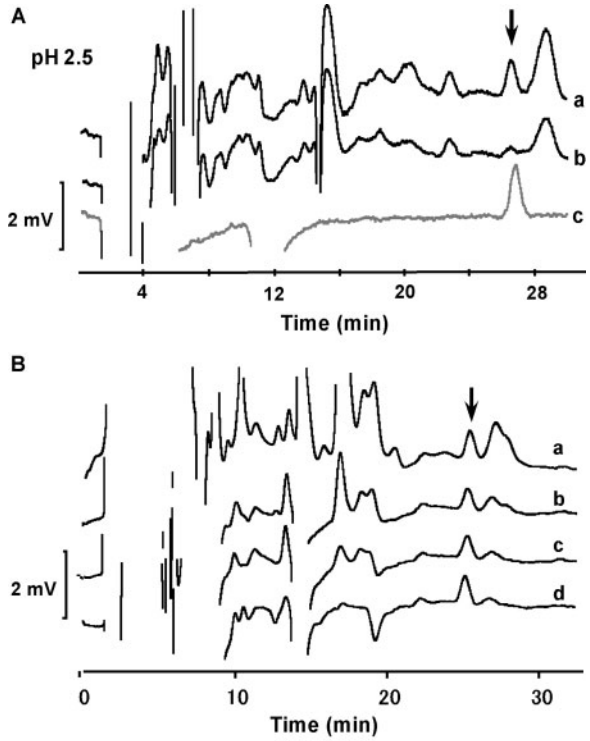

Fig. 3. **NTyr analysis of rat plasma samples at pH 2.5.** (A) Plasma samples from LPS-treated or control rats were analysed with HPLC–ECD using a highly acidic mobile phase at pH 2.5. (a) LPS-treated rat plasma; (b) control rat plasma and (c) 5 nM NTyr standard. (B) The profile of electrochemical responses for NTyr. NTyr standard (250 fmol) was analysed repeatedly (n=3) with a mobile phase at pH 2.5, varying the potential applied applied to the oxidation/detection cell. The applied potential to the reduction cell was at −600mV. (C) Improved specificity to NTyr by decreasing the applied potential to the oxidation/detection cell. The applied potential to the reduction cell was fixed at −600 mV, and that to the oxidation/detection cell was varied at (a) 200 mV, (b) 150 mV, (c) 100 mV and (d) 75 mV. The peak area of NTyr was well preserved, whereas other peaks decreased as the applied potential decreased.

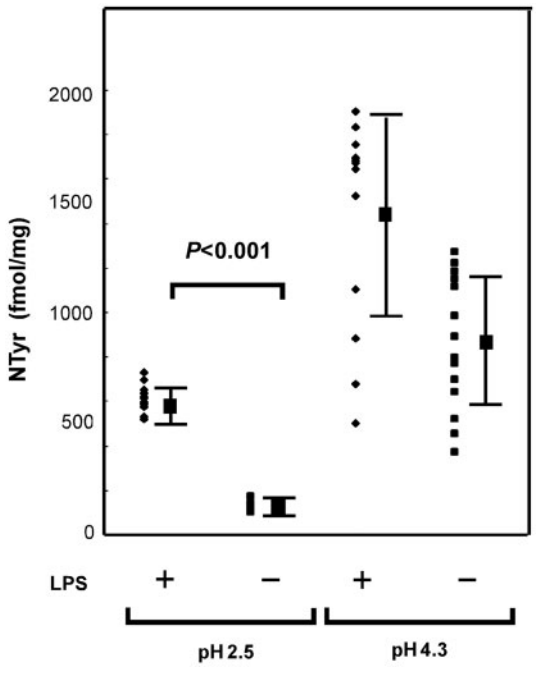

Fig. 4. **Reproducibility of NTyr measurement at pH 2.5 and pH 4.3.** The same samples, prepared from pooled rat plasma from LPS-treated or control rats, were analysed repeatedly (15 times) at both pH 2.5 and pH 4.3. The reproducibility of NTyr measurement was better using the new protocol at pH 2.5 than when using the former protocol at pH 4.3. Coefficient of variation was 9.1 (pH 2.5) *vs* 31.8 (pH 4.3) for LPS-treated rats and 24.1 (pH 2.5) *vs* 33.6 (pH 4.3) for control rats.

DISCUSSION

An accurate and reproducible method to measure NTyr is crucial to our understanding of the role of nitration reactions in pathophysiological processes. Such a method should also be applicable to biological samples, which contain various interfering substances. Our early attempts to determine the concentration of NTyr required repeated measurements for each sample, because of variable amounts of NTyr due to poor separation of NTyr from other components in plasma at pH 4.3.

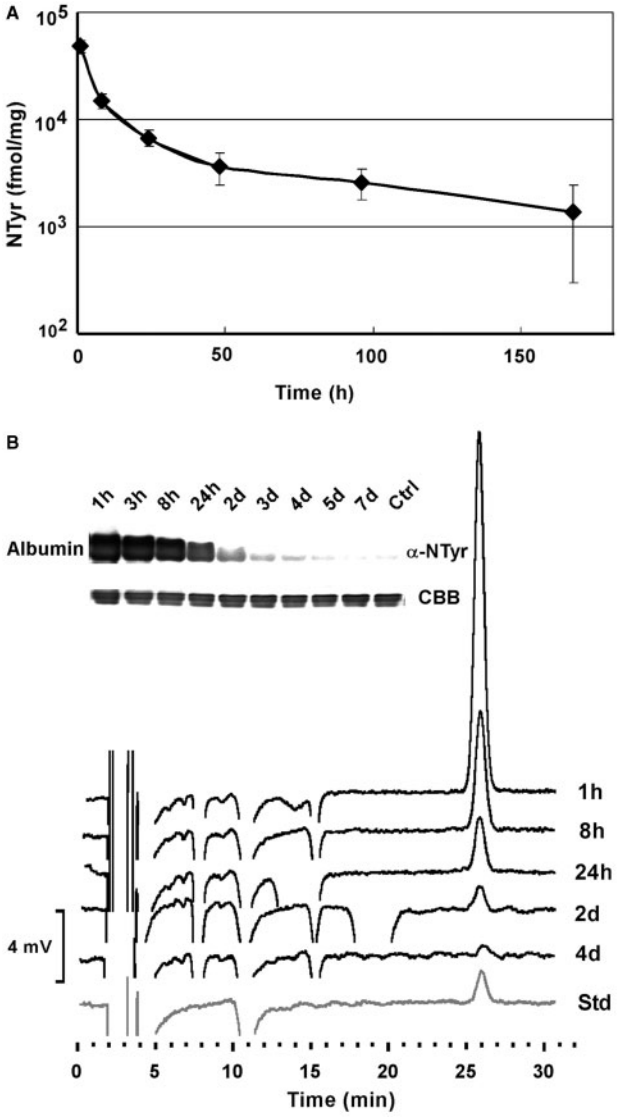

Fig. 5. **Elimination of nitrated plasma protein from the rat circulation.** (A) Semilogarithmic plot of the concentration of NTyr (fmol/mg plasma protein) *vs* time after an i.v. bolus infusion of nitrated plasma protein. The values shown are the mean ± standard deviation ($n = 4$). (B) Representative chromatograms are from one rat after administration of nitrated plasma protein. The same samples were subjected to western analysis for nitrated albumin with an anti-NTyr antibody. As control, the same samples were electrophoresed in parallel, stained with coomassie brilliant blue (CBB), and shown in their albumin bands.

To optimize the protocol, we tested more than 20 conditions for HPLC, and found that a single peak in the LPS-treated rat plasma always had the same retention time as for the NTyr standard (ranging from 8 to 33 min). We also found that the ratio of the peak in LPS-treated rat plasma and that of NTyr standard became constant, when the pH of the mobile phase was decreased to <3.0. We believe that this indicates successful separation of NTyr from other components in plasma, which have similar retention times as NTyr under the conventional assay conditions. Further evidence was provided during the optimization of the potentials applied to ECD, which showed that these peaks share the same electrochemical

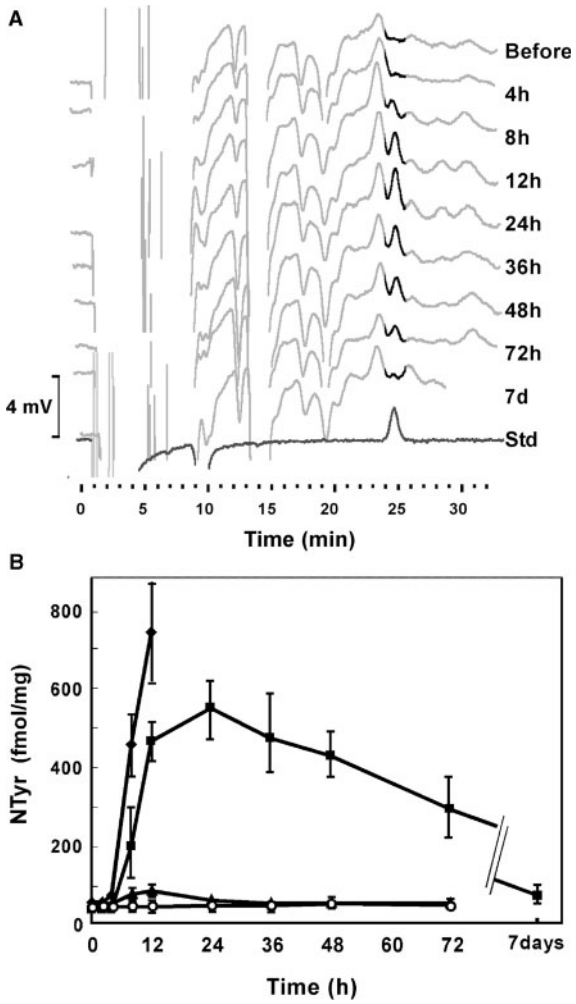

Fig. 6. **The time course of protein-bound NTyr in rat plasma after LPS administration.** (A) Chromatograms of HPLC–ECD to measure NTyr concentration at pH 2.5. Rat blood samples were collected from the tail vein before, 4, 8, 12, 24, 36, 48 and 72 h: and 7 days after LPS administration (5 mg/kg). Discrete peaks of NTyr were detected at the same retention time for the NTyr standard (Std). (B) Time course of protein-bound NTyr in rat plasma. Four groups of animals ($n = 3$ each) were administered 0 (○), 0.5 (△), 5 (□), or 10 (◇) mg/kg LPS, and blood samples were collected at each time point. The concentration of NTyr determined with HPLC–ECD. The values shown are the mean ± standard deviation. At the highest dose of LPS (10 mg/kg), two of the three animals died within 24 h.

properties because the peak ratio remained constant over a wide range of applied potentials. Therefore, we conclude that the combination of the mobile phase at pH 2.5 and decreased applied potentials to ECD achieves successful separation of NTyr in digested rat plasma protein. Interestingly, the differences in the concentration of NTyr in rat plasma with or without LPS treatment were comparable at pH 2.5 (239.6 fmol/mg) and at pH 4.3 (284.4 fmol/mg) (see Fig. 4). We speculated that this indicates proper separation of the NTyr peak from other components in plasma samples.

The use of a highly acidic mobile phase is a prerequisite for accurately quantifying NTyr concentration using HPLC–ECD. However, under the strongly acidic condition, tyrosine is nitrated by nitrite often present in biological samples (*32*, *33*). For example, normal human serum contains 20 μM nitrite. To deny the possible formation of NTyr as an artefact, we added sodium nitrite (50 μM) to digested control rat plasma protein and injected the mixture into the HPLC–ECD system with a mobile phase at pH 2.5. The digestion product of rat plasma protein contains 3 mM free tyrosine. We detected no artefactual NTyr even after incubating the mixture for 1 h at room temperature. Because that the plasma protein had been precipitated and washed twice with nitrite-free acetonitrile/acetate buffer, the sample was kept at pH 7.4 throughout sample preparation, and the separation of tyrosine and nitrite immediately started in the separation column, artefactual NTyr formation within the HPLC line with acidic mobile phase was below detectable level in our protocol.

The amount of protein-bound NTyr was significantly lower and the measurement more reproducible at pH 2.5 than at pH 4.3. It was 100–1000 fmol/mg in rat plasma protein at pH 2.5. Since the concentration of tyrosine in the same samples was about 0.3 μmol/mg plasma protein, the molar ratio of NTyr to tyrosine was 2.0 ± 0.19 μmol of NTyr/mol of tyrosine in plasma protein from rats treated with 5 mg/kg of LPS and 0.37 ± 0.15 μmol of NTyr/mol of tyrosine in untreated rats. Although we should consider the tyrosine released from pronase itself (1 mg of pronase to 5 mg of plasma protein), our results are the lowest values ever reported using HPLC–ECD to measure NTyr, except for the estimation by Shigenaga *et al*. (*19*). They used a sophisticated method to convert NTyr to N-acetyl-3-aminotyrosine before injecting the sample into HPLC–ECD. Their estimation is comparable to our results. In addition, our results are also similar to 4–18 and 1.55 μmol of NTyr/mol of tyrosine measured by LC-tandem MS (25) and GC-tandem MS (34), respectively. Cumulatively, our data suggest that the amount of protein-bound NTyr in normal rat plasma may be less than 1 μmol of NTyr/mol of tyrosine. However, definitive identification of a compound(s) in the peak of our interest by other methods e.g., mass spectrometry coupled with the LC or GC, is warranted.

The *in vivo* half-life of free NTyr is 68.5 ± 18.4 min, after a single bolus dose of free NTyr (*35*). However, little information is available on the half-life of protein-bound NTyr. We analysed the elimination of *in vitro*-nitrated plasma protein from the rat circulation, and measured the *in vivo* half-life of nitrated plasma protein as 63.4 ± 16.8 h during the prolonged elimination phase. The western analysis of nitrated albumin showed similar elimination kinetics. The half-life of normal albumin is 18 to 21 days, and the nitrated albumin had a much shorter half-life than its normal counterpart did. The reason for the faster elimination of nitrated albumin is unknown. Because the protein was nitrated at pH 3.0 *in vitro*, it is possible that nitrated plasma proteins are recognized and eliminated by a similar mechanism to that, which scavenges denatured plasma protein (*36*). It is likely that the actual half-life of *in vivo*-nitrated plasma protein is no shorter than the value we observed.

These results provide solid evidence that protein-bound NTyr persists long enough to be a biomarker for *in vivo* RNS production. We found consistently that protein-bound NTyr persists in rat plasma for a week after administration of 5 mg/kg LPS. Although the NTyr concentration in plasma depends on the balance between its production and elimination, the gradual decline of NTyr concentration, starting from 24 h after LPS administration, may indicate a constant rate of elimination of *in vivo*-nitrated protein from the circulation. To determine the fate of protein-bound NTyr *in vivo*, it is necessary to identify which protein species are nitrated after LPS administration.

In rat plasma protein, the abundance of protein-bound NTyr was <10 NTyr residues per 1×10^6 tyrosine residue. Despite of its rarity, recent studies demonstrate the biological importance of tyrosine nitration, which is a highly site-specific reaction. MacMillan-Crow *et al*. (*37*) demonstrates that nitration of manganese superoxide dismutase (Mn-SOD) completely inhibits its enzymatic activity. Clinically, the inhibition of Mn-SOD activity by iNOS-dependent nitration was reported in acutely rejecting cardiac transplants (*38*). The site-specific protein nitration also affects the function of apolipoprotein A-1 (*39*, *40*), angiotensin II (*41*), α-synuclein in Parkinson's disease (*42*), α-enolase in a transgenic mouse model of familial amyotrophic lateral sclerosis (*43*). Slight alteration of the protein-bound NTyr level in plasma protein might reflect an important aspect of the RNS generation and metabolism.

In conclusion, we have developed a new protocol to quantify protein-bound NTyr, and have demonstrated the time course of NTyr disappearance from plasma after administration of *in vitro*-nitrated protein or LPS. Because our new protocol does not require elaborate sample purification or derivatization procedures, it is suitable for quantification of NTyr in multiple samples, and might contribute to our understanding of the importance of RNS generation in disease processes.

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