Showcasing research by Professor Hubert Girault and colleagues at the Laboratoire d'Electrochimie Physique et Analytique, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland and the Chemistry Department, Fudan University, Shanghai, China.

Antioxidant promotion of tyrosine nitration in the presence of copper(II)

In vivo, copper(II) is a major source of reactive oxygen species (ROS) via Fenton Haber Weiss reactions. Interest in Alzheimer's disease has led to intensive studies into the generation of ROS catalyzed by copper(II) with or without the presence of β -amyloid ($\text{A}\beta$) peptides and biological antioxidants. We establish a potential connection between tyrosine nitration and copper(II) toxicity in neurodegenerative diseases by demonstrating that tyrosine nitration can be induced by ascorbic acid, nitrite and copper(II) or peptide coordinated copper(II) under aerobic conditions via an *in vitro* protocol.

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Copper(II) is known to catalyze the generation of reactive nitrogen species in the presence of hydrogen peroxide, nitrite or nitric oxide, leading to tyrosine nitration, a biomarker for free radical species associated diseases. Here, we find that biological antioxidants such as ascorbic acid can promote tyrosine nitration in the presence of copper(II) and nitrite under aerobic and weak acidic conditions. Tyrosine nitration is demonstrated on both the β -amyloid peptide and angiotensin I. These studies show that (i) ascorbic acid works as a pro-oxidant in the presence of copper(II) to induce oxidation and nitration on peptides, (ii) both free and coordinated copper(II) can catalyze peptide oxidation and nitration, (iii) nitration occurs under mild acidic conditions (pH = 6.0–6.5).

Introduction

Tyrosine nitration has been demonstrated as a biomarker for free radical species associated diseases, such as cardiovascular diseases,¹ Alzheimer's disease^{2–4} and Parkinson's disease.⁵ It is important to study the pathways and mechanisms of tyrosine nitration for an in-depth understanding of these pathologies. Two reactive nitrogen species, peroxyxynitrite anions (ONOO[−]) and nitrogen dioxide radicals ($\bullet$ NO₂), are considered to directly react with the tyrosine residues resulting in their nitration.^{6,7} ONOO[−] is generated *in vivo* by the very fast reaction between nitric oxide ($\bullet$ NO) and the superoxide anion (O₂[−]).^{8–10} Alternatively, enzyme–ONOO[−] complexes can be generated from the reaction of peroxidases with hydrogen peroxide (H₂O₂) and nitrite (NO₂[−]).¹¹ Different pathways lead to the production of $\bullet$ NO₂ both *in vitro* and *in vivo*, including the oxidation of nitric oxide ($\bullet$ NO) by oxygen,¹² the decomposition of ONOO[−],^{6,10,13} and the oxidation of nitrite (NO₂[−]) by hydrogen peroxide (H₂O₂) catalyzed by peroxidases¹⁴ or other species having similar peroxidase properties, such as superoxide dismutase (SOD)^{15–18} and Hemin-containing proteins.¹⁹

Recently, we have demonstrated that copper(II) and coordinated copper(II) are also able to catalyze the generation of $\bullet$ NO₂

from nitrite and H₂O₂.²⁰ A key complex of copper(II)–hydroperoxo can be generated from copper(II) and the hydroperoxide anion (HO₂[−]), and further decomposed to form copper(I), which is able to react with H₂O₂ to produce hydroxyl radicals ($\bullet$ OH) *via* Fenton-reactions. The radicals can be scavenged by NO₂[−] to form $\bullet$ NO₂. In the presence of tyrosine, tyrosine radicals (Tyr $\bullet$) are also formed, leading to tyrosine nitration.

In vivo, copper(II) is the major source of reactive oxygen species (ROS) *via* Fenton Haber Weiss reactions. Interest in Alzheimer's disease has led to intensive studies on the generation of ROS catalyzed by copper(II) with or without the presence of β -amyloid (A β) peptides and biological antioxidants, such as ascorbic acid.²¹ To date, many conflicting studies have been reported. A β peptides have been suggested to act either as antioxidants to scavenge ROS²² or as pro-oxidants to promote the generation of ROS.²³ Hureau *et al.* have reviewed comprehensively the A β mediated ROS production in the presence of copper ions.²⁴ *In vivo*, the concentration of copper(II) ions can be 15–20 μ M in plasma, $\sim$ 250 nM in cerebrospinal fluid (CSF) and as high as 250 μ M when they are released at the synapse.^{25,26} The concentration of copper ions in the brain increases with age. In Alzheimer's disease, the concentration of copper was found in cerebral amyloid deposits at an abnormally high concentration of 340 μ M.²⁷ Copper is also reported to bind strongly with A β peptides, where the binding happens mainly on the N-terminal part of the peptides, especially histidine residues (His13–His14).^{28–31} All in all, the literature suggests an important role of copper in aging, especially Alzheimer's disease. On the other hand, the literature also suggests tyrosine nitration as a biomarker in Alzheimer's disease.^{2–4} Therefore, the performance of copper(II) in tyrosine

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nitration under oxidative and nitrative stresses is of significant interest.

Herein, we have studied the tyrosine nitration catalyzed by copper(II) in the presence of nitrite, ascorbic acid and A β peptides under aerobic conditions. The reaction was performed *in vitro* under mildly basic (pH = 8.0), neutral and mildly acidic conditions (pH = 6.0, 6.5) at 37 °C or 25 °C. Both oxidation and nitration products were observed by electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) for the validation of nitration and oxidation sites on peptides. It was found that reactive oxygen species H₂O₂ and •OH were generated by the reduction of oxygen by ascorbic acid under the catalysis of copper(II). The ROS could be further scavenged by nitrite to induce the generation of •NO₂ for tyrosine nitration. The nitration was demonstrated on both β amyloid peptide and angiotensin I with free cupric ions or coordinated cupric ions. The concentrations of copper(II) used to catalyze the reactions ranged from 25 μ M to 5 mM, which covered the reported physiological copper(II) concentration in plasma, synapse and cerebral amyloid deposits.^{25–27} Reactant concentrations and reaction conditions were varied for mechanism study. The current work can be important for the study of tyrosine nitration and copper(II) toxicity for neurodegenerative diseases.

Experimental

Chemicals and materials

Angiotensin I (H₂N–DRVYIHPFHL–COOH, 98%) and β -amyloid peptide 1–16 (A β 16 H₂N–DAEFRHDSGYEVHHQK–COOH) were purchased from Bachem (Switzerland). Acetic acid (99.8%) was purchased from Merck. Copper(II) chloride (CuCl₂·2H₂O, 99%) was bought from Acros. Sodium nitrite (NaNO₂, 99%) and ammonium bicarbonate (NH₄HCO₃, $\geq$ 99.5%) were purchased from Fluka. Methanol (HPLC grade) was purchased from Applichem. L(+)-Ascorbic acid (99.7%) was bought from Riedel-de Haën. All these reagents were used as received without further purification. Deionized water (18.2 M Ω cm) was obtained from an ultra-pure water system (Milli-Q 185 Plus, Millipore) and used for all experiments.

In vitro oxidation and nitration of peptides

The nitration and oxidation of the peptides were performed by incubating angiotensin I or A β 16 with nitrite, ascorbic acid and copper(II) chloride in NH₄HCO₃ aqueous solution at 37 °C or 25 °C and under persistent stirring (500 rpm). NH₄HCO₃ was dissolved in water at various concentrations to form buffers. The pH of the solution containing both buffer and reactants was measured using Whatman® Panpeha™ pH indicator strips 0–14 (Sigma-Aldrich). The reactant concentrations and reaction time were varied to study the mechanism and kinetics of the nitration.

In the case of A β 16 peptide bound copper(II) catalyzed tyrosine nitration, the copper chloride (25 μ M) was first mixed with A β 16 peptide (100 μ M) to coordinate all cupric ions

with A β 16. Then, the other reactants were added for the subsequent oxidation and nitration of peptides.

Mass spectrometry characterization of the reaction products

Reaction products were collected and diluted 10 times in an ESI buffer (1% acetic acid in 50% methanol and 49% water) followed immediately by the analysis using ESI-MS (Thermo LTQ Velos). The buffer was used to adjust the solution pH to a low value to form positively charged ions of the modified and non-modified peptides. Methanol was added to decrease the solvent surface tension thereby easily initializing the ESI. The diluted sample was injected into a commercial standard ESI emitter at a flow rate of 10 μ l min^{−1}. The ESI was performed at a high voltage of 3.7 kV and a sheath nitrogen gas flow of 5 psi. The generated ions were detected using a linear ion trap mass spectrometer, where the scan rate was 10 000 Th s^{−1} for enhanced resolution. Mass spectrometer parameters were kept constant during all experiments for quantitative analysis. Collision induced dissociation (CID) was used for tandem mass spectrometry. The fragmentation was performed on selected parent ions with 20–30 of instrumental collision energy.

Results and discussion

Chemistry background for the tyrosine nitration catalyzed by copper(II) in the presence of ascorbic acid and nitrite

Although there are many conflicting reports on the performance of A β peptides in the generation of ROS, there is a basic agreement that copper(II) facilitates the reduction of oxygen to H₂O₂ in the presence of biological reductants, *e.g.* glutathione and ascorbic acid, *via* a two-step reaction:³²

where Red is the reductant and Ox is the oxidant. In the case of ascorbic acid (AA) as a reductant, the first step would be:

where DA is the dehydroascorbic acid. During the reaction, copper(II) works as a catalyst for the reduction of oxygen to hydrogen peroxide by ascorbic acid. However, copper(II) and copper(I) can further catalyze the decomposition of H₂O₂ according to our previous study on tyrosine nitration as follows:²⁰

Such a reaction mechanism has been previously suggested.³³ The first two steps in eqn (3) are essentially the Harber–Weiss cycle, while the last step is the Fenton reaction. According to previous studies of tyrosine nitration, one of the reactive nitrogen species that can induce direct nitration on tyrosine

is nitrogen dioxide ($\bullet\text{NO}_2$),²⁰ which can be generated from nitrite in the presence of hydroxyl radicals:

where Tyr is tyrosine and $\text{NO}_2\text{-Tyr}$ is nitrated tyrosine. Therefore, one can expect the nitration of tyrosine in the presence of copper(II), ascorbic acid, oxygen and nitrite.

Tyrosine nitration on angiotensin I and β amyloid 1–16 catalyzed by copper(II) ions

Two peptides, angiotensin I (Ang I) and β amyloid 1–16 (A β 16), were selected as model targets for the nitration on tyrosine. Angiotensin I is an extracellular peptidic pre-hormone involved in the physiological procedures that control blood pressure. This peptide contains one tyrosine residue. We chose Ang I as one of the targets for tyrosine nitration because of its easy characterization by mass spectrometry. By incubating angiotensin I with ascorbic acid, sodium nitrite (NaNO_2) and copper(II) in NH_4HCO_3 buffer (pH = 6) for 18 hours under persistent shaking, nitrated Ang I (Ang I + NO_2 , m/z = 448.0 Th, z = 3) and oxidized Ang I (Ang I + O, m/z = 438.3 Th, z = 3) were generated and detected by the ESI-MS together with the unmodified Ang I (m/z = 433.0 Th, z = 3), as shown in Fig. 1(a). The charge state for each ion was directly obtained from the isotopic peaks thanks to the sufficient MS resolution.

β -Amyloid (A β) peptides are of great interest in the mechanistic study of Alzheimer's disease. A β 16 contains one tyrosine residue and is reported to coordinate copper(II) ions.²⁹ Recent study shows that the nitration on tyrosine 10 of A β peptides accelerates its aggregation and plaque formation, where the nitration was induced by nitric oxide.³⁴ Replacing Ang I by A β 16 for the copper(II) catalyzed tyrosine nitration, nitrated A β 16 (A β 16 + NO_2 , m/z = 667.7 Th, z = 3) and oxidized A β 16 (A β 16 + O, m/z = 658.0 Th, z = 3) were also observed by mass spectrometry together with unmodified A β 16 (m/z = 652.7 Th, z = 3) as shown in Fig. 1(b). Different concentrations of copper(II) were employed for inducing Ang I and A β 16 nitration, indicating that copper(II) either as a trace or in abundant amounts can catalyze peptide nitration.

The collision induced dissociation (CID) tandem MS technique was used to characterize the structure of nitrated and oxidized peptides. As shown in Fig. 2(a), the nitrated b_4^* , b_5^* , b_6^* , b_8^* , b_9^* , y_7^* , y_8^* and y_9^* fragments were observed, indicating that the nitration happened on the 4th amino acid residue in the sequence, that is, tyrosine. The tandem MS spectrum of oxidized Ang I gave a more complex pattern: oxidized b_6^* , b_8^* , b_9^* and b_{10}^* fragments were observed while non-oxidized b_6 , b_8 , and a_9 were also generated, see Fig. 2(b). This fragmentation result suggested that either the 6th or the 9th amino acid residues in the sequence was oxidized, which are both histidine, and that the parent peak at m/z = 438.3 Th showed a mixture of

Fig. 1 (a) Mass spectrum of nitrated (Ang I + NO_2) and oxidized angiotensin I (Ang I + O) generated by incubating angiotensin I (Ang I, 25 mM) with CuCl_2 (1 mM), ascorbic acid (AA, 1 mM) and NaNO_2 (1 mM) in 1 mM NH_4HCO_3 (pH = 6) for 18 hours under persistent shaking; (b) mass spectrum of nitrated (A β 16 + NO_2) and oxidized β -amyloid 1–16 (A β 16 + O) generated by incubating β -amyloid 1–16 (A β 16, 0.1 mM) with CuCl_2 (0.025 mM), AA (1 mM) and NaNO_2 (1 mM) in 1 mM NH_4HCO_3 (pH = 6) for 18 hours under persistent shaking. The reaction was performed at 37 °C. The products were diluted 10 times in the ESI buffer (49% water/50% methanol/1% acetic acid) and infused at a flow rate of $10 \mu\text{L min}^{-1}$ into ESI-MS at an ionization voltage of 3.7 kV. The peaks without label are mainly attributed to contaminants of the synthesized Ang I and A β 16 and background contaminants in the ion trap except that a small peak after Ang I + NO_2 in Fig. 1(a) corresponds to the copper coordinated Ang I (m/z = 453.3 Th, z = 3).

the two oxidized products. Fig. 2(c) shows the MS/MS result of nitrated A β 16 where b -fragments were nitrated from b_{10}^* to b_{15}^* and y -fragments were nitrated from y_7^* to y_{15}^* , indicating that the tyrosine as the 10th amino acid residue in A β 16 was nitrated. The generation of nitrated or oxidized fragments was demonstrated by the observation of an increase in the molecular weight of 45 Da or 16 Da, respectively.

Tyrosine nitration influenced by reactant concentrations

According to previous studies, the intensity ratio of the peaks on mass spectra of Ang I and Ang I + NO_2 is proportional to the amount ratio of these two peptides in the sample with a slope at around 1.²⁰ Since tyrosine is an amino acid polar but uncharged, the nitration on the tyrosine residue of Ang I would not change the charge states of the peptide, and thus Ang I + NO_2 shows similar efficiency in the generation of triple protonated ions as Ang I. In this work, we demonstrated by standard addition calibration that the peak intensity ratio of Ang I and Ang I + O is also proportional to their amount ratio but with a slope at around 8, as is shown in ESI-1.† In Ang I, 3 basic amino acid residues (e.g. the 2nd amino acid R, the 6th and the 9th amino acids H) contribute the positive changes of the peptide in addition to its N-terminal. After oxidation on one histidine,

Fig. 2 Tandem mass spectra of (a) nitrated Ang I, (b) oxidized Ang I and (c) nitrated A β 16. The selected parent ion is at (a) $m/z = 447.9 \text{ Th} \pm 1.0 \text{ Th}$, or (b) $m/z = 438.3 \text{ Th} \pm 1.0 \text{ Th}$, or (c) $m/z = 667.7 \text{ Th} \pm 1.0 \text{ Th}$. The CID was performed with 20–30 of instrumental collision energy. *: (a and c) nitrated fragments and (b) oxidized fragments. The observed fragments are labeled on the spectra and summarized as insets in the figure.

the affinity of this amino acid residue to proton may be changed that influences the generation of triple protonated Ang I + O. According to the calibration, the intensities of nitrated, oxidized and unmodified Ang I on the mass spectra can be used to calculate the amount ratio among the three peptides as: $I_{\text{NO}_2\text{AngI}}/(I_{\text{NO}_2\text{AngI}} + I_{\text{AngI}} + 8I_{\text{OAngI}})$ for the ratio of nitrated Ang I (NO_2AngI) among the three peptides, $8I_{\text{OAngI}}/(I_{\text{NO}_2\text{AngI}} + I_{\text{AngI}} + 8I_{\text{OAngI}})$ for oxidized Ang I (OAngI) and $I_{\text{AngI}}/(I_{\text{NO}_2\text{AngI}} + I_{\text{AngI}} + 8I_{\text{OAngI}})$ for Ang I, where I is the peak

The kinetic study was made by incubating Ang I (0.25 mM) with copper(II) (0.025 mM), nitrite (1 mM) and ascorbic acid (1 mM) in NH_4HCO_3 buffer (pH = 6) at 37 °C under aerobic conditions and persistent shaking (500 rpm). We found that the oxidation was a rather fast reaction, reaching equilibrium of 58.5% Ang I + O ratio in 30 min (ESI-2†). In contrast, the nitration was very slow approaching equilibrium only after 55 hours of reaction, Fig. 3(a).

The influence of reactant concentrations on the nitration and oxidation of Ang I was also studied by incubating the peptide with various amounts of copper(II), nitrite and ascorbic acid in NH_4HCO_3 buffer (pH = 6) at 37 °C or 25 °C under aerobic conditions and persistent shaking (500 rpm). The reaction time was fixed as 18 hours for all experiments. Similar nitration and oxidation on the peptide were observed at both 37 °C and 25 °C, whereas the reaction at higher temperature was more efficient, Fig. 3.

As shown in Fig. 3(b), both nitration and oxidation increased with the ascorbic acid concentration. This can be easily explained as more hydroxyl radicals were produced when more ascorbic acid was added in the reaction system. However, the oxidation was much more stronger and suppressed the nitration. Even at very high concentrations of ascorbic acid, only a small fraction of Ang I was nitrated. Keeping constant the concentrations of copper(II) (0.025 mM) and ascorbic acid (1 mM), the nitration increased while the oxidation decreased with the nitrite concentration, Fig. 3(c), indicating that nitrite acts as a hydroxyl radical scavenger to prevent the histidine oxidation by $\bullet\text{OH}$ and to produce the nitrogen dioxide radical promoting tyrosine nitration. Indeed, the oxidation was very strong when the concentration of nitrite was low, where most Ang I was oxidized, Fig. 3(c). The role of nitrite as a hydroxyl radical scavenger was also demonstrated by fluorometric assay in a previous study.²⁰

A phenomenon of interest is that nitration increased while oxidation decreased strongly with the copper(II) concentration, Fig. 3(d). According to our previous studies,²⁰ copper(II) can be coordinated by Ang I and the binding happens close to the tyrosine residue of Ang I. The same binding site between Ang I and copper(II) was also reported by Kim *et al.*³⁵ In Fig. 1(a), the copper coordinated Ang I is observed as a small peak after the nitrated Ang I ((Ang I + NO₂)³⁺). When the copper(II) is in excess, free copper(II) ions largely exist together with Ang I coordinated copper(II) ions; while at low copper(II) concentration, most cupric ions are coordinated by Ang I. Therefore, the results in Fig. 3(d) suggest that a large amount of free copper(II) promotes nitration of Ang I and blocks the oxidation route, while Ang I coordinated copper(II) catalyzes preferably oxidation of Ang I. Kawakishi *et al.* also reported that catalytic amounts of copper(II) ion together with ascorbate can induce oxidation on histidine residues of angiotensin I.³⁶

To illustrate the role of oxygen, the same Ang I nitration as Fig. 1(a) was performed but under anaerobic conditions.

Fig. 3 (a) Ang I nitration in 1 mM NH_4HCO_3 (pH = 6) induced by ascorbic acid (1 mM), NO_2^- (1 mM) and CuCl_2 (0.025 mM) at 37 °C with various reaction times under persistent shaking. Ang I (0.25 mM) nitration and oxidation at 25 °C or 37 °C in 1 mM NH_4HCO_3 induced by different concentrations of (b) ascorbic acid, (c) nitrite and (d) copper(II) after 18 hours of reaction under persistent shaking. b: CuCl_2 = 0.025 mM, NO_2^- = 1 mM; c: CuCl_2 = 0.025 mM, ascorbic acid = 1 mM; d: ascorbic acid = 1 mM, NO_2^- = 1 mM. The left y-axis shows the Ang I oxidation while the right y-axis shows the Ang I nitration. Note the differences in different y-axis ranges. The error bars show the standard deviations. Three experiments were conducted to obtain the standard deviations.

Fig. 4 Mass spectrum of reaction products by incubating angiotensin I (Ang I, 0.25 mM) with CuCl_2 (1 mM), ascorbic acid (AA, 1 mM) and NaNO_2 (1 mM) in 1 mM NH_4HCO_3 (pH = 6) for 18 hours at 37 °C and under persistent shaking. The reaction was performed under the protection of nitrogen without oxygen. The products were diluted 10 times in the ESI buffer and infused at a flow rate of $10 \mu\text{L min}^{-1}$ into ESI-MS at an ionization voltage of 3.7 kV.

As shown in Fig. 4, only unmodified Ang I and copper coordinated Ang I were observed after 18 hours of reaction. The other peaks on the spectrum are attributed to contaminants of the

synthesized Ang I and background contaminants in the ion trap. These results supported the mechanism that ROS is generated by reducing oxygen in the presence of ascorbic acid and copper(II) to induce tyrosine nitration.

Based on the results in Fig. 3, Fig. 4 and previous mechanism studies,^{20,32} a mechanism for the tyrosine nitration in the presence of copper(II), ascorbic acid and nitrite is summarized in Scheme 1. The reduction of copper(II) to copper(I) by ascorbic acid is a key process in the generation of H_2O_2 and $\bullet\text{OH}$ radicals, where the latter can induce fast oxidation of histidine or slow nitration of tyrosine in the presence of nitrite. During the whole procedure copper(II) works as a catalyst, while the overall reaction can be viewed as oxidation of ascorbic acid, histidine and tyrosine by dissolved oxygen.

Scheme 1 Proposed mechanism for tyrosine nitration in the presence of nitrite, ascorbic acid and copper(II). AA: ascorbic acid; DA: dehydroascorbic acid; His: histidine; Tyr: tyrosine; O-His: oxidized histidine; $\text{NO}_2\text{-Tyr}$: nitrated tyrosine. The Cu^{2+} can be free cupric ions or coordinated cupric ions.

Tyrosine nitration catalyzed by coordinated copper(II) and under varied buffer conditions

During the copper(II) catalyzed tyrosine nitration on angiotensin I, both free copper(II) and coordinated copper(II) may involve in the reaction mechanism because of the affinity between copper(II) and Ang I. Considering the fact that the *in vivo* free copper concentration is rather low, the performance of peptide coordinated copper(II) in tyrosine nitration is of more interest when considering physiological conditions. β -Amyloid peptides were reported to coordinate strongly the cupric ions.^{28–31} In Fig. 1, the nitration on A β 16 has already been observed. Considering that the concentration of A β 16 was much larger than copper(II), A β 16 coordinated copper(II) catalyzed the tyrosine nitration in Fig. 1(b).

Over years, conflicting reports have been published on the role of β -amyloid peptides in $\bullet$ OH radical generation from copper(II), oxygen and ascorbic acid: both antioxidant and pro-oxidant properties have been proposed.^{22,23} Some reports even suggested that β -amyloid peptides could reduce copper(II) to copper(I) without the presence of ascorbic acid to initialize the generation of H_2O_2 .³⁷ Herein, A β 16 coordinated copper(II) was incubated with Ang I, nitrite and ascorbic acid at pH 6.0, 37 °C, under aerobic conditions and persistent shaking (500 rpm) for 18 hours. Besides the observation of nitrated and oxidized A β 16 peptides, much more efficient nitration on Ang I was obtained than the copper(II) catalyzed Ang I nitration, Fig. 5. The results show that copper(II) coordinated by peptides at a concentration as low as 25 μ M, which is actually close to the physiological concentration of cupric ions,²⁵ can catalyze efficiently oxidation or nitration of peptides. However, it is hard to suggest that A β 16 acts as an antioxidant or a pro-oxidant since less efficient oxidation was observed meanwhile.

Fig. 5 Ang I (0.25 mM) nitration and oxidation induced by ascorbic acid (1 mM), NO_2^- (1 mM) and $CuCl_2$ (0.025 mM) at 37 °C, with persistent shaking and various buffer conditions. NH_4HCO_3 at different concentrations was used to regulate the pH. For A β 16 Cu^{2+} induced nitration, $CuCl_2$ (0.025 mM) was mixed with the 0.1 mM A β 16 peptide before the addition of Ang I (0.25 mM), ascorbic acid (1 mM) and nitrite (1 mM) to ensure the coordination of copper(II) by A β 16 peptides. The reaction time was 18 hours. The left y-axis shows the Ang I oxidation while the right y-axis shows the Ang I nitration. Note the difference in different y-axis ranges. The error bars show the standard deviations. Three experiments were conducted to obtain the standard deviations.

The results in Fig. 1 and 3 were obtained under weak acidic conditions (pH 6.0). Influence of proton concentration on the peptide oxidation and nitration was further studied. The reaction was performed in NH_4HCO_3 buffer at various concentrations. As shown in Fig. 5, nitration happened under the weak acidic conditions (pH 6.0, 6.5) but not neutral or weak basic conditions. The pH conditions for many human tissues are around neutral, e.g. 7.4 for blood and brain. However, the local pH can be mildly acidic when there is physiological acidosis that often comes along with diseases, such as tumor and Alzheimer's disease.^{38,39} The altered neuron H^+ homeostasis was observed in common with the appearance of amyloid deposits.³⁹ It was reported that the Alzheimer's disease is complicated by cerebral acidosis of pH 6.6.⁴⁰ Indeed, there are studies on the pH influence of A β peptide aggregation induced by copper(II) showing that the aggregation emerged under weak acidic conditions.^{39,41}

Conclusion

In conclusion, we have demonstrated that tyrosine nitration can be induced by ascorbic acid, nitrite and copper(II) or peptide coordinated copper(II) under weak acidic and aerobic conditions via an *in vitro* protocol. The phenomenon is important for several reasons: (i) the concentration of peptide coordinated copper(II) as low as 25 μ M that is close to physiological concentration can already catalyze tyrosine nitration; (ii) nitration was suggested to be important for many diseases; (iii) accumulation of copper(II) was found in Alzheimer's disease. Our finding may establish a potential connection between tyrosine nitration and copper(II) toxicity for neurodegenerative diseases.

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