[Frontiers in Bioscience 8, a139-142, May 1, 2003]

MODIFICATIONS OF TYROSINE AND CATECHOLAMINES BY PEROXYNITRITE, NITRITE AND NITRATE

Wu-Nan Kuo, Joseph M. Kocis and Luvina K. Guruvadoo

Division of Science and Mathematics, Bethune-Cookman College, Daytona Beach, Florida

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1. ABSTRACT

At pH 7.0 in non-"deaired" potassium phosphate buffer, the reactions of L-tyrosine, L-dopa, dopamine, L-norepinephrine, and L-epinephrine with peroxynitrite (PN) or nitrite, generated colored products. These products displayed not only unique colors and patterns of mobility on silica thin layer chromatographic plates, but also varied increase of absorbance between 400 and 540 nm. In particular, with the treatment of nitrite, catecholamines exhibited longer bands and multiple colored spots due to the formation of multiple compounds. In addition, significantly increased mobilities were noted with nitrate-incubated catecholamines. These results imply the occurrence of various types of reactions, such as nitration and nitrosation, via the production of active intermediates of oxygen and/or nitrogen species during incubation.

2. INTRODUCTION

Nitric oxide (NO) is an important multifunctional signaling molecule (1). However, under oxidative stress it may react with superoxide free radicals to form peroxynitrite (PN), a potent oxidant (2-5), and subsequently be converted into nitrite/nitrate (1,5-7) thereby causing undesirable effects such as excessive and prolonged protein nitrosation. It has been speculated that tyrosine-nitration may disturb the tyrosine-phosphorylation/dephosphorylation cascade and inhibit an array of enzymes (3,8) thus leading to pathogenesis. 3-Nitrotyrosine residues of proteins, presumably due to excessive production of NO/PN, have been found in a myriad of diseased tissues (1) including Parkinson's (9), Alzheimer's (9), Huntington's (10), multiple sclerosis (11) and others. In addition, significant dietary and respiratory intake of nitrite/nitrate (1) may be linked to impairment of thyroid function, interference with vitamin A and E metabolism (12), and gastric inflammation in *Helicobacter pylori* infection (13). Furthermore, it has been implicated that nitrites and nitrates may react with certain amines to form carcinogenic nitrosamines (14). At high NO concentrations (mM range), dopamine undergoes nitrosation with subsequent nitration, whereas, at low NO concentrations (μM range) dopaminochrome is formed (12). The PN- and nitrite-induced oxidation of dopamine may result in the loss of dopaminergic cells (15). Unlike the above-mentioned studies requiring sophisticated instrumentation, this investigation employed simple and rapid methods, including thin layer chromatography and visible spectroscopy, to further explore the possible modifications of L-tyrosine and its catecholamine derivatives, after reaction with PN, nitrate and nitrite.

3. MATERIALS AND METHODS

3.1. Materials

L-Tyrosine, L-dopa, dopamine, L-epinephrine, L-norepinephrine, 3-nitrotyrosine, sodium nitrite, sodium nitrate, sodium dodecyl sulfate (SDS) and silica type G, all were supplied by Sigma Chemical Company, St. Louis, MO, USA. Peroxynitrite (PN) was purchased from Upstate Biotechnology, Lake Placid, NY, USA. Other chemicals for silica thin layer chromatography solvent system

Figure 1. Silica thin layer chromatogram of L-tyrosine and derivatives. Approximately 0.5-1.0 mg of samples were spotted after incubation in 200 mM potassium phosphate buffer (pH 7.0) at 25ºC for 45 minutes. Lane 1, L-tyrosine (alone); Lane 2, L-tyrosine incubated with 200 mM nitrite; Lane 3, 3-nitrotyrosine.

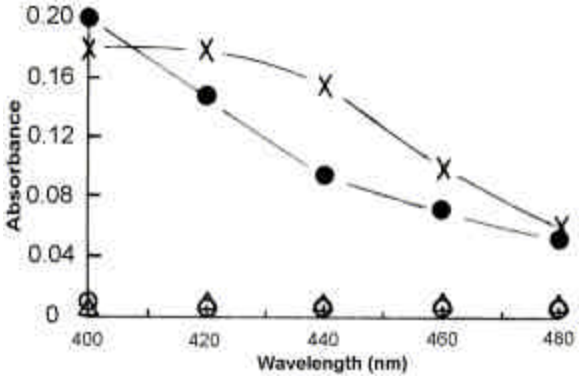

Figure 2. Absorption spectrum of L-tyrosine and derivatives formed in nitrite solution. Incubation mixtures were in a final volume of 300 µl of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM of nitrite and 10 mM L-tyrosine. The reactions were carried out at 25° C for 45 minutes. The incubations of L-tyrosine alone (o), L-tyrosine and nitrite (•), L-tyrosine and nitrate (Δ) and 3-nitrotyrosine alone (x).

preparation were acquired from Fisher Scientific, Pittsburgh, PA, USA.

3.2. Methods

Distilled/deionized H_2O, which may still contain trace metal cations, was used to prepare all solutions. The stock solutions of L-tyrosine, L-dopa, dopamine, L-epinephrine, L-norepinephrine, sodium nitrite, sodium nitrate and 3-nitrotyrosine were all prepared in appropriate concentrations of potassium phosphate buffer (pH 7.0). Prior to incubation, the buffer was not "deaired" (e.g., to drive off dissolved air from the solution via saturation with an inert gas such as argon).

After preincubating L-tyrosine or catecholamines with or without PN, nitrite or nitrate, the samples were spotted on silica thin layer chromatographic (TLC) plates which were developed in a solvent system containing toluene: 2-propanol: acetone: acetic acid (23:23:12:9). The developed plates were saturated in ammonium hydroxide vapor in a closed tank for 20 minutes and then detected with ninhydrin spray. Additionally, after preincubation the changes in L-tyrosine, catecholamines and their respective derivatives were monitored between 400 and 540 nm using a Bio-Tek µQuant (MQX200) microplate spectrophotometer.

4. RESULTS AND DISCUSSION

Since L-tyrosine can be converted into 3-nitrotyrosine by the reaction with PN (2), it is convenient to use a pure form of 3-nitrotyrosine (acquired from Sigma) as a positive control to compare along with L-tyrosine, nitrite-incubated L-tyrosine and nitrate-incubated L-tyrosine in the analysis by TLC. In the reaction mixtures, prior to being applied to TLC plates, the samples of L-tyrosine, nitrite-reacted L-tyrosine and 3-nitrotyrosine showed colorless, light yellow and dark yellow colored compounds, respectively. However, L-tyrosine incubated with nitrate showed no significant change of color. The TLC chromatogram developed by the solvent system containing toluene: 2-propanol: acetone: acetic acid (23:23:12:9) (Figure 1) further distinguished these three samples. 3-Nitrotyrosine (Figure 1, lane 3) exhibited a much greater mobility in comparison to nitrite-treated L-tyrosine (Figure 1, lane 2), which lacked significant mobility. Nitrate-treated L-tyrosine also lacked significant mobility (data not shown). After ninhydrin spray, 3-nitrotyrosine exhibited an intense yellow color (Figure 1, lane 3), whereas nitrite-treated L-tyrosine displayed a pinkish-brown color (Figure 1, lane 2) and nitrate-treated L-tyrosine showed no color change (data not shown). Hence, the production of distinct colored compounds observed before and after the chromatographic procedure implies that L-tyrosine indeed reacted with nitrite to form a product different from 3-nitrotyrosine, presumably by nitrosation on the aromatic ring of L-tyrosine; leaving the amino group intact for ninhydrin to react with. Moreover, both nitrite-treated L-tyrosine and 3-nitrotyrosine exhibited significantly increased absorbance between 400 and 480 nm, in comparison with that of L-tyrosine and nitrate-treated L-tyrosine, suggesting that L-tyrosine had reacted with nitrite but not nitrate (Figure 2).

L-Tyrosine is a precursor of catecholamines. In addition, tyrosine-phosphorylation and dephosphorylation plays a crucial role in the signal transduction cascade (1). Hence, a separate experiment was performed not only in the conversion of reduced 3-nitrotyrosine into aminotyrosine, but also further acetylation into acetaminotyrosine. Interestingly, these products also exhibited changes in the color and the mobility of thin layer chromatograms (data not shown). Nonetheless, the pathophysiological significance is yet to be evaluated.

In comparison with the color changes in L-tyrosine samples as mentioned above, more rapid and drastic color changes were observed in the incubated catecholamines. L-dopa incubated with nitrite or PN

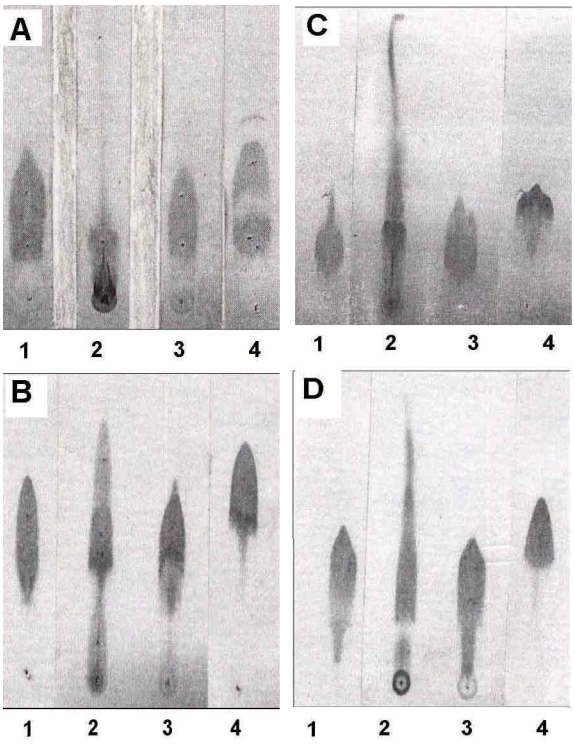

Figure 3. Silica thin layer chromatograms of catecholamines and derivatives. Approximately 0.5-1.0 mg samples were spotted after incubation in 200 mM potassium phosphate buffer (pH 7.0) at 25ºC for 35 minutes. (A) Lane 1, L-dopa (alone); Lane 2, L-dopa incubated with 10 mM nitrite; Lane 3, L-dopa incubated with 70 µM peroxynitrite; Lane 4, L-dopa with 10 mM nitrate. (B) Lane 1, dopamine (alone); Lane 2, dopamine incubated with 10 mM nitrite; Lane 3, dopamine incubated with 70 µM peroxynitrite; Lane 4, dopamine with 10 mM nitrate. (C) Lane 1, L-epinephrine (alone); Lane 2, L-epinephrine incubated with 10 mM nitrite; Lane 3, L-epinephrine incubated with 70 µM peroxynitrite, Lane 4, L-epinephrine with 10 mM nitrate. (D) Lane 1, L-norepinephrine (alone); Lane 2, L-norepinephrine incubated with 10 mM nitrite; Lane 3, L-norepinephrine incubated with 70 µM peroxynitrite; Lane 4, L-norepinephrine with 10 mM nitrate.

resulted in light yellow and dark yellow, respectively; dopamine incubated with nitrite or PN converted into dark yellow and orange/yellow, respectively; L-epinephrine incubated with nitrite or PN changed into pink/orange and dark orange, respectively; and L-norepinephrine incubated with nitrite or PN turned into light orange/pink and dark yellow, respectively. However, no significant change of color was observed in the catecholamine samples incubated with nitrate. The formation of the new products by the incubations of nitrite, PN, and nitrate with catecholamines were further confirmed via the changes of the color and the mobility in the TLC chromatograms (Figure 3), as well as the changes of absorbance (Figure 4). As observed in these samples of L-tyrosine (Figure 1), ninhydrin spray yielded colored products (Figure 3) indicating the amine group of the products also remained intact as reactants, and the modifications may occur on the aromatic ring of catecholamines during incubation. However, it cannot be excluded that ninhydrin spray failed to detect some products in TLC chromatograms, owing to the possible modification of amino group during preincubation. Apparently multiple products were generated after incubation with nitrite, as evident by the wide range in mobility of formed products (Figure 3, lanes 2). This was further confirmed by the separation of various products by ion exchange and affinity chromatography (data not shown).

Although PN-treatment did not lead to the significant alteration of mobility (Figure 3, lane 3), there were some differences in the color of the PN-incubated products: L-dopa, dopamine, L-epinephrine and L-norepinephrine turned light brown, pink/orange, brown and dark orange, respectively (Figure 3, lanes 3). In comparison, the untreated samples of L-dopa, dopamine, L-epinephrine and L-norepinephrine turned light pink, pink/orange, brown and light orange/pink, respectively (Figure 3, lanes 1). These results suggest that chemical modification(s) of catecholamines did occur after incubating with PN. Surprisingly, the preincubation with nitrate resulted in significantly increased mobilities (Figure 3, lanes 4), which were quite different from those of other samples (Figure 3, lanes 1-3), suggesting yet another/other chemical modification(s) of catecholamines. This was further supported by an additional experiment in which no increased mobility was noted when preincubation was omitted, and nitrate was spotted immediately under these (control) spots of untreated catecholamines on a thin layer chromatographic plate (data not shown).

The incubation of nitrite with L-dopa (Figure 4A), dopamine (Figure 4B), and L-epinephrine (Figure 4C) drastically increased the absorbance, especially at 400 nm, in comparison with the slight increase of L-norepinephrine sample (Figure 4D). However, no significant increase of PN-incubated samples was observed (Figure 4).

It has been reported that dopamine oxidation by nitric oxide may produce o-semiquinone and dopaminochrome (15). However, the reactions of catecholamines with PN, nitrite and nitrate may be far more complicated than expected. These reactions may be mediated through the production of intermediate active nitrogen/oxygen species, such as N_2O_3, $O_2^{\bullet -}$, and others (8). Since these reactions may represent severe cellular oxidative stress (8), it would be of great interest to develop simple, rapid and sensitive assays to monitor the level of catecholamine metabolites in serum due to the above mentioned reactions to correlate with pathogenic processes, particularly before the onset of Parkinson's disease.

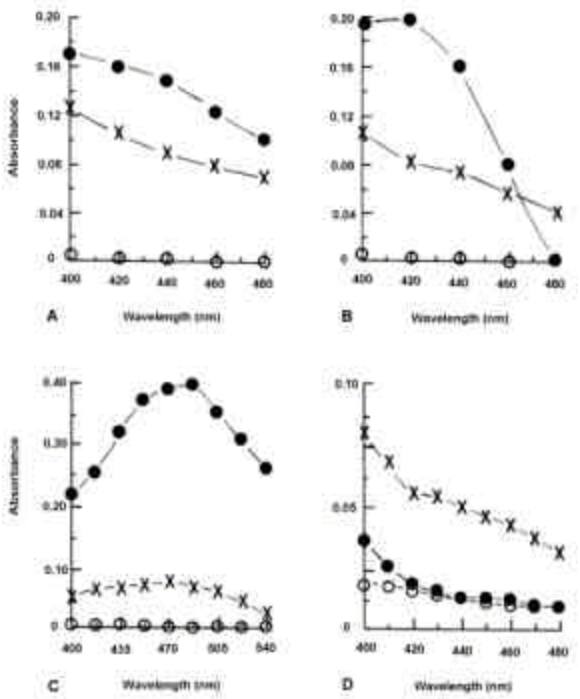

Figure 4. Absorption spectra of catecholamines and derivatives. Incubation mixtures were in a final volume of 300 µl of 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM of L-dopa, dopamine, 10 mM L-epinephrine, L-norepinephrine and 10 mM nitrite or 70 µM active peroxynitrite. The reactions were carried out at 25° C for 35 minutes. (A) The incubations of L-dopa alone (o), L-dopa and nitrite (•), and L-dopa and peroxynitrite (x); (B) The incubations of dopamine alone (o), dopamine and nitrite (•), and dopamine and peroxynitrite (x); (C) The incubations of L-epinephrine alone (o), L-epinephrine and nitrite (•), and L-epinephrine and peroxynitrite (x); (D) The incubations of L-norepinephrine alone (o), L-norepinephrine and nitrite (•), and L-norepinephrine and peroxynitrite (x).

5. ACKNOWLEDGMENTS

This work was supported by grant GM08119 from National Institutes of Health, USA. The authors wish to thank Dr. N. Denslow for insightful comments.

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Key Words: Tyrosine, Catecholamine, Dopamine, Peroxynitrite, Nitrate, Nitrite

Send correspondence to: Dr. Wu-Nan Kuo, Division of Science and Mathematics, Bethune-Cookman College, Daytona Beach, Florida 32114, Tel: 386-481-2697, Fax: 386-481-2657, E-mail: kuow@cookman.edu