The Kinetics of Peroxynitrite on Insulin Nitration Damage

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Research article

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Abstract

Background: Insulin is one of the most important versatile hormones that is central to regulating the energy and glucose metabolism in the body. There has been accumulating evidence supporting that diabetes was associated with peroxynitrite and protein nitration, and insulin nitration induced by peroxynitrite affected its biological activity.

Methods: In this paper, the kinetics of insulin nitration by peroxynitrite in physiological conditions was studied by the stopped flow technique.

Results: We determined the values of the reactive rate constants of peroxynitrite decomposition and peroxynitrite-induced tyrosine nitration in the presence of insulin. The activation energy of peroxynitrite decomposition and 3-nitrotyrosine yield in the presence of insulin is 48.8 kJ·mol$^{-1}$ and 42.7 kJ·mol$^{-1}$ respectively.

Conclusions: It is inferred that the glutamate residue of insulin accelerated peroxynitrite decomposition and tyrosine nitration by reducing the activation energy of reactions. The results could be beneficial for exploring the molecular mechanism of diabetes and offering a new target for diabetes therapies.

Background

Peroxynitrite is a major cytotoxic species in vivo that is a potent oxidizing and nitrating agent, which is able to react with a wide range of biomolecules including DNAs, peptides, proteins, sugars and lipids (Szabo & Ischiropoulos & Radi, 2007; Radi, 2018). There are growing evidences that peroxynitrite is involved in many diseases such as inflammation, atherosclerosis, cancere and diabetes (Radi 2013; Speckmann et al. 2016). Diabetes is a severe disease and its chronic complications threaten patient health and can even be fatal. It was reported that peroxynitrite is closely related to diabetes and its complications (Stadler, 2011). Delaney et al. and Mathews et al. found that the amount of the antioxidant (such as glutathione) content and antioxidant enzyme in human pancreatic β-cells were very low, and that the cells were easily damaged by oxidative substances such as free radicals. The infusion of ONOO$^-$ into mature pancreatic β-cells significantly increased the amount of protein nitration products in cells and also decreased glucose absorption. Moreover, high glucose levels could exacerbate endothelial cell injury and lead to the continuous generation of peroxynitrite to further increase the nitration damage (Delaney et al., 1996; Mathews et al., 2005). Clinical research indicates that the vascular complications caused by diabetes are the main reason for mortality in diabetes patients. Moreover, the occurrence and development of vascular complications are highly related to increased oxidation and nitration stress levels under high blood glucose conditions. Ceriello et al. and Tannous et al. found that the concentrations of ONOO$^-$ and 3-nitrotyrosine, a protein nitration product, were significantly increased in the plasma of diabetes patients (Ceriello et al., 2001; Tannous et al., 1999). Therefore, peroxynitrite-induced protein nitration damage could play a very important role in the pathogenesis of diabetes and its complications.
Insulin is the only hormone in human body that can simultaneously promote the synthesis of saccharides, lipids, and proteins and regulate metabolism in the human body. Insulin is composed by the A and B chains; chain A contains 21 amino acids and chain B contains 30 amino acids. The A and B chains are linked together by two disulfide bonds: A7-B7 and A20-B19. Chain A contains another intrachain disulfide bond at A6-A11. In the crystal structure, chain A contains two α-helices (A1-A8 and A12-A18) and an irregular turn. Chain B contains an α-helix (B9-B19), two β-sheets (B1-B5 and B24-B30), and two β-turns (B6-B9 and B20-B23)(Baker et al., 1988). Mature insulin has four tyrosine residues, three of which lie in the classic binding domain between insulin and its receptor: Tyr-A19, Tyr-B16, and Tyr-B26. Chi et al. indicated that the tyrosine nitration could influence the bioactivity of insulin. The receptor-binding capacity of mono-nitrated insulin was ~70% of that of insulin, and the hypoglycemic efficiency was 75% (Chi & Wang & Huang, 2005). This suggests that the nitrilation of insulin could lead to insulin resistance and diabetes. Thus, it is necessary to explore the reaction kinetics and molecular mechanism by which ONOO− nitrates insulin. A complete insulin molecular nitration model should also be established to provide the basis for exploring the biological effects of insulin nitration damage.

Methods

Preparation for peroxynitrous acid

The peroxynitrite solution was prepared according to the two-phase reaction system proposed by Rao and William(Uppu & Pryor, 1996). The mechanism involves the substitution reaction of the hydrogen peroxide anion (aqueous phase) on isoamyl nitrite (organic phase). The product, peroxynitrite, stays in the aqueous phase, and isoamyl alcohol and unreacted isoamyl nitrite form a new organic phase. The preparation steps were as follows: 1. Ten milliliters of 30% H2O2 solution was added into a 100-mL volumetric flask using a 10-mL pipette, diluted with a small amount of pure water, and cooled in an ice-water bath. Next, NaOH (18 mL, 5 mol·L−1) was added into the volumetric ask and diluted with pure water to 100 mL. 2. The above basic H2O2 solution (100 mL) was added into a 200 mL Florence flask, and 13 mL of isoamyl nitrite was also added to create a molar weight equal to H2O2. The reaction was then incubated at 0–4 °C for ~8 h. After the reaction was stopped, the peroxynitrite solution was separated using a 500-mL separating funnel, and the water solution was extracted by a two-fold volume of n-hexane to remove the organic phase. The resulting water solution was basic peroxynitrite solution. 3. In an ice bath, 20 g manganese dioxide powder was added to the peroxynitrite solution and the solution was shaken to complete the reaction. Then, a cellulose ester microporous filter membrane (0.45 µm) was used to remove the manganese dioxide powder. 4. The absorbance of the peroxynitrite was detected using UV-vis spectroscopy at 302 nm, and the concentration was calculated according to equation ε302 = 1670 M−1cm−1 (Uppu & Pryor, 1996). The concentration of peroxynitrite was measured twice and the mean value from the two measurements was used for analysis.

Kinetics Of Peroxynitrite Decomposition In The Presence Of Insulin
The fast-reaction kinetics curve of the peroxynitrite (0.1 mM) decomposition reaction in the presence of 0.1 mM insulin (dissolved in 0.1 M phosphate buffer pH 7.4) was detected using retention kinetics equipment, and the apparent rate constant was calculated from the kinetics curve using Bio-Kine 32 software. The UV-vis absorption wavelength for detecting peroxynitrite was set at 302 nm. The relative proportion parameters of the retention kinetics equipment were adjusted so that the reaction was conducted in the phosphate buffer (PB, 0.1 M, pH 7.4) containing 0.1 mM DTPA. Phosphate buffer was injected into sample injector 1, and insulin was dissolved in PB and injected into sample injector 2. Peroxynitrite was diluted 100-fold with water and injected into sample injector 3. The data were obtained after each experiment was performed seven times.

**Kinetics Of The Effects Of Peroxynitrite On Insulin Damage**

The fast reaction kinetics curve of the 3-nitrotyrosine generated by the nitration reaction between 0.1 mM peroxynitrite and 0.1 mM insulin (dissolved in 0.1 M PB, pH 7.4) was detected using retention kinetics equipment; the apparent rate constant was then calculated from the kinetics curve using Bio-Kine software. The UV-vis absorption wavelength for detecting 3-nitrotyrosine was 418 nm. The relative proportion parameters of the retention kinetics equipment were adjusted so that the reaction was conducted in PB (0.1 M, pH 7.4) containing 0.1 mM DTPA. PB was injected into sample injector 1, and insulin was dissolved in PB and injected into sample injector 2. Peroxynitrite was diluted 100-fold with water and injected into sample injector 3. The data were obtained after each experiment was performed seven times.

**Detecting The Activation Energy**

The apparent rate constants of peroxynitrite decomposition in the presence of insulin and the 3-nitrotyrosine generated by peroxynitrite-induced insulin nitration damage were detected at 4 °C, 15 °C, 25 °C, and 37 °C. The activation energy was calculated from the slope of the lnk-1/T profile according to the Arrhenius theorem: $k = Ae^{-(E_a/RT)}$ or $\ln k = \ln A - (E_a/RT)$, and, by plotting $\ln k$ versus $1/T$, a straight line was obtained, the slope of which can be used to calculate the activation energy $E_a$.

**Results**

**Kinetics of Peroxynitrite Decomposition in the Presence of Insulin**

Fast reaction kinetics were used to investigate the spontaneous decomposition of peroxynitrite and the decomposition reaction kinetics in the presence of insulin. The kinetic curve of peroxynitrite is shown in Fig. 1. The data showed the presence of insulin increased the decomposition of peroxynitrite. The decomposition reaction of peroxynitrite in PBS solution was a first-order reaction. Kinetics curve simulation software revealed that the apparent rate constant at 37 °C was 1.34 s$^{-1}$. The half-life of peroxynitrite spontaneous decomposition, as calculated using equation $t_{1/2} = \ln 2/k_{obs}$, was 0.52 s.
The kinetics curve of peroxynitrite decomposition in the presence of insulin is shown in Figs. 1-b. Data fitting revealed that the decomposition of peroxynitrite in the presence of insulin complied with monoexponential function, which was a first-order reaction (Fig. 2). The kinetics curve simulation software was used to calculate that the apparent rate constant at 37 °C was 2.05 s\(^{-1}\). The half-life of peroxynitrite in the presence of insulin, as calculated using the equation \(t_{1/2} = \ln 2/k_{obs}\), was 0.33 s. The presence of insulin increased the decomposition reaction rate constant of peroxynitrite and decreased the half-life, suggesting that insulin promoted the decomposition of peroxynitrite.

We have detected the reaction rate constants of peroxynitrite decomposition in the presence of different concentrations of insulin (0.05, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4 mM). When the insulin concentration changed, the reaction rate constant of peroxynitrite decomposition remained consistent (\(k_{obs} = 2.03 \pm 0.03\) s\(^{-1}\)), which suggests that changing the insulin concentration did not affect the decomposition of peroxynitrite. This further confirmed that the decomposition of peroxynitrite was a first-order reaction in the presence of insulin.

The Activation Energy of Peroxynitrite Decomposition in the Presence of Insulin

The apparent rate constants of the decomposition of peroxynitrite at 4 °C, 15 °C, 25 °C, and 37 °C in the presence of insulin was detected using retention fast reaction kinetics. Then, the logarithm of the reaction rate constants of peroxynitrite decomposition at different temperatures and a plot of the reciprocal of temperature were used to draw the Arrhenius curves of peroxynitrite composition (Fig. 3). According to the Arrhenius theorem, the activation energy (\(E_a\)) of peroxynitrite decomposition in the presence of insulin was 48.8 kJ·mol\(^{-1}\). The activation energy of the spontaneous decomposition of peroxynitrite was 80.8 kJ·mol\(^{-1}\). The comparison between the two values suggested that insulin decreased the activation energy of peroxynitrite decomposition and accelerated the rate of peroxynitrite decomposition.

Kinetics Of The Effects Of Peroxynitrite On Insulin Damage

Next, the signal of 3-nitrotyrosine, a product generated by peroxynitrite nitration damage to the tyrosine residues of insulin, was detected using a UV-detector to draw a kinetics curve for 3-nitrotyrosine generation (Fig. 4). In addition, the apparent rate constant of the generation of 3-nitrotyrosine by peroxynitrite nitration-induced insulin damage was detected at different temperatures (4 °C, 15 °C, 25 °C, and 37 °C). Then, the logarithm of the reaction rate constants of peroxynitrite decomposition at different temperatures and a plot of the reciprocal of temperature were used to draw the Arrhenius curves of 3-nitrotyrosine (Fig. 5). According to the Arrhenius theorem, the activation energy (\(E_a\)) of 3-nitrotyrosine was 42.7 kJ·mol\(^{-1}\). However, the activation energy of peroxynitrite nitrating free tyrosine is 75.3 kJ·mol\(^{-1}\). This comparison suggests that insulin decreased the activation energy of peroxynitrite-induced tyrosine nitration and accelerated the generation rate of 3-nitrotyrosine generated by peroxynitrite nitrating tyrosine.

Discussion
In this paper, the kinetics of insulin nitration by peroxynitrite in physiological conditions was studied by the stopped flow technique. The values are determined of the reactive rate constants of peroxynitrite decomposition and peroxynitrite-induced tyrosine nitration in the presence of insulin. The kinetic results of peroxynitrite decomposition in the absence and presence of insulin showed that insulin increased the decomposition reaction rate constant of peroxynitrite and decreased the half-life, suggesting that insulin promoted the decomposition of peroxynitrite.

Fast reaction kinetics and the Arrhenius theorem were used to determine that the activation energy of peroxynitrite decomposition in the presence of insulin was 48.8 kJ·mol\(^{-1}\) and that the activation energy of 3-nitrotyrosine generated by the peroxynitrite-induced nitration of insulin tyrosine residues was 42.7 kJ·mol\(^{-1}\). In the absence of insulin, the activation energy of the spontaneous decomposition of peroxynitrous acid is 80.8 kJ·mol\(^{-1}\) and the activation energy of the peroxynitrous acid-induced nitration of free tyrosine is 75.3 kJ·mol\(^{-1}\). In contrast, in the presence of insulin the activation energy of peroxynitrite decomposition was decreased by 32.0 kJ·mol\(^{-1}\) and the activation energy of 3-nitrotyrosine generation was decreased by 32.6 kJ·mol\(^{-1}\); the decrease of both parameters had a similar magnitude. This suggests that insulin promoted the decomposition of peroxynitrite and tyrosine nitration at the same time.

In the current study, we analyzed the kinetics of peroxynitrite decomposition and 3-nitrotyrosine generation when peroxynitrite was damaging insulin. As shown in Fig. 7, the decomposition of peroxynitrite occurred concurrently with 3-nitrotyrosine generation, and both reactions finished within 3 s. When examining the nitration activity of peroxynitrite on 4-glycolic acid, Beckman et al. found that the nitration rate of 4-glycolic acid was very close to the spontaneous decomposition rate of peroxynitrite.(Beckman et al., 1992). Thus, they concluded that tyrosine nitration and peroxynitrite decomposition shared similar reaction mechanisms. Gunaydin et al. applied quantum computational chemistry to demonstrate that the rate-limiting reaction of tyrosine nitration was the decomposition of peroxynitrite(Gunaydin & Houk, 2009); therefore, both reactions were in theory conducted via a similar pathway. Therefore, we deduced that the decomposition of peroxynitrite shared a similar reaction pathway and mechanism with 3-nitrotyrosine generation during peroxynitrite-induced insulin damage.

During peroxynitrite-induced insulin damage, we analyzed the reason for the decrease in the activation energy of peroxynitrite decomposition and 3-nitrotyrosine generation. Souza et al. studied protein-selective modifications and found that the selective modification of tyrosine in proteins was closely related to the protein microenvironment of tyrosine residues(Souza et al., 1999). Hollenberg et al. used site-directed mutagenesis to demonstrate that Glu-49, which is adjacent to P450, in cytochrome C mediated the selective nitration of Tyr-190(Lin et al., 2003; Lin et al., 2005). During a study on the SOD-catalyzed nitration of peroxynitrite on tyrosine residues, Crow et al. found that the nitration of peroxynitrite was closely related to the protein environment(Crow et al., 1997). Acidic amino acids such as glutamic acid can promote the nitration of tyrosine residues. Based on quantum computational chemistry, Zhang et al. demonstrated that glutamic acid anions could promote the nitration of tyrosine
residues by increasing their ionization, stabilizing the intermediate, and mediating proton transfer (Zhang & Huang, 2008).

Chain A of insulin contains two tyrosines, Tyr-14 and Tyr-19, and there are two acidic amino acids (Glu-4 and Glu-17) nearby, as illustrated in Fig. 7. Similarly, chain B also two tyrosines (Tyr-16 and Tyr-26) with two acidic amino acids (Glu-4 and Glu-17) nearby (Fig. 8). It is presumed that the presence of glutamic acid residues near tyrosine in insulin facilitates the reaction between tyrosine residues and peroxynitrite. Furthermore, it promotes 3-nitrotyrosine generation and peroxynitrite decomposition by decreasing the activation energy simultaneously.

Conclusions

In this work, the kinetics of insulin nitration by peroxynitrite in physiological conditions was studied by the stopped flow technique. The results showed that the presence of insulin increased the decomposition reaction rate constant of peroxynitrite and decreased the half-life, suggesting that insulin promoted the decomposition of peroxynitrite. Meanwhile, kinetics of the effects of peroxynitrite on insulin damage by monitoring 3-nitrotyrosine generation. The values are determined of the reactive rate constants of peroxynitrite decomposition and peroxynitrite-induced tyrosine nitration in the presence of insulin. The activation energy of peroxynitrite decomposition and 3-nitrotyrosine yield in the presence of insulin is 48.8KJ·mol⁻¹ and 42.7 kJ·mol⁻¹ respectively. It is inferred that the glutamate residue of insulin accelerated peroxynitrite decomposition and tyrosine nitration by reducing the activation energy of reactions. The results could be beneficial for exploring the molecular mechanism of diabetes and offering a new target for diabetes therapies.

Abbreviations

3-NT, 3-nitrotyrosine; Tyr, tyrosine; PB, phosphate buffer; DTPA, diethylenetriaminepentaacetic acid; UV-vis, ultraviolet–visible.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Competing interests

The authors declare no conflict of interest.

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Authors’ contributions

Zhang C.X. carried out the experiments and drafted the manuscript. Sun F.X. participated in drafting the manuscript. Zhang C.J. participated in the data processing. Luo Y.J. participated in the design of the experiments.

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**Figures**
Figure 1

Kinetics curve of peroxynitrite decomposition at 302 nm.

a) without insulin

b) 0.1mM insulin
Figure 2

Logarithmic plot of the kinetics curve of peroxynitrite decomposition in the presence of insulin at 302 nm.

Figure 3

Arrhenius curves of peroxynitrite decomposition in the presence of insulin.
Figure 4

Kinetics curve of the generation of 3-nitrotyrosine caused by peroxynitrite-induced insulin damage.
Figure 5

Arrhenius curve of the generation of 3-nitrotyrosine caused by peroxynitrite-induced insulin damage.
Figure 6

Kinetics curve of peroxynitrite decomposition and 3-nitrotyrosine generation during peroxynitrite-induced insulin damage.

Figure 7
Spatial structure of insulin chain A.

**Figure 8**

Spatial structure of insulin chain B.