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Research Article

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Effects of carbon dioxide on damage of amino acids induced by peroxynitrite

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ABSTRACT

Peroxynitrite is a potent oxidant to nitrate tyrosine of protein, induce DNA strand breaks and promote the lipid peroxidation in vivo, which can result in serious diseases, such as cardiopathy, neurodegenerative disorders and even cancer. However, carbon dioxide can react with peroxynitrite and change the toxicological properties of peroxynitrite, which is in equilibrium with bicarbonate anion in the extracellular milieu (25 mM in plasma). Due to reaction mechanisms of peroxynitrite with amino acids are the bases to explore the damage of protein induced by peroxynitrite. Here, by using stopped flow instrument, UV-visible spectra, fluorescence spectroscopy and high performance liquid chromatography, we investigated the kinetics reaction of carbon dioxide and peroxynitrite in the different environment and the effects of carbon dioxide on damage of amino acids caused by peroxynitrite. The results showed when the temperature was 37°C and pH 7.4, two-order rate constant of peroxynitrite react with $NaHCO_3$ was 2.3×10^3 $M^{-1}\cdot s^{-1}$. The damage of amino acids aroused by peroxynitrite was weakened in 0.1 M phosphate buffer solution with 25 mM $NaHCO_3$ and the damage inhibition ratio of tryptophan and tyrosine reached to 27% and 50%, respectively. Moreover, carbon dioxide could also reduce the fluorescence quenching of tyrosine induced by peroxynitrite.

Keywords: carbon dioxide, peroxynitrite, amino acids, kinetics, inhibition ratio.

INTRODUCTION

Protein consists of amino acids. Damage of protein caused by peroxynitrite is mainly manifested by modification of its amino acid residues. Therefore, reaction mechanisms of peroxynitrite with amino acids are the bases to explore the damage of protein induced by peroxynitrite. Peroxynitrite ($ONOO^-$) can impact normal physical function of protein by nitration and oxidation of amino acid residues, leading to serious biological consequences [1-3], such as neurodegenerative disorders and cardiovascular disorders [4-6]. In recent years, researchers found it was amino acids with sulfur group (cysteine, methionine) and amino acids with aromatic group (tryptophan, tyrosine) reacted with peroxynitrite. A large number of 3-nitrotyrosine were found in the tissues of many diseases, included Parkinson's disease, stroke and cardiovascular disorders, which was identified as the key product of tyrosine and peroxynitrite [7-10]. In addition to 3-nitrotyrosine, 3, 3'-dimerization tyrosine and 3, 4'-dihydroxyphenylalanine (dopa) also were generated in the reaction of tyrosine with peroxynitrite [11]. The reaction product of tryptophan and peroxynitrite was 6-nitro-tryptophan [12-15]. Moreover, since one of the most abundant constituents of the extracellular milieu is bicarbonate anion (25 mM in plasma) which is in equilibrium with carbon dioxide, carbon dioxide can react with peroxynitrite and change the toxicological properties of peroxynitrite. On the basis of above results, we made use of stopped flow instrument, UV-Vis spectra and HPLC to assay the kinetics reaction of carbon dioxide and peroxynitrite in the different environment, the effects of carbon dioxide on the damage of amino acids and fluorescence spectroscopy to detect fluorescence quenching of tyrosine aroused by peroxynitrite. This research provided kinetic parameters of peroxynitrite with carbon dioxide and the damage inhibition ratio of tryptophan and tyrosine in 0.1 M phosphate buffer solution with 25 mM $NaHCO_3$. In this paper, 25 mM bicarbonate anion can mimic the microenvironment of the vivo, which is more accurate to reflect the toxicological properties of peroxynitrite in the human body. It has positive significance to the prevention and treatment of the related diseases caused by peroxynitrite.

EXPERIMENTAL SECTION

Materials

Tryptophan and tyrosine were obtained from the Beijing east central joint chemical plant and Beijing chemical reagent company. Peroxynitrite was prepared from the reaction of hydrogen peroxide and isoamyl nitrite according to reported procedures [16]. Hydrogen peroxide was decomposed by manganese (IV) dioxide. The stock solutions of peroxynitrite in 0.1 M NaOH were stored at -20 □ and used within 3-4 weeks after synthesizing. Peroxynitrite concentrations were determined prior to each experiment by measuring the absorbance at 302 nm (ε_{302} = 1.67 $mM^{-1} \cdot cm^{-1}$). All other chemicals were analytical grade or the highest grade commercially available. All stock solutions were prepared in ultra-pure water.

Stopped flow instrument

Stopped flow measurements were performed by using a Bio-Logic stopped flow spectrophotometer (SFM 300, MOS-250). The 0.1 M phosphate buffer solution (PBS), 25 mM Sodium Bicarbonate (SB), 0.1 M phosphate buffer solution with 25 mM $NaHCO_3$ and peroxynitrite were separately placed in the different syringes. Reactions of peroxynitrite were studied in different buffer by using a rapid-mixing stopped flow diode array spectrophotometer to monitor the reaction from 300 nm to 500 nm. All experiments were carried out at 25°C and 37°C. The instrument was equipped with thermostat water bath which could control the temperature of the reaction.

UV-Vis spectra analysis

The concentrations of tryptophan were 0.4 mM, which were dissolved in 0.1 M phosphate buffer solution and 0.1 M phosphate buffer solution with 25 mM $NaHCO_3$, respectively. Different concentration of peroxynitrite was added in amino acid solution with a constant rate with stirring for 12 h. Tyrosine was dissolved as the same method. They were detected at full wavelength with U-3010 UV-Vis (Hitachi, Japan).

Fluorescence spectroscopy analysis

The reactions of different concentrations of peroxynitrite with tyrosine were stirred for 12 h in 0.1 M phosphate buffer solution and 0.1 M phosphate buffer solution with 25 mM $NaHCO_3$, respectively. The fluorescence intensities were recorded at excitation wavelength of 265 nm with F-4500 fluorescence spectrophotometer (Hitachi, Japan).

High performance liquid chromatography analysis

The concentrations of tryptophan were 0.4 mM, which were dissolved in 0.1 M phosphate buffer solution and 0.1 M phosphate buffer solution with 25 mM $NaHCO_3$, respectively. Different concentration of peroxynitrite was added in amino acid solution with a constant rate with stirring for 12 h. Tyrosine was dissolved as the same method. They were separated and detected at 280 nm with e2695 high performance liquid chromatography (HPLC, Waters). Chromatographic conditions: mobile phase was consisted with water and acetonitrile (8:92); flowing rate was 0.7 $mL \cdot min^{-1}$; detector was the UV-visible CCD detector.

RESULTS AND DISCUSSION

Stopped flow instrument

Kinetics experiments had been used to elucidate the effect of carbon dioxide on the decomposition rate of peroxynitrite. Kinetic curves showed the concentration change of $NaHCO_3$ could influence the decomposition rate of peroxynitrite (Fig.1), which was faster in the presence of $NaHCO_3$. The decomposition rate of peroxynitrite was fastest in 0.1 M phosphate buffer solution with 25 mM $NaHCO_3$.

The apparent rate (k_{obs}) was obtained by fitting with first-order reaction equation in the decomposition reaction of peroxynitrite. Eq. 1 is the kinetic constants equation:

$$k_{obs} = k\ [HCO_3^-] \quad (1)$$

where k_{obs} expresses the apparent rate, k is the rate constant of bicarbonate anion and peroxynitrite reaction. According to Eq. 1, we could calculate and obtain k of peroxynitrite react with bicarbonate anion in different conditions (Fig.2).

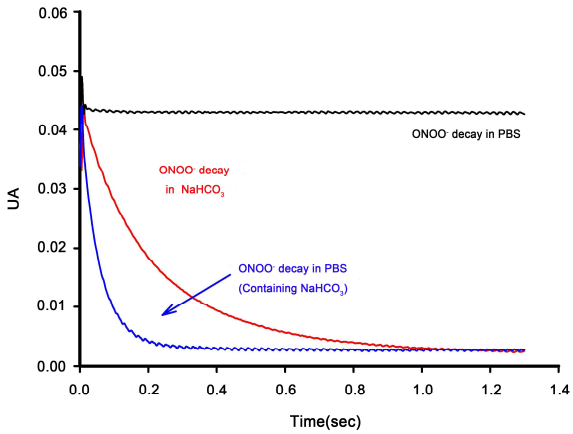

Fig.1: Kinetic traces of peroxynitrite decomposition in different buffer

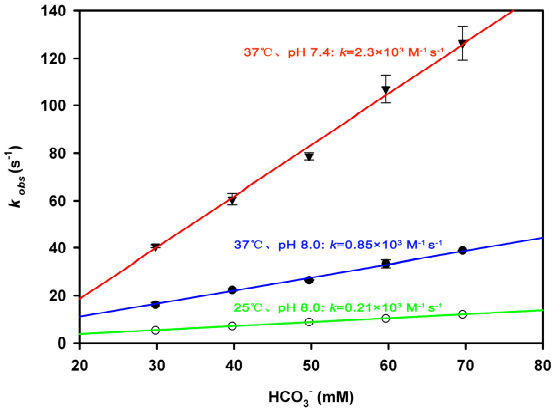

Fig.2: k_{obs} of peroxynitrite react with bicarbonate anion in different conditions

A linear equation was obtained by mapping with k_{obs} and the corresponding concentration of $NaHCO_3$. From the Fig.2, we can see the slope of k_{obs} to $NaHCO_3$ concentration in 37°C (blue function image) was higher than in 25°C (green function image). We calculated: first, when the temperature was 37°C and pH 7.4, two-order rate constant of peroxynitrite react with $NaHCO_3$ was $k = 2.3\times10^3\ M^{-1}\cdot s^{-1}$; second, when the temperature was 37°C and pH 8.0, two-order rate constant of peroxynitrite react with $NaHCO_3$ was $k = 0.85\times10^3\ M^{-1}\cdot s^{-1}$; third, when the temperature was 25°C and pH 8.0, two-order rate constant of peroxynitrite react with $NaHCO_3$ was $k = 0.21\times10^3\ M^{-1}\cdot s^{-1}$. The above data illustrated that when pH was fixed, the reaction rate of peroxynitrite and $NaHCO_3$ in 37°C was faster than in 25°C. The reaction rate of peroxynitrite and $NaHCO_3$ in neutral solution also was faster than in alkaline solution. The results illustrated carbon dioxide could promote the decomposition of peroxynitrite.

UV-Vis spectra analysis

The response spectra of peroxynitrite and Trp/Tyr were determined in the absence and presence of $NaHCO_3$ by UV-Vis spectrophotometer (Fig.3). The absorption peak of Trp and Tyr both emerged at 280 nm. When peroxynitrite reacted with Trp/Tyr, the new absorption peak of Trp and Tyr emerged at 357 nm and 352 nm, respectively, which rose along with the concentration of peroxynitrite increasing. It explained Trp/Tyr could react with peroxynitrite and appear the nitrated product.

As can be seen from the Fig.3 (a) and Fig.3 (b), the increase rate of new absorption peak of Trp was slower in the presence of $NaHCO_3$, which indicated $NaHCO_3$ made the nitration product decrease. It illustrated that $NaHCO_3$ can inhibit the damage of Trp induced by peroxynitrite. When the concentration of peroxynitrite was 10.8 mM, the inhibition of nitration product of Trp generated up to 27%. As can be seen from the Fig.3 (c) and Fig.3 (d), the increase rate of new absorption peak of Tyr was slower in the presence of $NaHCO_3$, which indicated $NaHCO_3$ also influenced the oxidation rate of Tyr catalyzed by peroxynitrite. When the concentration of peroxynitrite was 10.8 mM, the inhibition of nitration product of Tyr generated up to 50%. These variable findings suggested that the presence of $NaHCO_3$ might modulate the oxidative chemistry of Trp/Tyr with peroxynitrite.

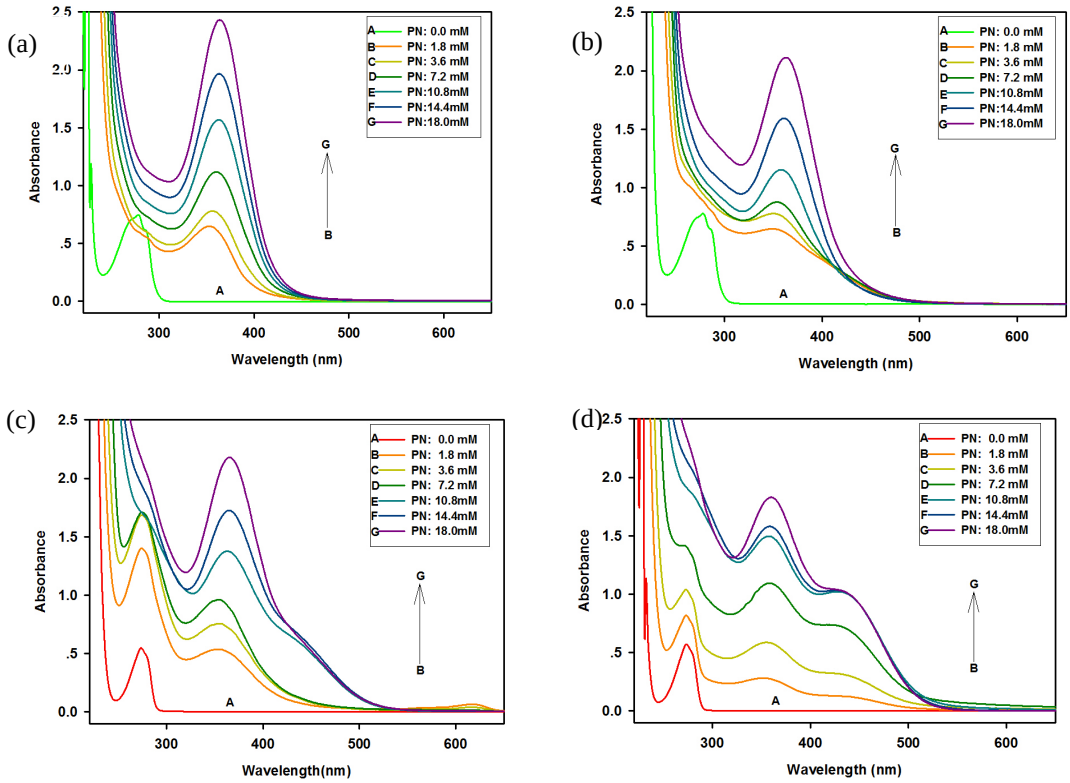

Fig.3: UV-Vis spectrum of Trp/Tyr react with peroxynitrite

(a) 0.4 mM Trp + 0.0/1.8/3.6/7.2/10.8/14.4/18.0 mM $ONOO^-$ in 0.1 M PBS
(b) 0.4 mM Trp + 0.0/1.8/3.6/7.2/10.8/14.4/18.0 mM $ONOO^-$ in 0.1 M PBS with 25 mM $NaHCO_3$
(c) 0.4 mM Tyr + 0.0/1.8/3.6/7.2/10.8/14.4/18.0 mM $ONOO^-$ in 0.1 M PBS
(d) 0.4 mM Tyr + 0.0/1.8/3.6/7.2/10.8/14.4/18.0 mM $ONOO^-$ in 0.1 M PBS with 25 mM $NaHCO_3$

Fluorescence spectroscopy with Tyr

The reaction of peroxynitrite and tyrosine had an analysis with fluorescence spectrum in 0.1 M phosphate buffer solution and 0.1 M phosphate buffer solution with 25 mM $NaHCO_3$, respectively (Fig.4). As can be seen from the Fig.4, fluorescence intensity of Tyr was quenching with the concentration of peroxynitrite increasing. By comparing the change of two pictures in Fig.4, fluorescence of Tyr was higher in the presence of $NaHCO_3$, which indicated $NaHCO_3$ retarded the damage rate of Tyr caused by peroxynitrite.

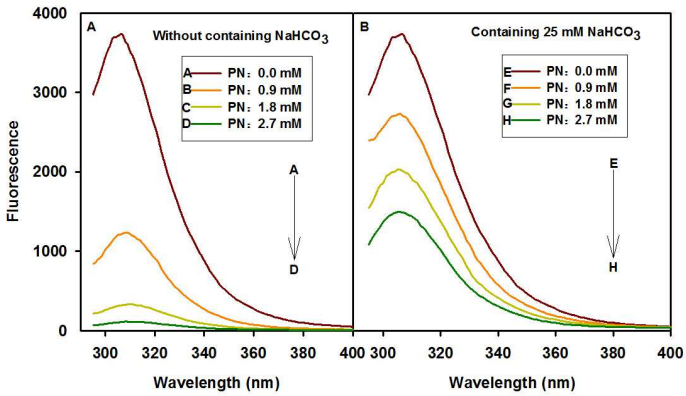

Fig.4: Fluorescence spectrum of Tyr react with peroxynitrite

We made a bar-chart from the data of Fig.4 (Fig.5). From the bar-chart, we can see clearly the difference between the absence and present of $NaHCO_3$ in the reaction, which explicated $NaHCO_3$ could inhibit the fluorescence quenching of Tyr caused by peroxynitrite at a glance.

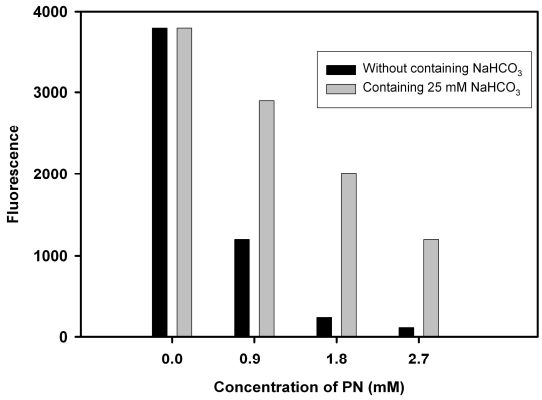

Fig.5: Fluorescence quenching of Tyr induced by peroxynitrite

High performance liquid chromatography analysis

Tryptophan/tyrosine and peroxynitrite reacted for 12 h, which were identified by high performance liquid chromatography and researched the effect of carbon dioxide on damage of Trp/Tyr induced by peroxynitrite. The reaction product of 0.4 mM Trp and different concentrations of peroxynitrite were separated in the absence and present of $NaHCO_3$, respectively (Fig.6). The peak of Trp decreased with the increase of peroxynitrite concentration. When the detection wavelength was 280 nm, a new chromatographic peak emerged 11 min, which had a spectral wavelength at 357 nm (Fig.6). Spectrum chart of the peak was illustrated in the small picture of Fig.6 (a) and (b), which was the product of tryptophan damaged by peroxynitrite. The peak was higher in 0.1 M phosphate buffer solution with 25 mM $NaHCO_3$, which indicated $NaHCO_3$ could retard the damage of Trp caused by peroxynitrite.

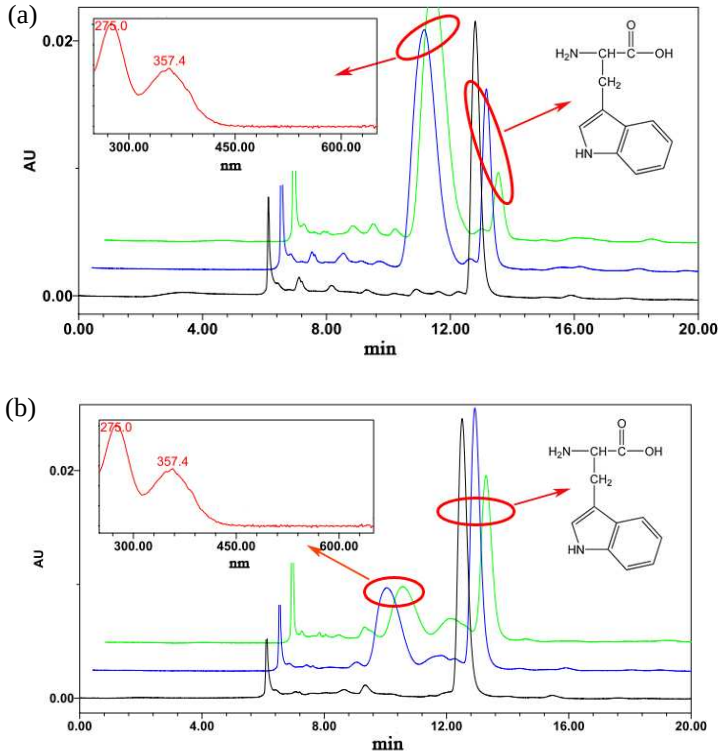

Fig.6: Retention time of Trp reacted with peroxynitrite (detection at 280 nm)

(a) 0.4 mM Trp + 0.0/1.7/8.5 mM $ONOO^-$ in 0.1 M PBS

(b) 0.4 mM Trp + 0.0/1.7/8.5 mM $ONOO^-$ in 0.1 M PBS with 25 mM $NaHCO_3$

In order to make a further research to the damage of Trp caused by peroxynitrite and the influence of $NaHCO_3$ on this reaction, we made a quantitative analysis of the above reaction and the results were shown in Table-1. The detection wavelength was 280 nm and the initial concentration of Trp was 100.0%. The Table-1 showed that Trp decreased with the increase of peroxynitrite concentration, at the same time, the corresponding nitration product increased. When peroxynitrite concentration changed from 0.0 mM to 34.0 mM, Trp decreased 70.0% in the system without $NaHCO_3$, however, Trp decreased 66.0% in the system contained 25 mM $NaHCO_3$. The above data showed the reaction system with $NaHCO_3$ promoted the decomposition of peroxynitrite to inhibit the damage of tryptophan

caused by peroxynitrite.

Table-1 Nitration of Trp induced by peroxynitrite

Peroxynitrite (mM)	Trp (%)	Trp(SB) (%)	Product (μM)	Product(SB) (μM)
0.0(Control)	100.0	100.0	0.0	0.0
1.7	85.4	89.2	26736.0	17745.0
8.5	71.9	76.6	119607.0	104169.0
17.0	54.6	60.8	206713.0	177029.0
34.0	30.6	34.3	327636.0	275345.0

The reaction product of 0.4 mM Tyr and different concentrations of peroxynitrite were separated in the absence and present of $NaHCO_3$, respectively (Fig.7). The peak of Tyr decreased with the increase of peroxynitrite concentration. When the detection wavelength was 280 nm, a new chromatographic peak emerged at 11.8 min, which had a spectral wavelength at 352 nm (Fig.7). Spectrum chart of the peak was illustrated in the small picture of Fig.7 (a) and (b), which was the product of tyrosine damaged by peroxynitrite. It was 3-NO_2-Tyr (3-NT), which was authenticated by 3-NO_2-Tyr calibration curve. The peak was higher in 0.1 M phosphate buffer solution with 25 mM $NaHCO_3$, which indicated $NaHCO_3$ could retard the damage of Tyr caused by peroxynitrite. Moreover, there was a peak at a retention time of 13 min in the presence of $NaHCO_3$. Its spectrum chart was different from spectrum chart of 3-NO_2-Tyr (Fig.7 (b)), which indicated that there was another nitrated product in the system of $NaHCO_3$. The spectral wavelength of the product was 355 nm.

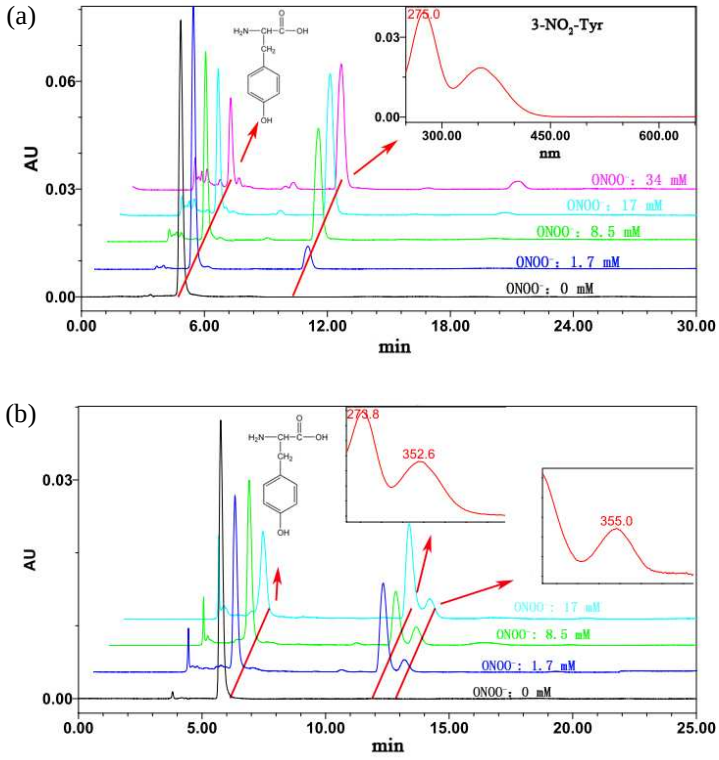

Fig.7: Retention time of Tyr reacted with peroxynitrite (detection at 280 nm)
(a) 0.4 mM Tyr + 0.0/1.7/8.5/17 mM $ONOO^-$ in 0.1 M PBS
(b) 0.4 mM Tyr + 0.0/1.7/8.5/17 mM $ONOO^-$ in 0.1 M PBS with 25 mM $NaHCO_3$

In addition, we also made a quantitative analysis of the above reaction and the results were shown in Table-2. The detection wavelength was 280 nm and the initial concentration of Tyr was 100.0%. The Table-2 showed that Tyr decreased with the increase of peroxynitrite concentration, at the same time, the corresponding nitration product increased. When peroxynitrite concentration changed from 0.0 mM to 34.0 mM, Tyr decreased 68.4% in the system without $NaHCO_3$, however, Tyr decreased 57.7% in the system contained 25 mM $NaHCO_3$. The above data indicated the reaction system with $NaHCO_3$ promoted the decomposition of peroxynitrite to inhibit the damage of tyrosine caused by peroxynitrite.

Table-2 Nitration of Tyr induced by peroxynitrite

Peroxynitrite (mM)	Tyr (%)	Tyr(SB) (%)	3-NT (μM)	3-NT(SB) (μM)
0.0(Control)	100.0	100.0	0.0	0.0
1.7	97.0	98.9	36.6	29.6
8.5	67.0	73.3	167.1	146.4
17.0	48.2	61.6	210.9	180.2
34.0	31.6	42.3	283.1	266.1

We can conclude that the reaction product of peroxynitrite and Trp/Tyr in the presence of $NaHCO_3$ was obviously less than in the absence of $NaHCO_3$, which can promote the decomposition of peroxynitrite so as to inhibit the damage of Trp/Tyr caused by peroxynitrite.

CONCLUSION

In this study, stopped flow, UV-Vis spectra, fluorescence spectroscopy and HPLC were applied to investigate the effect of carbon dioxide on damage of Trp/Tyr aroused by peroxynitrite. Kinetic curves showed two-order rate constant of peroxynitrite react with $NaHCO_3$ was $2.3\times10^3\ M^{-1}\cdot s^{-1}$ in 37°C, which was faster than in 25°C. In addition, the reaction rate of peroxynitrite and $NaHCO_3$ in neutral solution was faster than in alkaline solution. The spectra experimental results revealed that the injury extent of Trp/Tyr induced by peroxynitrite will be weakened in the presence of $NaHCO_3$ system. The injury inhibition rate of tyrosine and tryptophan could reach to 27% and 50%, respectively. Carbon dioxide could also reduce the fluorescence quenching degree of tyrosine. Finally, we can conclude that if there was carbon dioxide in the reaction system, it can promote the decomposition of peroxynitrite so as to inhibit the injury of Trp/Tyr caused by peroxynitrite.

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REFERENCES

[1] GL Squadrito; WA Pryor. *Chem-Biol. Interac.*, **1995**, 96(2), 203-206.
[2] MP Murphy; MA Packer; JL Scarlett; SW Martin. *General Pharmacology*, **1998**, 31(2), 179-186.
[3] JM Souza; G Peluffo; R Radi. *Free Radic. Biol. Med.*, **2008**, 45(4), 357-366.
[4] H Ischiropoulos. *Arch. Biochem. Biophys.*, **1998**, 356(1), 1-11.
[5] SR Danielson; JK Andersen. *Free Radic. Bio. Med.*, **2008**, 44(10), 1787-1794.
[6] R Gonzalo; R Peluffo. *Cardiovasc. Res.*, **2007**, 75, 291-302.
[7] B Halliwell. *FEBS Lett.*, **1997**, 411(2-3), 157-160.
[8] F Yamakura; H Taka; T Fujimura; K Murayama. *J. Biol. Chem.*, **1998**, 273(23), 14085-14089.
[9] S Park; TJ Geddes; JA Javitch; DM Kuhn. *J. Biol. Chem.*, **2003**, 278(31), 28736-28742.
[10] D Pietraforte; AM Salzano; G Marino; M Minetti. *Amino Acids*, **2003**, 25(3-4), 341-350.
[11] R Radi. *Proc. Natl. Acad. Sci. U S A*, **2004**, 10(12), 4003-4008.
[12] Y Ishida; H Takiuchi; A Matsushima; Y Inada. *Biochim. Biophys. Acta.*, **1978**, 536(1), 70-77.
[13] B Alvarez; H Rubbo; M KIRK. *Chem. Res. Toxicol.*, **1996**, 9(2), 390-396.
[14] S Padmaja; MS Ramazenian; PL Bounds. *Redox Report*, **1996**, 2(3), 173-177.
[15] F Yamakura; T Matsumoto; H Taka. *Adv. Exp. Med. Biol.*, **2003**, 527, 745-749.
[16] RM Uppu; WA Pryor. *Anal. Biochem.*, **1996**, 236(2), 242-249.