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Lipopolysaccharide-induced increase in plasma nitrotyrosine concentrations in rats

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Abstract

Since the production of peroxynitrite may contribute to the pathophysiology of endotoxemia or sepsis, the quantities of the produced peroxynitrite were evaluated in rats after lipopolysaccharide (LPS) treatment by measuring plasma nitrotyrosine concentrations with a new method. The intraperitoneal administration of LPS caused a persistent increase in plasma nitrotyrosine concentrations, which reached a maximum with 6-fold level of the base line (105 pmol ml^{-1}) at 24 h and gradually declined to 3-fold level of the base line at 7 days. However, plasma concentrations of nitrite and nitrate peaked at 18 h, returning to base line within 48 h. The effect of LPS on the increase in plasma concentration of nitrotyrosine was dose-dependent and consistent with that of nitrite and nitrate concentrations. On the other hand, intravenous injection of nitrotyrosine revealed a rapid clearance with a plasma half-life of 1.67 h. These results indicate that the elevation of plasma nitrotyrosine concentrations may persist for more than a week after LPS treatment, and that the determination of plasma nitrotyrosine concentrations may be useful to detect the previous peroxynitrite-dependent oxidative damages. © 1997 Elsevier Science B.V.

Keywords: Nitrotyrosine; Lipopolysaccharide; Nitrate; Nitrite; Peroxynitrite; Endotoxemia

1. Introduction

Nitric oxide (NO) is a short-lived cytotoxic mediator that has been implicated in the pathogenesis of endotoxin-induced tissue injury and septic shock [1–3]. Administration with bacterially derived lipopolysaccharide (LPS) causes the induction of nitric oxide synthase isoform (type II), resulting in the production of large quantities of NO in many cells including macrophage and vascular smooth muscle [4–6]. The subsequent interaction of NO with superoxide (O_2^-) forms peroxynitrite ($ONOO^-$), a powerful oxidant capable of causing pathological damages in conditions such as endotoxemia or sepsis, inflammation, atherosclerosis, and ischemia-reperfusion [7–11]. Peroxynitrite can decompose to give various products that nitrate aromatic amino acids existing as free forms or components of proteins. Nitration on the *ortho* position of tyrosine is a major reaction. Therefore, the plasma concentration of nitrotyrosine (NO_2–Tyr) may reflect the degree of peroxynitrite (NO and superoxide)-dependent damages around vascular tissues, since it is difficult to demonstrate the other oxidative products of peroxynitrite [7–10,12,13]. However, there is a possibility that plasma tyrosine

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may be nitrated by the peroxidase–hydrogen peroxide system during inflammatory processes [14].

Although the elevated plasma levels of NO_2–Tyr in patients of rheumatoid arthritis were detected by reversed-phase high-performance liquid chromatography (HPLC) with a direct measurement of its own ultraviolet absorbance (274 nm), normal levels of NO_2–Tyr in human plasma were below the detection limit of 0.2 μM [7,8]. Thus, we have developed a highly sensitive and simple HPLC method for determination of plasma concentrations of NO_2–Tyr with a precolumn derivatization of the amino acid by 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) [15].

Therefore, in the present experiments, we investigated time- and dose-dependent alterations of plasma NO_2–Tyr concentrations after LPS treatments in rats, compared to the changes in plasma concentrations of nitrite and nitrate (the stable oxidation products of NO), in order to clarify whether plasma NO_2–Tyr concentration may be an useful marker for peroxynitrite (NO and superoxide)-dependent tissue damages.

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide (serotype 055:B5), 3-nitro-L-tyrosine (NO_2–Tyr) and α-methyl-L-*p*-tyrosine were purchased from Sigma (St. Louis, MO). 4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) was from Dojindo (Kumamoto, Japan). Other chemicals were of analytical reagent grade and obtained from Wako (Osaka), Cica-Merck (Tokyo) and companies listed above.

2.2. LPS treatment and sample preparation

Male Wistar rats (200–250 g) were obtained from SLC (Shizuoka, Japan) and allowed to acclimate to their surroundings for 1 week before experiments. The experiments were conducted in the accordance with the guideline for animal experimentation in Faculty of Medicine, Tottori University. LPS was dissolved in the sterile saline and administered intraperitoneally into rats at a dose of 10 mg kg^{-1} of the body weight or the designated dose. Under pentobarbital anesthesia (50 mg kg^{-1}), blood samples (ca. 200 μl) were obtained by cardiac puncture 15 h or the indicated time after the LPS treatment and collected in microfuge tubes containing heparin (40 U ml^{-1}). A part of plasma (60 μl) was mixed with ethanol (140 μl) containing methyltyrosine (150 pmol), as an internal standard. After the centrifugation at 10000 × g for 10 min, the resulting supernatant was kept at −80°C until the chromatographic analysis for NO_2–Tyr. The remaining plasma was used for assay of nitrite and nitrate concentrations.

In a clearance experiment, authentic NO_2–Tyr (7.2 μmol kg^{-1}) was intravenously given into rats from a tail vein. Blood samples were taken 3, 6, 12, 24, and 36 h after the injection, and treated as described above.

2.3. Determination of plasma concentrations of NO_2–Tyr, nitrite and nitrate

Plasma NO_2–Tyr concentrations were determined as described previously [15]. Briefly, the stored samples (100 μl) were derivatized with NBD-F (1 mg) in 0.1 M sodium borate buffer (pH 8.7) at 60°C for 2 min. After the addition of 0.1 M HCl, aliquot (100 μl) was injected onto a reversed-phase column (Wakosil 5C18, 250 × 4.6 mm ID, Wako), which was eluted with 0.1 M sodium phosphate buffer (pH 7.2): methanol (45:55, v/v). Calculation of NO_2–Tyr concentration in each sample was based on the ratio of the fluorescent intensity of NO_2–Tyr to that of methyltyrosine, an internal standard. The values are expressed as pmol per ml of plasma.

Plasma nitrite and nitrate concentrations were colorimetrically determined by using the Griess reaction of a nitrite–nitrate assay kit-C (Dojindo, Kumamoto, Japan) according to the attached literature. Total NO_x (nitrite and nitrate) and nitrite concentrations were obtained with and without the treatment of nitrate reductase, respectively. Nitrate concentrations were calculated from the equation ([nitrate] = [total NO_x] − [nitrite]). The concentrations are expressed as nmol per ml of plasma.

2.4. Statistical analysis

The result of the experiments is expressed as the mean ± SD. For the statistical analysis, Tukey's test

was adopted to compare the source of data obtained from multiple groups after ANOVA was performed. Differences were considered significant when P values were smaller than 0.05.

3. Results and discussion

3.1. Time-dependent effects of LPS treatment

Administration of LPS (10 mg kg^{-1}) elicited a persistent increase in plasma NO_2–Tyr concentrations in rats. The plasma NO_2–Tyr concentration increased by 4–5 fold over the first 48 h, and gradually declined to the level of 3-fold of the basal level (105 ± 37 pmol ml^{-1}) at 7 days after the LPS treatment (Fig. 1A). The basal concentration of NO_2–Tyr corresponded to ca. 0.1% of the plasma tyrosine levels in rats. The elevations of NO_2–Tyr concentrations were significant ($P < 0.01$) at any time after LPS treatment, compared to the basal level. However, the increase in plasma NO_x concentration was transient. The plasma NO_x concentration peaked at 12 h, returning to the base line (54.1 ± 22.6 nmol ml^{-1}) within 48 h after (Fig. 1B). As shown in Fig. 2, [nitrite] comprised 20 to 30% of [total NO_x] (the remainder being [nitrate]).

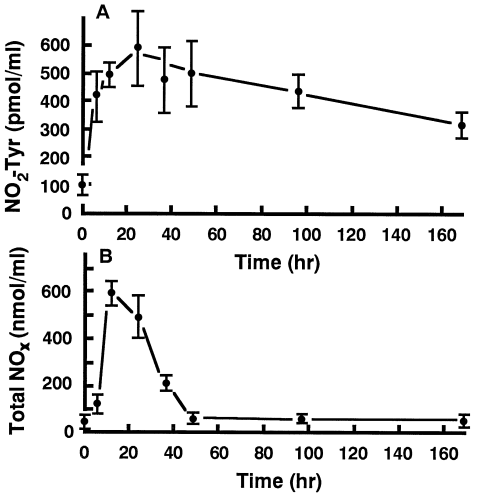

Fig. 1. Time course of plasma concentrations of nitrotyrosine (NO_2–Tyr) and total nitrite and nitrate (NO_x) after lipopolysaccharide (LPS) treatment. LPS (10 mg kg^{-1}) was administered intraperitoneally into rats at time zero, followed by determining plasma concentrations of NO_2–Tyr (panel A) and total NO_x (panel B) at the designated times using HPLC and the Griess reaction, respectively. Data are expressed as the mean ± SD; $n = 4$–9.

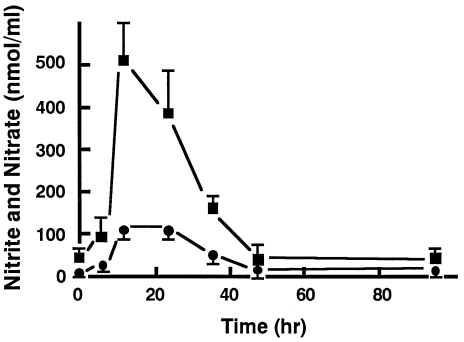

Fig. 2. Time course of LPS-induced increases in plasma concentrations of nitrite (circles) and nitrate (squares) in rats. After LPS injection, plasma concentrations of nitrite and total NO_x were measured by the Griess reaction without and with nitrate reductase-treatment, respectively. Nitrate concentrations were calculated by the subtraction of nitrite from the total NO_x. Data are expressed as the mean ± SD; $n = 4$–9.

3.2. Dose-dependent effects of LPS treatment

In order to detect the maximum effects on plasma concentrations of both NO_2–Tyr and NO_x, blood was taken 15 h after LPS administration at a dose of 0, 1, 3.16, or 10 mg kg^{-1}. Fig. 3 shows approximately parallel effects of LPS on both plasma concentrations, although their effects were not saturated due to the toxicity of LPS. The data indicate that NO_2–Tyr

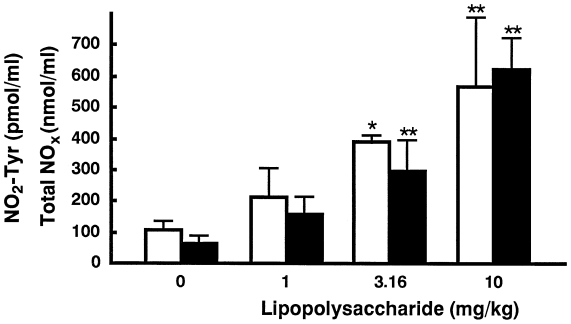

Fig. 3. Dose-dependent effects of LPS on plasma concentrations of NO_2–Tyr and total NO_x. 15 h after the injection of LPS (0–10 mg kg^{-1}), plasma concentrations of NO_2–Tyr (open columns) and total NO_x (shaded columns) were determined. Data are expressed as the mean ± SD; $n = 4$–6. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with that of vehicle treatment (LPS, 0 mg kg^{-1}).

and NO_x may be derived from the same substance that LPS treatment produced through the increase in NO from the induced NO synthase in various cells [4–6]. Since bacterial toxins stimulate leukocytes to generate superoxide radicals in addition to NO, a part of the generated NO reacts with superoxide anions to produce peroxynitrite, which subsequently nitrate tyrosine existing as a free from or protein-residue [7,12,13]. Therefore, it is necessary to clarify whether the discrepant results during elimination phases of NO_2–Tyr and NO_x may be attributed to the differences in their clearance rates in plasma.

3.3. Clearance of NO_2–Tyr in plasma

In order to investigate a clearance rate of NO_2–Tyr in rat plasma, the plasma concentrations were measured after intravenous injection of authentic NO_2–Tyr (7.2 μmol kg^{-1}). Pharmacokinetic analysis of data in Fig. 4 revealed that the plasma half-life of NO_2–Tyr was 1.67 ± 0.17 h, which is consistent with those of physiological amino acids and amino acid-related drugs such as levodopa and methyldopa [16–18]. However, plasma NO_2–Tyr concentrations during the declining phase (Fig. 1A) indicate that NO_2–Tyr is eliminated from rat plasma with a half-life of 7.8 ± 0.7 days, which is similar to those of plasma proteins such as albumin (1–2 weeks) [19,20]. Since peroxynitrite gives adducts of nitro group to tyrosine residues in proteins, in addition to free tyrosine, the degradation of nitrated plasma proteins may explain the delayed clearance of plasma NO_2–Tyr. However, nitrated proteins are reported to be degraded more rapidly than non-nitrated proteins [21]. Moreover, there is a possibility that the elimination system of plasma NO_2–Tyr may be disturbed by the LPS treatment. Therefore, the persisted elevation of plasma NO_2–Tyr concentrations after LPS treatment may be due to continuous supplement from the degradation of nitrated plasma proteins and due to the slow clearance induced by the oxidative stress, although further experiments are necessary to clarify the mechanism.

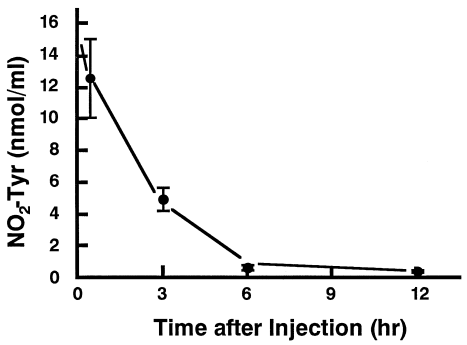

Fig. 4. Plasma clearance of NO_2–Tyr. After NO_2–Tyr (7.2 μmol kg^{-1}) was intravenously given into rats, the plasma concentrations were determined at the indicated time. Data are expressed as the mean ± SD; $n = 8$. Each pharmacokinetic analysis demonstrated the plasma half-life (1.67 ± 0.17 h) and distribution volume (0.386 ± 0.060 l kg^{-1}) of NO_2–Tyr.

3.4. Conclusion

Although plasma NO_2–Tyr is formed not only by peroxynitrite-dependent mechanism but also by the oxidation of nitrite through peroxidase–hydrogen peroxide system [14], it is accepted that NO_2–Tyr is produced during the peroxynitrite-dependent oxidation processes such as endotoxemia or sepsis, inflammation, atherosclerosis, and ischemia-reperfusion [7–11]. In addition, the existence of plasma metabolite of NO_2–Tyr may indicate that the plasma concentration of NO_2–Tyr may not reflect the whole nitration products derived from peroxynitrite [22,23]. Therefore, the present results suggest that a single treatment of LPS causes a sustained increase in plasma NO_2–Tyr for more than a week, although plasma NO_x returned to the basal level within 48 h, and that the measurement of plasma NO_2–Tyr concentration may be useful for the past evidence of peroxynitrite (NO and superoxide)-dependent oxidation stress including endotoxemia or sepsis.

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