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Determination of Nitrotyrosine by HPLC-ECD and Its Application

Nobushige ISHIDA[1)], Telhisa HASEGAWA[1)], Kazutaka MUKAI[1)], Masanobu WATANABE[2)] and Hirohito NISHINO[2)]

[1)]Equine Research Institute, Japan Racing Association, 321–4 Tokami-Cho, Utsunomiya 320–0856 and [2)]Eicom Corporation, 24–2 Shimo Toba Enmenda-Cho, Fushimi-ku, Kyoto 612–8474, Japan

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ABSTRACT. 3-nitrotyrosine, a product of tyrosine nitration, is a useful indicator of oxidative damage. We modified the previously reported HPLC-electrochemical detection (ECD) method: specifically, a through-type porous carbon electrode was used as a reducing electrode instead of the mercury-gold amalgam electrode, because the response of the latter changes over time. A combination of reverse-phase HPLC and electrochemical detector passed through –800 mV reduction potential and subsequently under +250 mV oxidation potential allows measurement of 3-nitrotyrosine. The detection limit of this assay was less than 10 fmol. In mice to which lipopolysaccharide (LPS) was administered intraperitoneally, plasma 3-nitrotyrosine levels were elevated, corresponding to LPS dosage. These findings suggest that the improved HPLC-ECD method can be used as a specific and sensitive assay of biological 3-nitrotyrosine and can be applied clinically.

KEY WORDS: HPLC-ECD, LPS, 3-nitrotyrosine, peroxynitrite.

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In recent years, the biological activity of nitric oxide (NO) has been actively investigated [2, 6]. Although NO is a relatively stable inorganic radical, its chemical reactivities differ from those of oxygen radicals. Biologically produced NO is subjected to various oxidation reactions, eventually taking the forms of relatively stable ions such as NO_2^- and NO_3^-. However, during these processes, NO also reacts with coexisting molecular oxygen (O_2), oxygen radicals (active oxygen), or heavy metals (Fe, Cu) to produce highly reactive nitrogen oxides that exert actions different from NO.

Some pathways associated with NO signal transduction are cGMP dependent, while others are not cGMP dependent. The former includes signal transduction mechanisms such as endothelium-dependent relaxation [2]. In the latter group, rather than NO itself, nitration and nitrosylation caused by NO-derived reactive NOx, such as peroxynitrite ($ONOO^-$), N_2O_3, and N_2O_4 (NO_2) are involved in a wide variety of biological phenomena [1, 4, 11].

Several recent studies have shown that the nitration of tyrosine, caused by compounds such as $ONOO^-$, is closely involved with intracellular and intercellular signal transduction and cell death by suppressing biological phosphorylation and inducing apoptosis. Therefore, establishing an assay for biologically-synthesized 3-nitrotyrosine is important not only for ascertaining the degree of the biological synthesis of reactive nitrogen oxides such as $ONOO^-$, but also for analyzing the biological activity of these reactive nitrogen oxides.

The HPLC-electrochemical detector has been used to detect and measure various biological substances due to a detection sensitivity comparable to that of fluorescence detectors, and because it maintains linearity across a wide range of concentrations [9]. Among the studies on 3-nitrotyrosine detection utilizing this method [3, 7, 9, 10], the dual-mode electrochemical detection method reported by Sodum *et al.* [10] has the highest sensitivity. However, with their method, responses tend to change the mercury surface periodically and redraw the calibration curve. As a result, continuous use is not possible with their method . With the method of Oshima *et al.* [7] in which a platinum column is used to reduce 3-nitrotyrosine to 3-aminotyrosine, as the catalytic capability of the platinum column decreases, its reducing ability also declines. To overcome these disadvantages, plasma 3-nitrotyrosine detection was investigated using the HPLC-electrochemical detection (HPLC-ECD) method, using a through-type porous carbon electrode as a reducing electrode, which allows continuous usage.

When the whole body or cells is exposed to LPS, excessive amounts of NO and O_2^- are produced. These molecules react to produce $ONOO^-$, inducing the nitration of tyrosine and subsequently the production of 3-nitrotyrosine [8, 12, 13]. Since endotoxin shock sometimes poses a problem in racing horses, we administered lipopolysaccharide (LPS) to mice and investigated the relationship between LPS dosage and 3-nitrotyrosine production by the present HPLC-ECD method.

MATERIALS AND METHODS

Nitrotyrosine detection system: Nitrotyrosine was detected by HPLC-EC detector in which a through-type (porous carbon) electrolytic cell and a glassy carbon amperometric electrochemical detector were aligned serially (Eicom). A SC-5ODS 3 × 150 mm column (Eicom) was used as the separating column. Nitrotyrosine in the column eluate was subjected to electrolytic reduction in the through-type electrolytic cell. The resulting products were subjected to oxidation in the amperometric electrochemical detector.

Standard samples: Commercially available 3-nitrotyrosine (Sigma) was dissolved in 0.01 M PBS (pH: 7.2) to prepare 10^{-6}–10^{-10} M solutions.

The tested parameters included: Reduction voltage, oxidation voltage, mobile phase pH, and methanol concentration.

Protein sample preparations: Commercially available BSA (Sigma) and BSA treated with peroxynitrite (Calbiochem) were adjusted (protein concentration: 2.5 mg/m*l* and protease [PRONASE, Calbiochem] concentration: 0.5 mg/m*l*) and subjected to hydrolysis at 50°C for 18 hr.

Animal experiments:

1) Animals: Sixteen 8-week-old male BALB/c mice were used.

2) LPS administration: Either 0, 1, 5, or 10 μg/g.B.W. of LPS (Sigma), dissolved in physiological saline, was administered intraperitoneally to four mice.

3) Sample preparation

At 15 hr after LPS administration, a whole blood sample was collected and heparinized plasma obtained by centrifugation. Following the addition of 22.5 μl of plasma, 422.5 μl of 0.01 M PBS (pH: 7.2), and 50 μl of PRONASE (10 mg/m*l*), the resulting solution was subjected to hydrolysis at 50°C for 18 hr. After the completion of hydrolysis, 500 μl of the resulting solution was centrifuged to remove very little amount of not-reacted proteins and other debris at 4°C and 15,000 g for 10 min using an ultrafiltration unit, Vivaspin 500 (VIVASCIENCE, molecular cutoff, 10000). Then 50 μl of the centrifuged eluate was subjected to HPLC-EC detector to quantify 3-nitrotyrosine.

RESULTS AND DISCUSSION

Detection of 3-nitrotyrosine: The optimal HPLC conditions were determined with standard 3-nitrotyrosine. The mobile phase was a 100 mM phosphate buffer solution (pH: 5.0) to which various concentrations of methanol were then added. The results indicated that the separation of 3-nitrotyrosine from plasma components was most favorable at a methanol concentration of 5%. The flow rate of the mobile phase was set at 0.5 m*l*/min and the column temperature at 25°C. Following HPLC, the optimal electrochemical reduction voltage was shown to be –800 mV vs Au and the optimal oxidation voltage to be +250 mV vs Ag/AgCl (Fig. 1).

Figure 2 shows the relationship between 3-nitrotyrosine concentrations and peak areas with an oxidation voltage of +250 mV. The results showed that the detection limit of the present assay, at less than 10 fmol, to be remarkably sensitive. Its sensitivity was better than that reported by Sodum *et al.* [10], and continuous measurement was possible. Its detection specificity was also assessed by examining the effects of reduced voltage on peaks. Since 3-nitrotyrosine can be detected using a low oxidation voltage of +250 mV, we were able to establish a stable and reproducible assay with reduced coexisting peaks. In order to detect 3-nitrotyrosine bound to other amino acids, it is necessary to break down proteins into amino acids. We used protease to hydrolyze proteins. Figure 3 shows the results of HPLC-ECD conducted under various conditions: a) BSA was hydrolyzed using protease (control); b) BSA, treated with $ONOO^-$ was hydrolyzed using protease; and c) the ECD response of the standard 3-nitrotyrosine. These findings show that 3-nitrotyrosine can be isolated and detected finely even in the presence of other amino acids.

LPS administration to mice: Plasma samples collected from mice receiving LPS intraperitoneally were subjected to hydrolysate using protease and then subjected to HPLC-ECD (Fig. 4). Figure 5 shows the relationship between the LPS dose and the 3-nitrotyrosine production. Higher LPS doses tended to result in greater 3-nitrotyrosine production, consistent with the severity of the clinical conditions of the

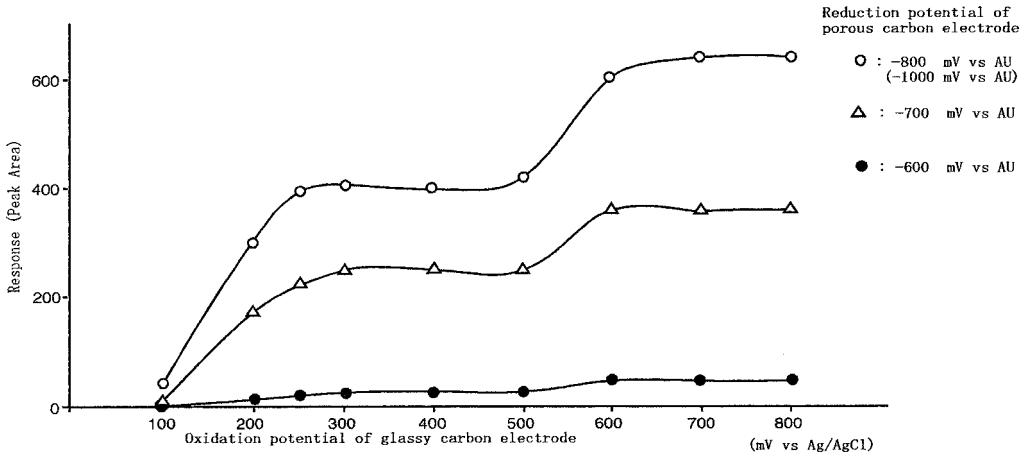

Fig. 1. Hydrodynamic voltammograms of 3-nitrotyrosine. Samples of fixed volume and concentration of 3-nitrotyrosine standard were submitted repeatedly to reverse-phase HPLC-ECD, with varying potentials applied to either the upstream or downstream electrodes. Responses of the upstream porous carbon electrode at various reduction potentials and the downstream glassy carbon electrode at various oxidation potentials were plotted. The mobile phase was a 100 mM phosphate buffer solution (pH: 5.0) containing methanol concentration of 5%.

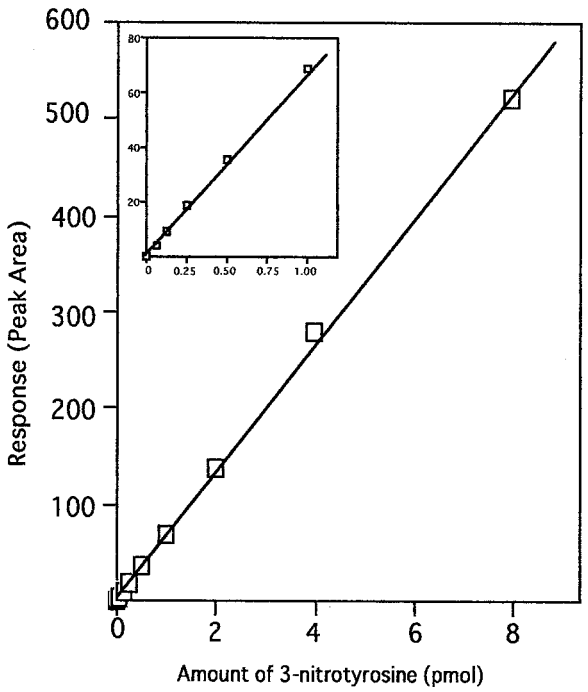

Fig. 2. HPLC-ECD response to serial dilution of 3-nitrotyrosine obtained with applied potential of +250 mV.

mice. One study reported that cells and tissues produced more NO and O_2^- when treated with higher doses of LPS [5]. NO and O_2^- induce the production of $ONOO^-$, which then induces tyrosine nitration. Thus, it is possible to estimate the severity of oxidative damage caused by the excessive production of $ONOO^-$ by measuring the level of 3-nitrotyrosine productions.

In conclusion, the present study showed that the highly specific and sensitive HPLC-ECD nitrotyrosine assay makes it possible to estimate the severity of biological oxidative damage by measuring the level of 3-nitrotyrosine. In the future, we plan to investigate the severity of oxidative damage (using 3-nitrotyrosine as an indicator) in horses suffering from endotoxin shock, colic, or other diseases or in severely fatigued horses after strenuous exercise.

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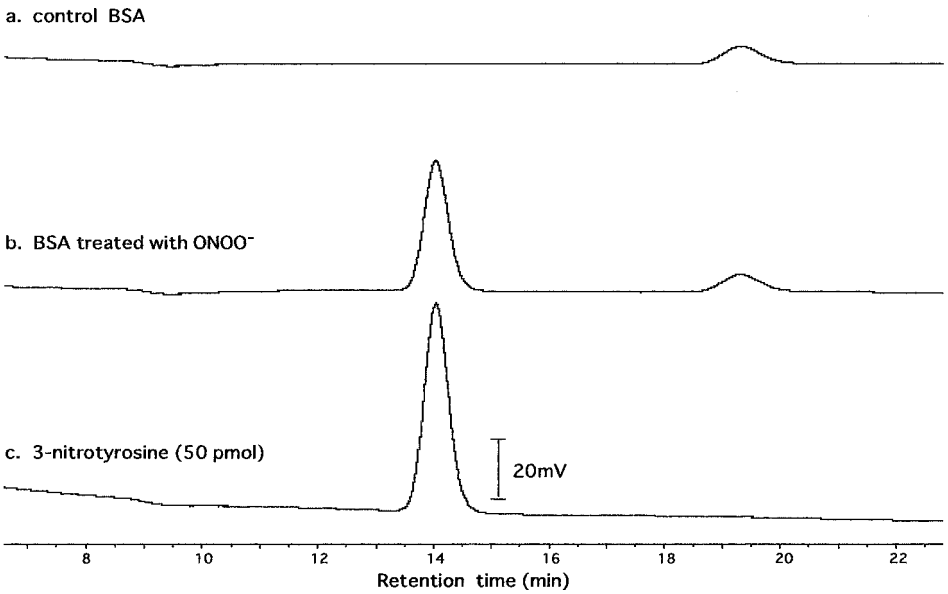

Fig. 3. HPLC-ECD analysis of (a) protease hydrolysate of control BSA, (b) protease hydrolysate of BSA treated with $ONOO^-$ (2 mM), and (c) authentic 3-nitrotyrosine (50 pmol) detected with applied potential of +250mV.

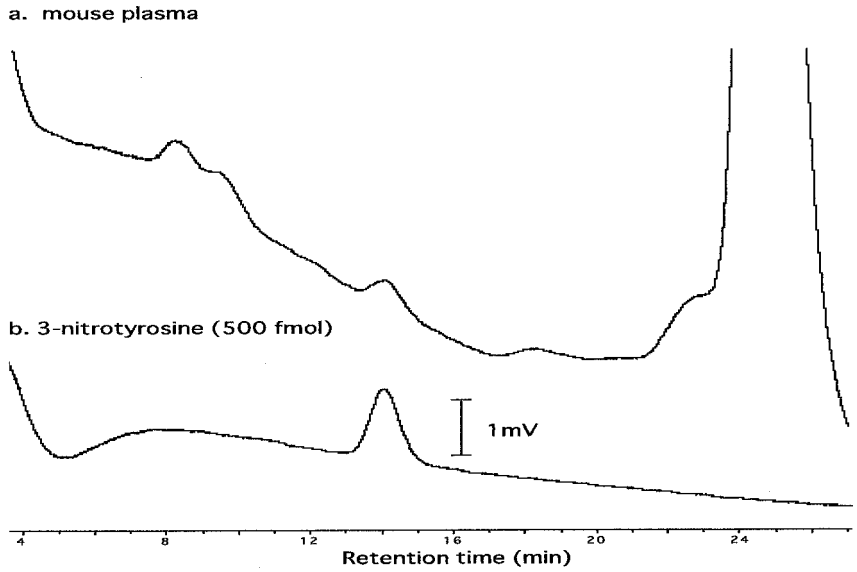

Fig. 4. Nitrotyrosine formation in mouse plasma after the 10 μg/g.B.W. of LPS treatment. (a) protease hydrolysate of plasma analyzed by HPLC-ECD at +250 mV, (b) authentic 3-nitrotyrosine (500 fmol).

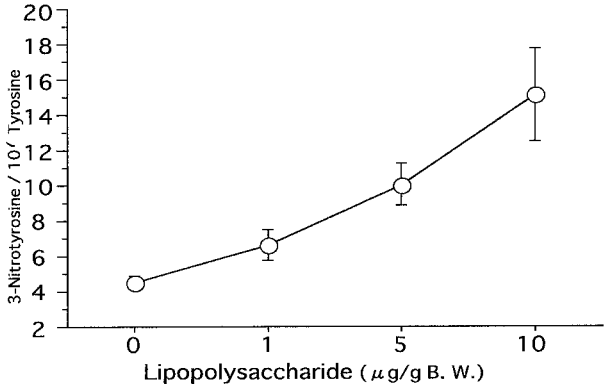

Fig. 5. Levels of 3-nitrotyrosine in the plasma proteins of mice treated with various amounts of LPS .

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