ELSEVIER

Journal of Chromatography B, 676 (1996) 153–158

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Identification of 3-nitro-L-tyrosine, a product of nitric oxide and superoxide, as an indicator of oxidative stress in the human brain

Wakako Maruyama[a], Yoshio Hashizume[b], Kazuo Matsubara[c], Makoto Naoi[d,*]

[a]*Department of Neurology, Nagoya University School of Medicine, Tsurumai-cho, Showa-ku, Nagoya 466, Japan*
[b]*Institute for Medical Science of Aging, Aichi Medical University, Nagakute-cho, Aichi-gun, Aichi, 480-11, Japan*
[c]*Department of Legal Medicine, Shimane Medical University, Showa-cho 89-1, Izumo 693, Japan*
[d]*Department of Biosciences, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya 466, Japan*

Received 20 April 1995; revised 29 August 1995; accepted 19 September 1995

Abstract

3-Nitro-L-tyrosine synthesized from L-tyrosine by peroxynitrite, a product of superoxide and nitric oxide, was identified for the first time in human brains. By quantitative analysis using high-performance liquid chromatography with multi-electrochemical detectors, 3-nitro-L-tyrosine concentration in the gray matter was higher in the cerebrum than in the cerebellum; 0.96 and 0.29 nmol/g wet weight, respectively. On the other hand, L-tyrosine concentration was not different. 3-Nitro-L-tyrosine in the brain may be used as an indicator of oxidative stress induced by reactive oxygen species and nitric oxide.

Keywords: 3-Nitro-L-tyrosine; Nitric oxide; Superoxide

1. Introduction

Nitric oxide (NO) is produced in the endothelial cells and neurons by nitric oxide synthetase and plays important roles in humans under many physiological and pathological conditions. It is known to function as an endothelium-derived vascular relaxing factor (EDRF) or to be involved in the signal transduction in the brain. Recently NO itself or an oxidant derived from NO were proposed to be cytotoxic. NO contains an unpaired electron that can combine with free radicals such as superoxide (O_2^-), and NO and O_2^- produce a strong oxidant, peroxynitrite ($ONOO^-$) in vivo [1]. This reaction is suggested to be involved in the breakdown of NO/EDRF and to reduce vascular relaxation, and thus might enhance tissue ischemia. On the other hand, an O_2^--metabolizing enzyme, superoxide dismutase (SOD), was reported to prolong the half-life of NO/EDRF [2]. $ONOO^-$ is a relatively long-living oxidant with a half life of 1.9 s at pH 7.4, and can diffuse into the cellular targets [3]. $ONOO^-$ decomposes into strong oxidants, hydroxyl radical ($OH\cdot$) and nitrogen dioxide [3], and $ONOO^-$ oxidizes sulfhydryl residues in tissue proteins and inhibits the function of enzymes such as the mitochondrial electron transport chain [4,5]. It was reported that $ONOO^-$ mediates the oxidation of lipoprotein, especially low density lipoprotein, in the arteries and it was supposed to generate atherosclerosis [6,7].

$ONOO^-$ might play a major role in ischemic

*Corresponding author.

0378-4347/96/$15.00
SSDI 0378-4347(95)00400-9

injury by limiting vascular relaxation and direct tissue damage. The brain is known to be especially vulnerable to chronic ischemic injury, and $ONOO^-$ is supposed to be involved in some pathological process by the above-mentioned mechanism. A method for the quantitation of $ONOO^-$ in the brain is needed, however the direct detection of this unstable molecule in human material has been impossible so far.

Recently $ONOO^-$ has been reported to react with L-tyrosine (L-Tyr) to produce 3-nitro-L-tyrosine (NO_2-Tyr) [8], and thus NO_2-Tyr might be applied as a marker for $ONOO^-$ mediated tissue damage in humans. NO_2-Tyr was detected in the atheroma deposits of human coronary arteries by an immunohistochemical technique using anti-NO_2-Tyr antibody [9]. However, quantitative analysis of NO_2-Tyr in the human brain has never been reported.

In this paper NO_2-Tyr was identified in the human brain for the first time and determined by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD). The significance of NO_2-Tyr is discussed in relation to NO-mediated oxidative stress in the brain.

2. Experimental

L-Tyr, NO_2-Tyr and sodium octanosulfonate (SOS) were purchased from Sigma (St. Louis, MO, USA). Three persons without neurological diseases were autopsied 15–30 h after death and the half hemispheres were immediately stored at −80°C until analysis. There were two males and one female and their ages were 74, 68, and 45 years. Gray matter and white matter of the cerebrum and the cerebellum were punched out and used for analysis. The samples were added to 10 volumes per wet weight of 100 m*M* perchloric acid containing 100 μM ethylenediaminetetraacetic acid disodium salt (EDTA) and sodium metabisulfite, and sonicated by a Branson sonicator for 30 s with a 50% duty cycle at a power level of 20 W. Then the sample was centrifuged at 22 000 *g* for 10 min, and filtered through a Millipore HV filter (pore size 0.45 μm). A 70-μl volume of sample was applied to an HPLC apparatus. The HPLC apparatus with an array of sixteen electrodes for ECD (CEAS, ESA, Chelmsford, MA, USA) was used for analysis [10]. Twelve of the sixteen electrodes were set at 60 mV to 720 mV with 60 mV intervals. The mobile phase was 90 m*M* sodium acetate–35 m*M* citric acid buffer, pH 4.35, containing 130 μM EDTA and 460 μM SOS. The column used was a prepacked reversed-phase column, Inertsil ODS-3 (GL Science, Tokyo, Japan, 250×4.6 mm I.D.). The flow-rate was 0.6 ml/min and the temperature was set at 22°C. Peak identification was performed from the retention time and the reactivity to the two or more electrodes with different voltages. To confirm the identification of the NO_2-Tyr, 35 pmol of authentic NO_2-Tyr was spiked into the samples. Using a different HPLC system, the peaks in samples were identified as NO_2-Tyr. The mobile phase was 0.1 *M* sodium-phosphate buffer, pH 3.0, containing 16.7% of methanol and 2 m*M* of SOS and 100 μM of EDTA, and the column used was an Inertsil ODS-3 column (GL Sciences, Tokyo, Japan, 150×4.6 mm I.D.). The flow-rate was 0.5 ml/ml. A coulometric electrochemical detector, Coulochem-II (ESA) was used for the measurement. The voltage of a guard cell, Model 5020, was set at 700 mV and that of first and second electrode of an analytical cell, Model 5010, were set at 500 mV and at 725 to 775 mV, respectively. Quantitation of the compounds was performed by comparison of the peak area to that of the standards.

3. Results

The synthesis pathway of NO_2-Tyr from L-tyrosine reacted with $ONOO^-$ is shown in Fig. 1.

Fig. 2A shows the typical chromatogram of authentic standard solution (0.7 nmol) of L-Tyr (arrow 1) and NO_2-Tyr (arrow 2). L-Tyr reacted widely with the electrodes 10–12 (600–720 mV) and NO_2-Tyr reacted with electrode 12 most strongly. Fig. 2B is the chromatogram of a sample prepared from human cerebrum gray matter. The peaks corresponding to L-Tyr (arrow 1) and NO_2-Tyr (arrow 2) were identified. The detection limit of NO_2-Tyr was 0.062 pmol (S/N=3). A 35-pmol amount of authentic NO_2-Tyr was added to the sample and it was shown to co-elute with the peak. (Fig. 3A and Fig. 3B). The retention time and the relative ratio of the peak areas detected with the electrode 12/11 were

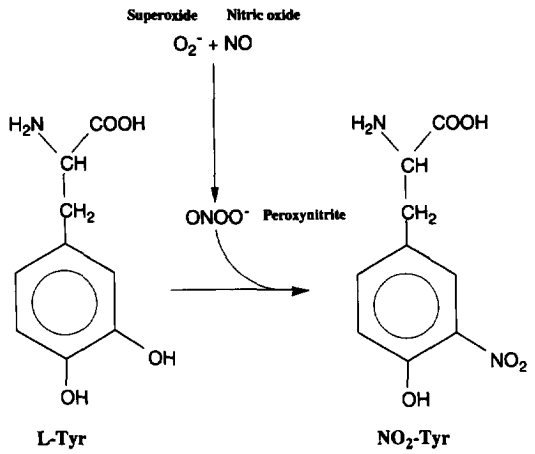

Fig. 1. Synthesis of 3-nitro-L-tyrosine from L-tyrosine and peroxynitrite.

29.98 min and 2.54 in the sample, which corresponded with those of the standard, which were 29.52 min and 3.99. On the other hand, in the cerebellum gray matter the amount of NO_2-Tyr was smaller than that in the cerebrum (Fig. 3C). The concentration of L-Tyr and NO_2-Tyr, and relative concentration of NO_2-Tyr/L-Tyr in the cerebrum and the cerebellum are summarized in Table 1. The concentration of L-Tyr was not different among the four regions in the brain, while that of NO_2-Tyr was higher in the cerebrum than in the cerebellum, but the difference was not statistically significant. The concentration of NO_2-Tyr was almost the same between the gray and white matter in cerebrum and cerebellum, respectively. The samples were analyzed by another HPLC system using a Coulochem-II; the retention time and the relative response set at 750 mV or 725 mV (750 mV/725 mV) were 28.87 min and 2.07 for the samples, and 28.92 min and 2.21 for the standard.

4. Discussion

Recently a HPLC–multi-ECD system was used for analysis of neurotransmitters, such as biogenic amines and neuropeptides, and amino acids, in human serum, urine and cerebrospinal fluid [11]. This analysis system is an improvement on the conventional HPLC-ECD system with a single detector. By use of an array of multi-ECD electrodes, the peak can be identified more precisely, not only by comparison of the retention time but also by the reactivity to the two or more electrodes. In addition, the contaminated compounds, which are oxidized with lower voltages, can be oxidized coulometrically and completely at electrodes set in a series of graded oxidation voltages. The "screening" effects make it easier to separate a compound from the concomitant peaks, especially when the compound reacts against the electrode with high voltage, like NO_2-Tyr and L-Tyr [10].

This paper is the first report to identify NO_2-Tyr in the human brain, and the concentration was estimated to be about 960 n*M* and 280 n*M* for the cerebrum and cerebellum, respectively. By using HPLC with multi-ECD, the compound could be identified easily with a minimum of sample preparation, and the detection limit was lower than in previous reports. By HPLC with ultraviolet detection, the detection limit was reported to be 0.2 μM (14 pmol/70 μl) [12] and by gas chromatography, 2.2 pmol [13]. The production of NO_2-Tyr is dependent on the concentrations of both L-Tyr and nitrat-

Table 1
Concentrations of L-Tyr and NO_2-Tyr in the human brain

	L-Tyr (nmol/g wet weight)	NO_2-Tyr (nmol/g wet weight)	NO_2-Tyr/L-Tyr (%)
Cerebrum			
Gray matter	591±68	0.959±0.024	0.161±0.029
White matter	598±110	0.962±0.017	0.163±0.029
Cerebellum			
Gray matter	526±161	0.285±0.261	0.049±0.035
White matter	367±139	0.276±0.248	0.067±0.042

Each value represents mean±S.D. of triplicate measurements of three samples.

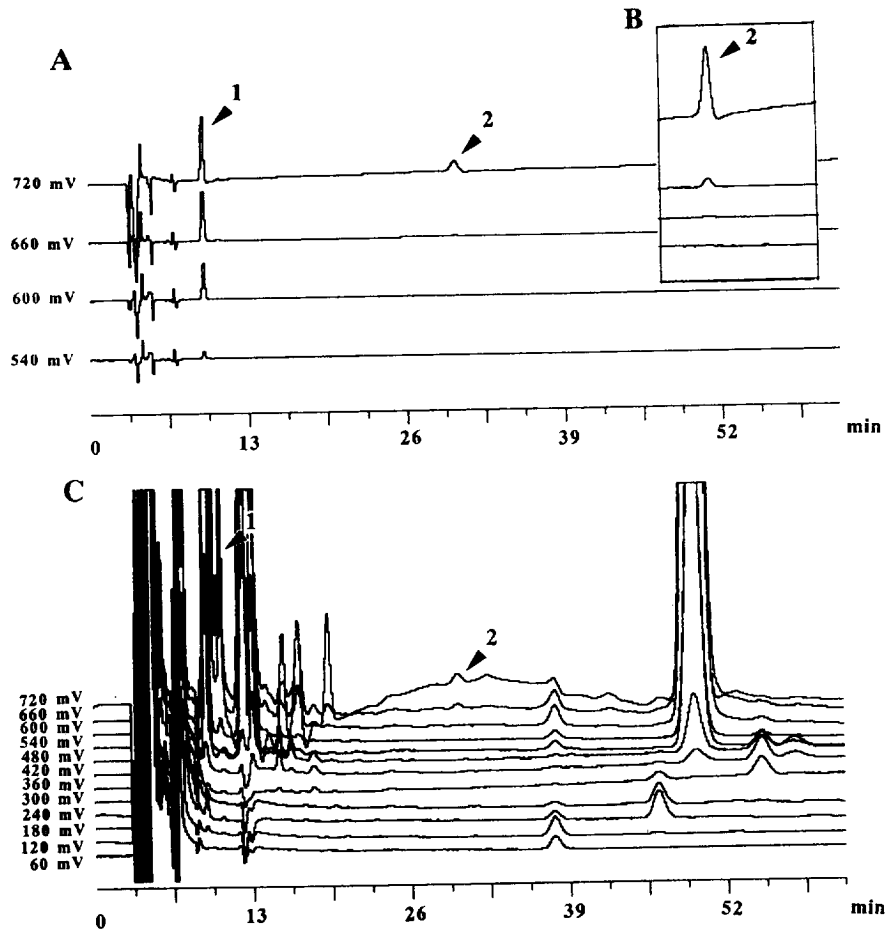

Fig. 2. Chromatograms of standards and samples. (A) and (B) Chromatogram of authentic standard (70 pmol) of L-tyrosine (arrow 1) and 3-nitro-L-tyrosine (arrow 2). Full scale was set at 1 μA (A) or at 200 nA (B). (C) Chromatogram of 70 μl cerebral gray matter sample. Peaks corresponding to L-tyrosine (peak 1) and 3-nitro-L-tyrosine (peak 2). Full scale was set at 100 nA. The amount of 3-nitro-L-tyrosine was calculated to be 7.98 pmol.

ing agents [13]. In the human brain the L-Tyr concentration was calculated to be 350–600 μM (Table 1). The concentration of $ONOO^-$ has never been determined, but the concentration of NO was reported to be 100–400 nM in the endothelium and smooth muscle [14] and that of O_2^- might be much lower [15]. The large difference in the concentration of L-Tyr and these reactive oxygen species suggests that the production of NO_2-Tyr is dependent on the production of $ONOO^-$ from NO and O_2^-. In this study a higher concentration of NO_2-Tyr was detected in the cerebrum, suggesting that $ONOO^-$-mediated oxidative stress may be more severe in the cerebrum than that in the cerebellum. $ONOO^-$ might, therefore, be related to different vulnerability in the brain regions by oxidative damage induced by NO· and O_2^-.

On the other hand, NO-mediated oxidative stress has been suggested to be involved in the tissue damage not only by ischemic injury but also in many autoimmune diseases. $ONOO^-$ is suggested to be the major product of activated macrophage-derived NO [16], and NO synthesis was reported to increase in experimental autoimmune encephalomyelitis in rats, a model for multiple sclerosis in the human [17], and in the model for insulin-dependent diabetes mellitus [18]. Recently it was reported that NO_2-Tyr could be detected in the serum and synovial fluid from some

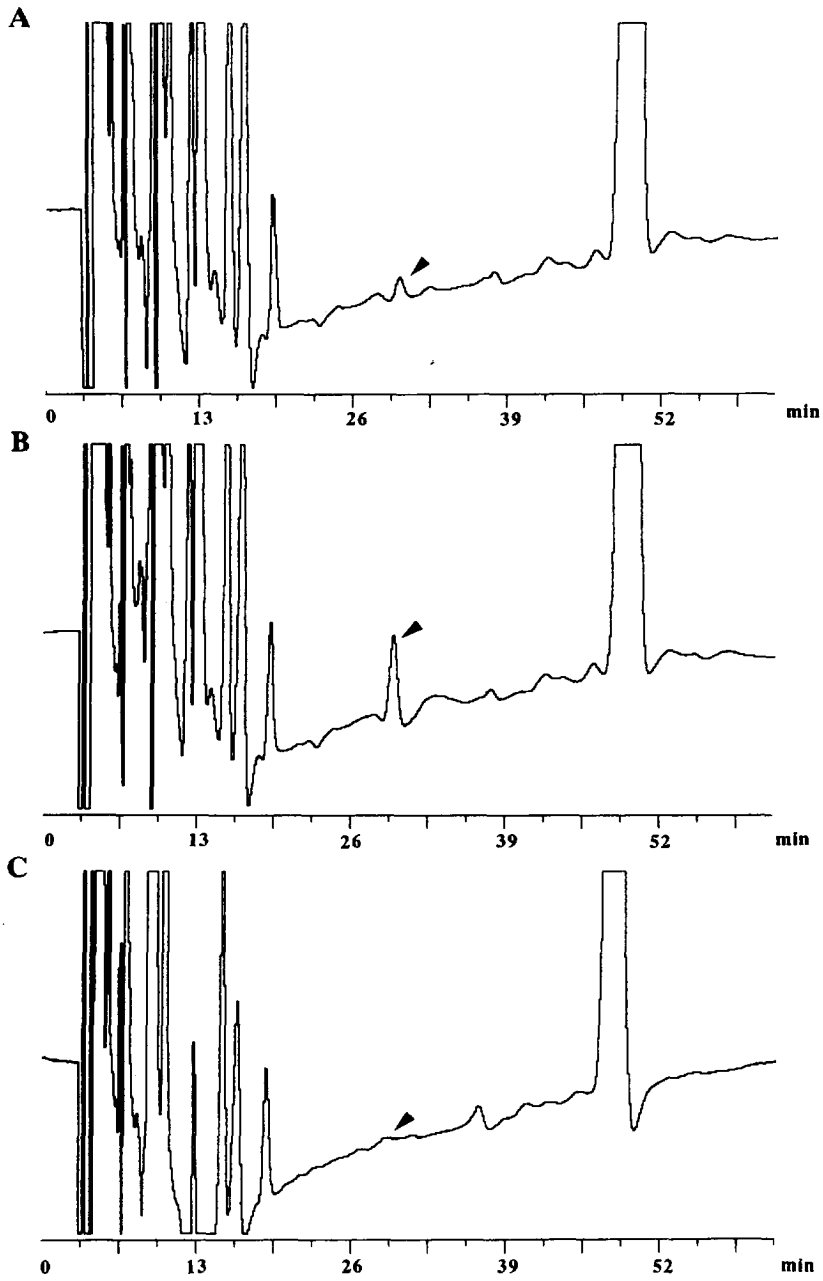

Fig. 3. Chromatogram of electrode 12 (720 mV). Arrow indicates peaks corresponding 3-nitro-L-tyrosine. (A) 70 μl of the sample prepared from cerebral gray matter. The amount of 3-nitro-L-tyrosine was calculated to be 7.98 pmol. (B) The same cerebral gray matter sample spiked with 35 pmol of 3-nitro-L-tyrosine. (C) 70 μl of the sample prepared from cerebellar gray matter. The amount of 3-nitro-L-tyrosine was calculated to be 3.28 pmol.

patients with rheumatoid arthritis, and its concentration was ca. 0.5 μM, but it could not be detected in the serum and synovial fluid from normal subjects [12]. This newly-devised assay method of NO_2-Tyr might improve the diagnosis of these diseases and the evaluation of therapeutic effects because of its

high sensitivity and simple sample preparation. By application of this method, a further study on NO_2-Tyr as an indicator of oxidative stress induced by NO and O_2^- in humans is planned.

Acknowledgments

This work was supported by a Grant-in Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture, Japan.

References

[1] R.E. Huie and S. Padmaja, Free Rad. Res. Commun, 18 (1993) 195.
[2] M.E. Murphy and H. Sies, Proc. Natl. Acad. Sci. U.S.A., 88 (1991) 10860.
[3] J.S. Beckman, T.W. Beckman, J. Chen, P.A. Marshall and B.A. Freeman, Proc. Natl. Acad. Sci. U.S.A., 87 (1990) 1620.
[4] R. Radi, J.S. Beckman, K.M. Bush and B.A. Freeman, J. Biol. Chem., 266 (1991) 4244.
[5] R. Radi, M. Rodriguez, L. Castro and R. Telleri, Arch. Biochem. Biophys., 308 (1994) 89.
[6] C. Roger, T.M. Brock, L-Y. Chang, J. Crapo, P. Briscoe, D. Ku, W.A. Bradley, S.H. Gianturco, J. Gore and B.A. Freeman, Proc. Natl. Acad. Sci. U.S.A., 91 (1994) 1044.
[7] R. Radi, J.S. Beckman, K.M. Bush and B.A. Freeman, Arch. Biochem. Biophys., 288 (1991) 481.
[8] A. Van der Vilet, C.A. O'Neill, B. Halliwell, C.E. Cross and H. Kaur, FEBS Lett., 339 (1994) 89.
[9] J.S. Beckmann, Y-Z., Ye, P.G. Anderson, J. Chen, M.A. Accavitti, M.M. Tarpey and C.R. White, Hoppe-Seyler's Biol. Chem., 375 (1994) 81.
[10] M. Naoi, W. Maruyama, I.N. Acworth, D. Nakahara, H. Parvez, in S.H. Parvez, M. Naoi, T. Nagatsu, and S. Parvez (Editors), Methods in Neurotransmitter and Neuropeptide Research Part I, Elsevier, Amsterdam, 1993, p. 1.
[11] W. Maruyama, M. Naoi, T. Takahashi, H. Watanabe, Y. Konagaya, K. Mokuno, S. Hasegawa and D. Nakahara, J. Neural Transm. [GenSect], 90 (1992) 183.
[12] H. Kaur and B. Halliwell, FEBS Lett., 350 (1994) 9.
[13] H. Ohshima, I. Brouet, M. Friesen and H. Bartsch, in I.K. O'Nell, J. Chen, and H. Bartsch (Editors), Relevance to Human Cancer of N-Nitro Compounds, Tobacco Smoke and Mycotoxins, International Agency for Research on Cancer, Lyon, 1991, p. 443.
[14] T. Malinski and Z. Taha, Nature, 358 (1992) 676.
[15] J.A. Imlay and I. Fridovich, J. Biol. Chem., 266 (1991) 6957.
[16] H. Ischiropoulos, L. Zhu and S. Beckman, Arch. Biochem. Biophys., 298 (1992) 446.
[17] D. MacMicking, D.O. Willenborg, M.J. Weidemann, K.A. Rockett and W.B. Cowden, J. Exp. Med., 176 (1992) 303.
[18] K.D. Kröncke, V. Kolb-Bachofen, B. Berschick, V. Burkart and H. Kolb, Biochem. Biophys. Res. Commun., 175 (1991) 752.