ESR studies on reaction of saccharide with the free radicals generated from the xanthine oxidase/hypoxanthine system containing iron

Gui-min Luo[a,*], Dan-hua Qi[a], Ying-guang Zheng[a], Ying Mu[a], Gang-ling Yan[a], Tong-shu Yang[a], Jia-Cong Shen[b]

[a] *Key Laboratory for Molecular Enzymology and Engineering of Ministry of Education, Jilin University, 125 Jiefang Road, Changchun 130023, PR China*

[b] *Key Laboratory for Supramolecular Structure and Spectroscopy of Ministry of Education, Jilin University, 119 Jiefang Road, Changchun 130023, PR China*

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Abstract The free radicals generated from the iron containing system of xanthine oxidase and hypoxanthine (Fe-XO/HX) were directly detected by using spin trapping. It was found that not only superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$), but also alkyl or alkoxyl radicals ($R^{\bullet}$) were formed when saccharides such as glucose, fructose and sucrose were added into the Fe-XO/HX system. The generated amount of $R^{\bullet}$ was dependent on the kind and concentration of saccharides added into the Fe-XO/HX system and no $R^{\bullet}$ were detected in the absence of saccharides, indicating that there is an interaction between the saccharide molecules and the free radicals generated from the Fe-XO/HX system and saccharide molecules are essential for generating $R^{\bullet}$ in the Fe-XO/HX system. It is expected that the toxicity of $R^{\bullet}$ would be greater than of hydrophilic $O_2^{\bullet-}$ and $OH^{\bullet}$ because they are liposoluble and their lives are longer and the active sites of biomolecules are closely related with lipophilic phase, thus they can damage cells more seriously than $O_2^{\bullet-}$ and $OH^{\bullet}$. The $R^{\bullet}$ generated from the saccharide containing Fe-XO/HX can be effectively scavenged by selenium containing abzyme (Se-abzyme), indicating Se-abzyme is a promising antioxidant.

Key words: Alkyl/alkoxyl radical; Free radical; Xanthine oxidase/hypoxanthine system; Saccharide; Electron spin resonance; Selenium containing abzyme

1. Introduction

The xanthine oxidase/hypoxanthine (XO/HX) system generates superoxide anion ($O_2^{\bullet-}$) [1], but the iron containing XO/HX (Fe-XO/HX) system generates hydroxyl radical ($OH^{\bullet}$) [2]. We have directly detected the radicals from the Fe-XO/HX system using electron spin resonance (ESR) spin trapping [3] and found for the first time that when sucrose, glucose, and fructose were added into the Fe-XO/HX system respectively, the system not only produced $OH^{\bullet}$, but also generated alkyl/alkoxyl radical($R^{\bullet}$), and the concentration of the generated $R^{\bullet}$ was increased with the increase of saccharide concentrations, and no $R^{\bullet}$ was detected in the absence of the saccharides, indicating that there is an interaction between the saccharide molecules and the free radicals generated from the Fe-XO/HX system and the saccharide molecules are essential for generation of $R^{\bullet}$ in the Fe-XO/HX system. Although autoxidation of glucose under physiological condition has been reported [4], so far there is no report on the oxidation of other saccharides in the Fe-XO/HX system. Because there is the Fe-XO/HX system in vivo under physiological condition, it is important to study the oxidation of saccharides in the Fe-XO/HX system for evaluating the diabetes pathogenesis and developing respective antioxidant drugs [5].

2. Materials and methods

2.1. Materials

HX, XO, D-glucose, D-fructose, sucrose, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and tetra-pyridyl-1-oxide-*N*-*t*-butyl nitrone (POBN) were obtained from Sigma. Mitochondria (MT) were isolated and prepared from bovine heart according to [6]. Selenium containing abzyme (Se-abzyme) with glutathione peroxidase (GPX) activity was prepared according to [7]. All other reagents were of analytical grade.

2.2. The Fe-XO/HX system

The radical generation system was used, which consists of 0.02 mM HX, 0.3 mM ethylenediamine tetraacetic acid (EDTA), 0.01 mM $FeCl_3$, 0.016 U/ml XO, 0.02 M Tris–HCl buffer, pH 7.4, 0.15 M KCl. Total volume of the system was 1 ml, and the reaction was initiated by adding XO and carried out at 37°C in the water bath with shaking. HX and $FeCl_3$ were prepared before use. The system was also used for damaging MT in the presence and absence of saccharides.

2.3. Spin trapping

The ESR spectra were measured with a Bruker ESR spectrometer. The settings of the spectrometer were as follows: microwave frequency, 9.77 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation intensity, 0.125 mT; central magnetic field, 348.0 mT; scan range, 10.0 mT. Spin traps were DMPO and POBN, which concentration was 0.1 M. In order to quantitatively determine the change of the relative concentrations of radicals generated from the XO/HX system, the standard quartz glass tube with a diameter of 1 mm was used in all spin trapping experiments and 0.25 μl of DMPO and POBN was taken each time.

*Correspondence author. Fax: (86)-431-8923907.
E-mail: gmluo@mail.jlu.edu.cn

Abbreviations: MT, mitochondria; XO, xanthine oxidase; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; POBN, tetra-pyridyl-1-oxide-*N*-*t*-butyl nitrone; XO/HX, xanthine oxidase/hypoxanthine system; HX, hypoxanthine; Fe-XO/HX, xanthine oxidase/hypoxanthine system containing iron; Se-abzyme, selenium containing abzyme; DETPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; GPX, glutathione peroxidase; $R^{\bullet}$, alkyl or alkoxyl radical; $O_2^{\bullet-}$, superoxide anion; $OH^{\bullet}$, hydroxyl radical

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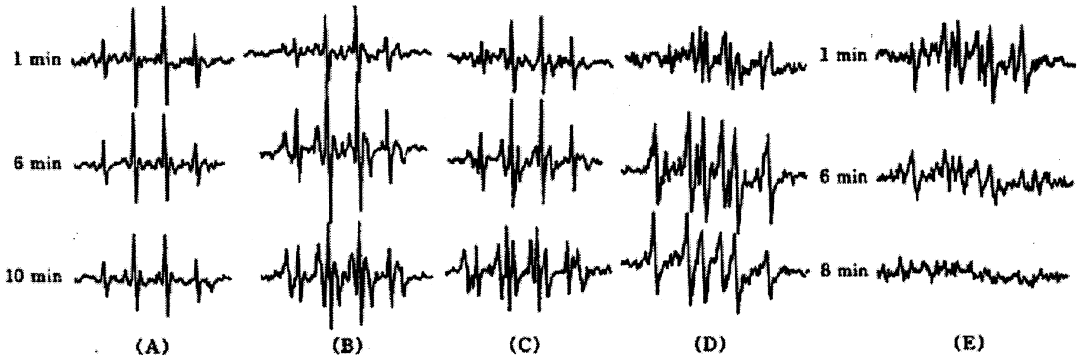

Fig. 1. ESR spectra of radicals trapped by DMPO in the Fe-XO/HX system in the presence of saccharide. (A) Without saccharide; (B) 15 mM fructose; (C) 15 mM glucose; (D) 15 mM sucrose; (E) 15 mM sucrose and 10^{-3} U/ml Se-abzyme.

The procedure was as follows: the reaction solution with constant temperature was taken and mixed with the spin trap in the small container, then XO was added to initiate the reaction and the sample was immediately drawn into a standard quartz glass tube to scan and record the ESR spectra. For examining the protection of Se-abzyme, the same procedure was carried out with exception of adding a certain concentration of Se-abzyme before initiating the reaction.

3. Results

3.1. The free radicals generated from Fe-XO/HX system

The Fe-XO/HX system was used to generate radicals and DMPO was used as a spin trap and the ESR spectra of the radicals trapped by DMPO were shown in Fig. 1A. The 1:2:2:1 quartet shown in Fig. 1A is a typical signal of the adduct formed by DMPO and $OH^{\bullet}$ ($a_N = a_H = 1.49$ mT). The intensity of the quartet was slightly decreased with time, but no other kind of spectrum peak was observed, indicating that only $OH^{\bullet}$ was generated from the Fe-XO/HX system.

3.2. The radicals produced by saccharides in the Fe-XO/HX system

The fructose, glucose, and sucrose were added into the Fe-XO/HX system respectively and the ESR spectra trapped by DMPO were shown in Fig. 1B–D. In comparison with Fig. 1A it was found that the 6-line ESR spectrum with equivalent intensity ($a_H = 2.26$ mT; $a_N = 1.58$ mT) was produced in addition to the original quartet spectrum of DMPO-OH. It was shown by analysis of the spectra that the 6-line ESR spectrum results from the $R^{\bullet}$ trapped by DMPO. It was known from Fig. 1B–D that the generation rate of $R^{\bullet}$ and its concentration were dependent on the kind of saccharide and the fastest rate of $R^{\bullet}$ generation was obtained in the presence of sucrose (see Fig. 1D). It was also found that the intensity of the 6-line spectrum was dependent on the concentration of the added saccharide. Fig. 2A,B showed the 6-line spectra of the radicals trapped by DMPO at different time when 75 and 15 mM of fructoses were added into the Fe-XO/HX system respectively. For the former, the 6-line spectra appeared at the beginning of the reaction, but for the latter, the 6-line spectra appeared much later than the former. The ratios between the relative intensities of $R^{\bullet}$ and $OH^{\bullet}$ at different time were calculated using the spectrum intensities of $R^{\bullet}(\downarrow)$ and $OH^{\bullet}(*)$ and listed in Table 1. Table 1 shows that the intensity of $R^{\bullet}$ was directly related to the fructose concentration in the Fe-XO/HX system. The higher the fructose concentration was, the faster and the more the $R^{\bullet}$ was generated.

Table 1
Effect of different concentration of fructose on the relative concentration ratio between $R^{\bullet}$ and $OH^{\bullet}$ produced at different times in the Fe-XO/HX system

Time (min)	$[R^{\bullet}]/[OH^{\bullet}]$	
	75 mM (fructose)	15 mM (fructose)
1	0.40:1	0.40:1
6	2.0:1	0.45:1
11	3.0:1	0.83:1

3.3. ESR spectra of the radicals trapped by POBN

In order to further demonstrate that the $R^{\bullet}$ could be generated by saccharides in the Fe-XO/HX system, the experiment above was repeated using lipid spin trap, POBN, and the same result was obtained (Fig. 3A). The ESR spectrum consisted of 6 lines with $a_H = 0.23$ mT; $a_N = 1.58$ mT, which is typical for the POBN-R spin adduct[8], indicating that there is $R^{\bullet}$ in the saccharide containing Fe-XO/HX system indeed.

3.4. Quenching of the radicals in the saccharide containing Fe-XO/HX system

When 10^{-3} U/ml of Se-abzyme with GPX activity was added into the sucrose containing Fe-XO/HX system and DMPO was used as spin trap, the generation of $R^{\bullet}$ was effectively inhibited (Fig. 1E). It is shown in Fig. 1E that the radicals basically disappeared at an interval of 8 min for the reaction, indicating that the ability for Se-abzyme to scavenge $R^{\bullet}$ free radicals is very strong.

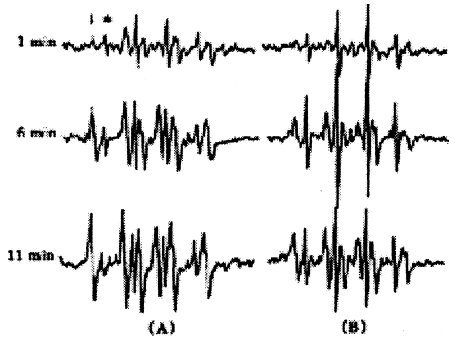

Fig. 2. ESR spectra of the radicals trapped by DMPO when the different concentrations of fructose were added into the Fe-XO/HX system. (A) 75 mM fructose; (B) 15 mM fructose.

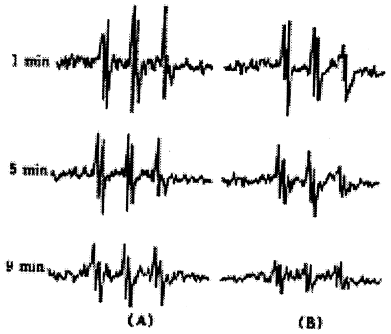

Fig. 3. ESR spectra of R• radicals trapped by POBN in Fe-XO/HX system in the presence of glucose. (A) 15 mM glucose; (B) 15 mM glucose and Se-abzyme (10^{-3} U/ml).

When 10^{-3} U/ml of the Se-abzyme was added into the glucose containing Fe-XO/HX system and POBN was used as a spin trap, the concentration of R• decreased by more than 50% on the basis of calculation of the ESR spectrum at an interval of 9 min for the reaction (Fig. 3B), indicating that the Se-abzyme could effectively scavenge R• free radicals.

3.5. The course of generating R• in the saccharide containing Fe-XO/HX system

In general, it is not easy to detect $O_2^{\bullet-}$ in the Fe-XO/HX system because $O_2^{\bullet-}$ in this system is fast converted to OH• by the Fenton reaction, thus, only OH• could be trapped by DMPO. In order to know the details of the reaction, diethylenetriaminepentaacetic acid (DETPA) instead of Fe^{3+} was added into the XO/HX system and the signal of DMPO-OOH was detected immediately. Fig. 4 shows the ESR spectra of the radicals trapped by DMPO in the DETPA and glucose containing XO/HX system. It is shown in Fig. 4A that $O_2^{\bullet-}$ was basically generated at 1–3 min, the ESR spectrum of $O_2^{\bullet-}$ was converted to the 1:2:2:1 quartet of OH• at 5 min, and the spectrum was becoming complex at 11 min. Analysis showed that the 6-line spectrum with equivalent intensity gradually appeared in addition to the original spectrum of OH•. In order to demonstrate this conjecture we used a computer to simulate the experimental spectra (Fig. 4B). The spectrum parameters obtained from the simulated spectra are as follows: DMPO-OH: $a_H = a_N = 1.49$ mT; DMPO-OOH: $a_N = 1.43$ mT, $a_{H1} = 1.13$ mT, $a_{H2} = 0.13$ mT; DMPO-R: $a_N = 1.58$ mT, $a_H = 2.26$ mT. The results are consistent with [9].

3.6. MT damage enhanced by saccharide and protection of MT by Se-abzyme

As we know now, MT are important cellular sites of both production of reactive oxygen species and oxidative damage by these species. When MT are attacked by free radicals, changes of their composition, morphology, structure, integrity and function take place. These changes are similar to what happens in cardiac diseases. We used the XO/HX, free radical generation system, to imitate the abnormal state of environment in vivo and demonstrated that MT damage was enhanced by saccharide in the Fe-XO/HX system and Se-abzyme could protect MT against the damage using the MT swelling as a measure of MT damage (Fig. 5). Swelling was measured as the decrease of absorbance of reaction mixture at 520 nm. The decrease of the absorbance indicates the increase

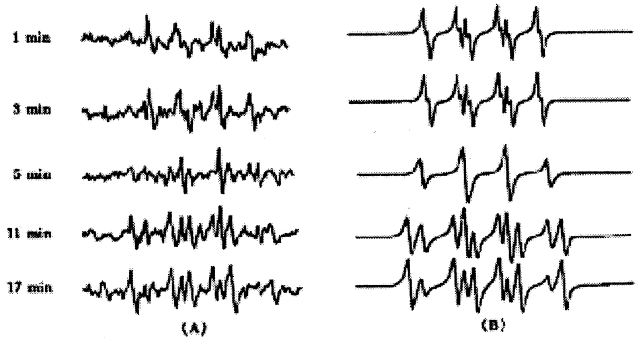

Fig. 4. ESR spectra of the radicals trapped by DMPO at different time when metal chelating agent DETPA was added into the glucose containing XO/HX system. (A) Experimental spectra; (B) simulated spectra. 1 min, $O_2^{\bullet-}$; 3 min, $O_2^{\bullet-}$; 5 min, OH•; 11 min, [R•]:[OH•] = 1.5:1; 17 min, [R•]:[OH•] = 2:1.

of the MT swelling and the decrease of the MT integrity. It is shown in Fig. 5 that at the same conditions (the MT were damaged for 5 min in the Fe-XO/HX system) the absorbance at 520 nm for control group was basically constant, while the absorbance for damage group was considerably decreased with time, indicating that the MT swelling was considerably increased. When the Fe-XO/HX system contains 15 mM of sucrose, the system gave MT more serious damage. But the swelling for the protection group, which contained a certain concentration of Se-abzyme, was apparently decreased, and the MT swelling was decreased with increase of Se-abzyme concentration. This observation is consistent with the result shown in Fig. 1E, indicating that this Se-abzyme possesses a great antioxidative ability and it could scavenge free radicals and prevent MT from oxidative damage by active oxygen. This laid the experimental foundation for the application of Se-abzyme as a medicine.

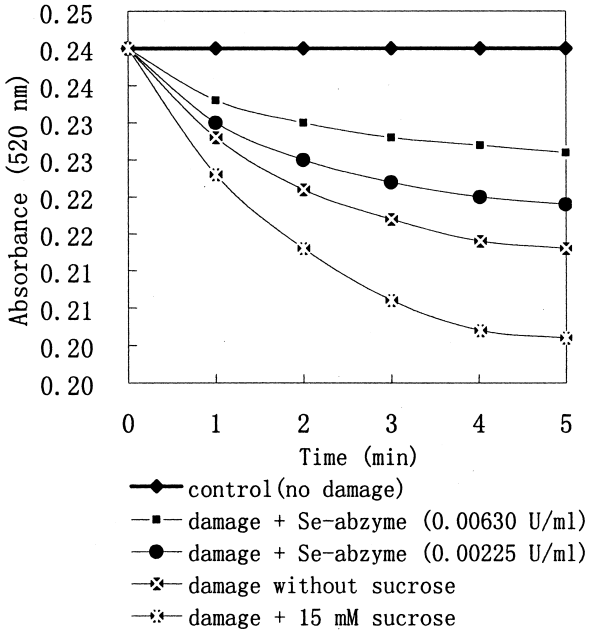

Fig. 5. Effect of Se-abzyme on the swelling of the MT.

4. Discussion

It is shown in Fig. 4 that at the beginning of the reaction the main $O_2^{\bullet-}$ was generated and the $O_2^{\bullet-}$ was gradually converted into $OH^{\bullet}$ with time. A large amount of $R^{\bullet}$ appeared in 11 min and the ratio of the radical concentrations, $[R^{\bullet}]$:$[OH^{\bullet}]$, was 1.5:1 at this time and the ratio was 2:1 in 17 min. When iron ions were in the XO/HX system, no $O_2^{\bullet-}$ was detected and $OH^{\bullet}$ and $R^{\bullet}$ appeared rapidly. Moreover, no $R^{\bullet}$ was detected in the Fe-XO/HX system in the absence of saccharides. Thus, the experimental results and the simulated spectra demonstrated that not only $OH^{\bullet}$ and $O_2^{\bullet-}$, but also $R^{\bullet}$ could be generated from saccharide containing Fe-XO/HX (or XO/HX) system.

On the basis of the results above and related references we propose the following mechanism for generating $R^{\bullet}$ in the saccharide containing Fe-XO/HX system:

$$HX + 2\,O_2 + 2\,H_2O \xrightarrow{XO} \text{uric acid} + 3\,O_2^{\bullet-} + 4\,H^+ \quad (1)$$

$$HX + O_2 + 2\,H_2O \xrightarrow{XO} \text{uric acid} + 2\,H_2O_2 \quad (2)$$

$$3O_2^{\bullet-} + 4\,H^+ \rightarrow 2\,H_2O_2 + O_2 \quad (3)$$

$$H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + OH^{\bullet} + OH^- \text{ (Haber} - \text{Weiss reaction)} \quad (4)$$

$$O_2^{\bullet-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (5)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^- \text{ (Fenton reaction)} \quad (6)$$

$$\text{Saccharide chain (R)} - H + OH^{\bullet} \rightarrow \text{saccharide derivatives} + R^{\bullet} + H_2O \quad (7)$$

XO catalyzes the reaction of HX and O_2 to give $O_2^{\bullet-}$ and H_2O_2, H_2O_2 reacts with $O_2^{\bullet-}$ to gradually form $OH^{\bullet}$ or Fe^{3+} catalyzes the reaction between H_2O_2 and $O_2^{\bullet-}$ to generate $OH^{\bullet}$. $OH^{\bullet}$ extracts the hydrogen from saccharides to generate $R^{\bullet}$. Because glucose is an aldose, fructose is a ketose and a molecule of sucrose is made up from a molecule of glucose and a molecule of fructose, it is expected that the extraction of hydrogen from the three kinds of saccharides by $OH^{\bullet}$ would take place at different sites of the three kinds of saccharides, thus the alkyl/alkoxyl radicals they produced would have different properties and play different roles in damage of MT. The study of the structures of the alkyl/alkoxyl radicals is now under way.

Because $R^{\bullet}$ is liposoluble and its life is longer and the active sites of biomolecules are closely related with lipophilic phase, it is expected that $R^{\bullet}$ has a greater toxicity than hydrophilic $O_2^{\bullet-}$ and $OH^{\bullet}$. In fact, we have found that the bovine myocardial MT were damaged more seriously by the Fe-XO/HX system containing sucrose than by the Fe-XO/HX without sucrose. It was first reported in China that injection of a low dose of D-galactose into mice could induce changes which resembled accelerated aging [10,11]. Recently, they demonstrated that excessive galactose caused glycation reactions in vivo, resulted in crosslinking of tissues, and finally facilitated aging [12]. But the biochemical mechanism of how the D-galactose acts in vivo to facilitate aging still needs to be further studied. We think the mechanism could be involved in the generation of $R^{\bullet}$. Because there is the Fe-XO/HX system in vivo under physiological conditions and our Fe-XO/HX system is similar to the Fe-XO/HX system in vivo under physiological conditions, our results should be helpful in evaluating the hypothesis that 'Eating excessive sucrose facilitates aging', and Se-abzyme could be an effective antioxidant.

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