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Original Article

Protoporphyrin IX catalyzed hydrogen peroxide to generate singlet oxygen

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Abstract: Aim: To study the role of protoporphyrin IX (pPIX) in mitochondrial metabolism of hydrogen peroxide (H_2O_2). Methods: O_2^- specific fluorescent markers DMA (9,10-dimerthylanthracence) and SOSG (Singlet Oxygen Sensor Green reagent) were used for measurement of singlet oxygen ($^1O_2$). Catalyzing conversion of H_2O_2 into $^1O_2$ by pPIX was monitored in vitro under varied H_2O_2 content, temperature, and PH value in the reaction. Ex vivo mitochondrial model was used to analyze effects of ferrochelatase (FECH) and high energy X-rays on this catalytic reaction. Results: In complete dark, measurable $^1O_2$ was generated when 1.5 mM of H_2O_2 was incubated with 24 μM of pPIX H_2O_2 at 37°C for 3 hours. Mitochondrial yield of H_2O_2 was 0.11±0.03 nmole/mg/min. Mitochondrial FECH significantly improve the catalytic ability of pPIX converting H_2O_2 into $^1O_2$. At presence of high-energy X-ray, incubation of 14.4 μM of pPIX with 0.54 μM of H_2O_2 also generated $^1O_2$, during which the fluorescence density of 1.05 μM of DMA decreased by 41.5% ($P < 0.05$). This conversion was not observed when pPIX was replaced with structurally similar hematoporphyrin. Conclusion: pPIX can catalyze conversion of H_2O_2 into $^1O_2$.

Keywords: Protoporphyrin, singlet oxygen, catalysis

Introduction

More than 90% of energy for cells is supplied by mitochondria via oxidative phosphorylation. In the oxidation process, a portion of electrons escaping from the respiratory chain participate in the conversion of oxygen at concentration of 0.4% to 4% into superoxide radical (O_2^-) [1-3]. The generated O_2^- species are converted to hydrogen peroxide (H_2O_2) by dismutase SOD [4], and H_2O_2 is further broken down into water and oxygen by peroxidase [5].

Porphyrins play a key role in biological process. They are the core structure and the catalytic center of a variety of key enzymes. Porphyrins are necessary for many metabolism process, such as synthesis of chlorophyll and heme, peroxidase mediated breakdown of H_2O_2, and metabolism of oxygen and carbon dioxide. The pPIX, a mitochondrial protoporphyrin, is a precursor compound for plants, algae and bacteria to synthesize chlorophyll, and for animals, birds and bacteria to synthesize heme.

In the present study, we reported that the porphyrin can catalyze hydrogen peroxide to form highly toxic singlet oxygen, which has great potential for the treatment of various human diseases.

Materials and methods

Materials

Protoporphyrin IX (pPIX), PBS-EDTA, xanthine, xanthine oxidase, SOD, catalase, N-Methyl Proto IX, vanillic acid, flavone, Sodium amalgam, Tris-EDTA, various organic solvents, lead tetraacetate, DMSO and singlet oxygen-specific fluorescent compound DMA (9,10-dimerthylanthracence) were purchased from Sigma (Shanghai). SOSG (Singlet Oxygen Sensor Green reagent) was purchased from Molecular Probes Inc. The major equipments used included 970-CRT fluorescence spectrophotometer (Shanghai Jing Branch Shanghai Analytical Instrument Co., Ltd.), MM50 high-energy cyclotron (IBA, Sweden), TG16KR-II desktop high-speed refrigerated centrifuge (Changsha East Wang Experimental Instrument Co., China).

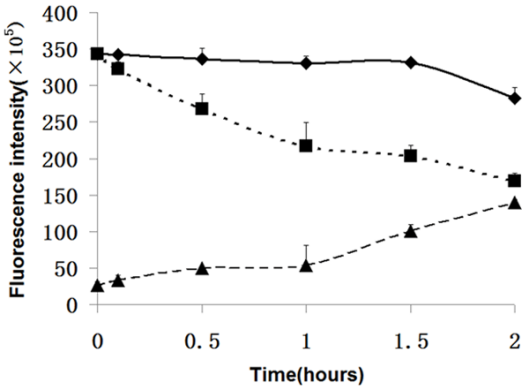

Figure 1. pPIX catalyzed generation of $^1O_2$. 24 μM of pPIX was incubated with 24.3 mM of H_2O_2 at 37°C in the dark. Generation of $^1O_2$ was detected with SOSG (triangular dotted line), and DMA (rhomboid solid line for 4°C, and rectangular dotted line for 37°C). n = 6.

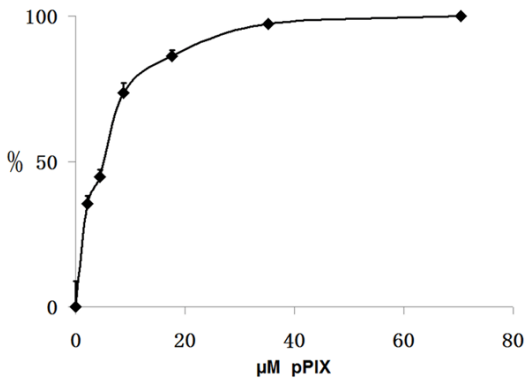

Figure 2. Dose response of conversion of H_2O_2 into $^1O_2$ with a range of pPIX concentrations. 24.3 mM of H_2O_2 was incubated with pPIX at a range of pPIX concentrations, 37°C in dark for 3 hours.

Preparation of protoporphyrinogen IX (Protogen IX) and Protogen IX-DMA solution

As described elsewhere [6], 3 mg of pPIX was dissolved in 25 ml of 20% ethanol. The reduction reaction was carried out in a closed dark locket with N_2 gas, i.e. 800 μl of Proto IX was placed in a 15-ml screw-cap test tube, and the tube was filled with pure N_2, followed by adding 0.5 g of sodium amalgam. The Protogen IX was filter after reaction, and diluted to 9 ml for immediate use. To prepare Protogen IX-DMA solution, 1.2 mg of DMA was dissolved in 20 ml of DMSO dissolved and 200 ml of water was added. The DMA was further diluted by adding 50 ml of DMSO to 30 ml of the DMA stock solution. 1 ml of the diluted DMA solution was added to the 9 ml of Protogen IX to make a total of 10 ml of Protogen IX-DMA solution.

Preparation of other fresh reagents

Peroxydicarbonate amide (Carbamide Peroxide) containing 33% H_2O_2 was diluted with ddH_2O to prepare solutions of a variety of concentrations. DMA solution was prepared by dissolving it in DMF followed by dilution with ddH_2O. pPIX-DMA solution (14.41 μM of pPIX in 2.12 μM of DMA) was prepared by dissolving 3 mg of pPIX in 20 ml DMF, further diluted with 250 ml distilled water and mixed with 100 ml of DMA solution. SOSG was dissolved in methanol, as instructed by the manual, and diluted with ultrapure water.

For ex vivo experiments, 3 mg of pPIX was dissolved in 5 ml of DMF, diluted with 20 ml of PBS (containing 0.1 mM EDTA, PH 7.4). 10 ml of the pPIX solution was further diluted with 80 ml of PBS (containing 0.1 mM EDTA, PH 7.4). Proto IX-DMA solution was prepared by dissolving 1.2 mg of DMA in 20 ml of DMF, diluted with 200 ml of water. DMA solution was prepared by mixing 30 ml of the solution with 50 ml of DMF. 10 ml of DMA solution was added to 90 ml of pPIX solution to make a total of 100 ml pPIX-DMA solution.

Preparation of mitochondria

2 g of liver tissue from a fed young Sprague-Dawley rat was cut into small pieces, homogenized in 40 ml of pre-cooled 0.25 M sucrose ten times at 4°C. Impurities, unbroken cells and cell nuclei were removed by 800 g × 10 min, centrifugation at 4°C. Crude mitochondrias were harvested by 7000 g × 10 min at 4°C centrifugation. The crude mitochondrial components were washed 3 times with 0.25 M sucrose, freezing and thawing 3 times, and stored at -30°C. Protein concentration was determined by Coomassie brilliant blue method. The control specimen was heat inactivate at 100°C, 5 minutes.

In vitro experiments

2 ml of pPIX-DMA was mixed with 2 mL H_2O_2 at different concentrations in test tubes, with 0.1 mL SOSG solution. The varied condition was stated in the results section. All experiments were carried out in dark.

Ex vivo experiments

Yield of H_2O_2 from mitochondria was determined as described elsewhere [7]. To measure

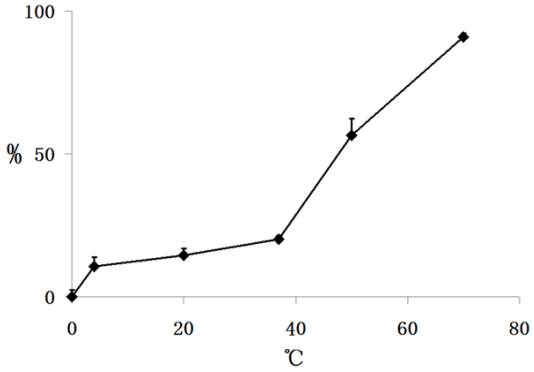

Figure 3. Effects of reaction temperature on the pPIX catalyzed reaction. 24.3 mM of H_2O_2 was incubated with 8.80 μM of pPIX at the temperatures as specified. The production of $^1O_2$ was monitored with 2.12 μM of DMA.

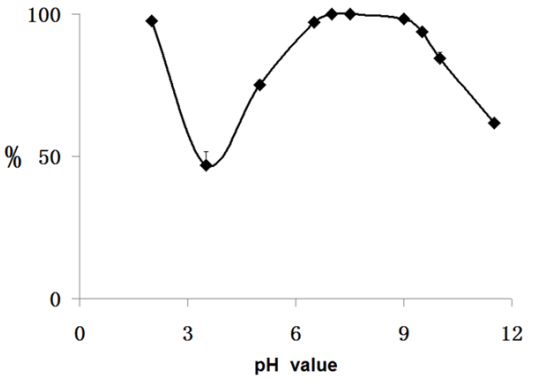

Figure 4. Effects of PH value on the pPIX catalyzed reaction. 24.3 mM of H_2O_2 was incubated with 8.80 μM of pPIX at 37°C in dark for 3 hours. The production of $^1O_2$ was monitored with 2.12 μM of DMA.

activity of FECH, 1 ml Protogen IX-DMA solution was mixed with 1 mL mitochondria preparation and 1 ml PBS, 37°C in the dark for 2 hours. The same conditions were used for measuring PPO (Protoporphyrinogen oxidase) activity except that 1 mL pBS was replaced with 1 ml of Tris-EDTA. DMA solution with 1 mL DMA solution, 1 ml PBS and 1 mL heat inactivated mitochondria was used as a control.

X ray irradiation experiments

The conditions were the same as those of the in vitro experiments. The loaded test tubes were irradiated with X-ray at different doses 37°C. The specimens were analyzed immediately after irradiation.

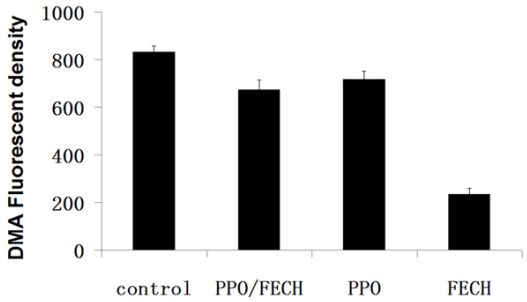

Figure 5. Effects of FECH on the pPIX catalyzed reaction. The reaction contained protogen IX at 1.3 μM (5.2 nmole), DMA at 0.27 Um (1.1 nmole), and mitochondrial proteins 1.0 mg, with or without FEC. Heat inactivated mitochondrial protein preparation was used as a negative control. Production of $^1O_2$ was monitored with 2.12 μM of DMA.

Measurement of singlet oxygen

DMA fluorescence intensity was measured with excitation (Ex) wavelength = 370 nm, and emission (Em) wavelength = 432 nm. SOSG fluorescence was measured at ex480 nm/em527 nm.

Results

Conversion of H_2O_2 into $^1O_2$ catalyzed by pPIX

We detected $^1O_2$ when 24 μM of pPIX was incubated with H_2O_2 at a concentration of as low as 1.5 mM. In absence of pPIX, no $^1O_2$ could be measured concentrations even H_2O_2 concentration was increased up to 500 mM (**Figure 1**). Provided H_2O_2 concentration at 24.3 mM, the conversion reaction was positively correlated with the concentration of pPIX (**Figure 2**) and incubation temperature (**Figure 3**). In addition, neutral PH value was most favorable for the reaction (**Figure 4**). Also as shown in **Figure 1**, oxidization of DMA and SOSG by $^1O_2$ quenched and enhanced their fluorescence, respectively.

Effects of mitochondrial FECH on $^1O_2$ production

Our data showed that the mitochondrial H_2O_2 yield was 0.11 ± 0.03 nmole/mg/min, comparable to others' results [7]. In the mitochondria, PPO catalyzes conversion of Protogen IX to pPIX [8], and FECH catalyzes conversion of pPIX to heme [9]. Tris-EDTA inhibits FECH activity, as evidenced by the similar results from treatments with Tris-EDTA and DMA control, which

Table 1. Effects of FECH on the pPIX catalyzed reaction (n = 6)

DMA control[1]	FECH	Inactivated FECH[2]	Lead tetraacetate[3]	VA[4]
823.2±94.7	234.7±24.0*	832.7±25.8#	710.7±55.7#	402.0±23.4#

Note: [1]DMA control, containing 2 mL of PBS/0.1 mM EDTA and 1 mL of Proto IX-DMA solution. [2]100°C 5 min inactivated. [3]The mitochondrial protein preparation was pre-incubated with 33.3 μM of lead tetraacetate, 37°C for 1 hour, prior to being mixed with other reaction components. [4]In Tris-EDTA containing 0.2 mM of vannillic acid. *P < 0.0001, comparing to that of DMA control. #P < 0.0001, comparing to that of FECH treatment.

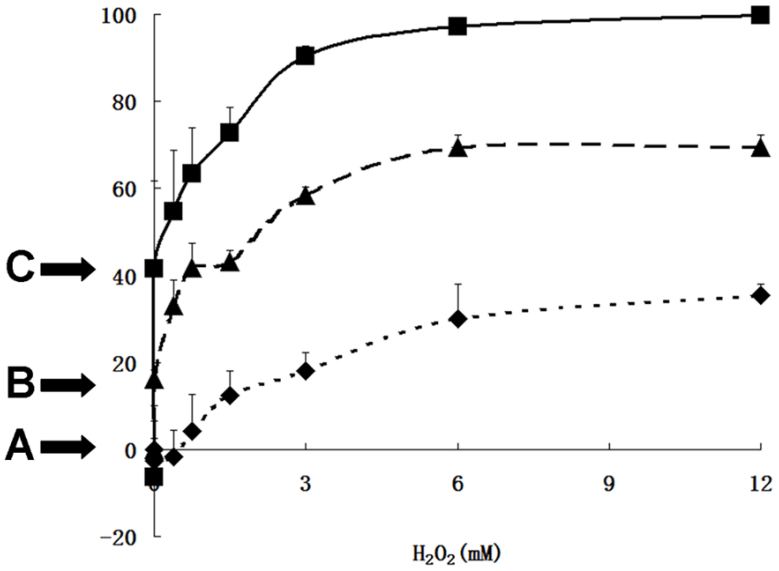

Figure 6. Effects of irradiation on the pPIX catalyzed reaction. In presence of 14.4 μM of pPIX and 1.05 μM of DMA, the reaction was treated with 0.54 μM of H_2O_2 (A), 1 Gy irradiation of 6MV X-ray (B) or 1 Gy irradiation of 45 MV X-ray (C). The production of $^1O_2$ was monitored with 2.12 μM of DMA.

contained heat inactivated mitochondrial proteins) (**Figure 5**). In contrast, when FECH added, DMA fluorescence signals were significantly decreased (**Figure 5**), indicating that pPIX could catalyze conversion of H_2O_2 to $^1O_2$, which was greatly enhanced by FECH (**Table 1**). Conversion of H_2O_2 to $^1O_2$ by pPIX was also significantly inhibited by heat inactivation of FECH, Pb (an FECH antagonist), or H_2O_2 antagonist vanillic acid.

Effects of X-ray irradiation on pPIX catalyzed production of $^1O_2$

No $^1O_2$ was produced in presence of only H_2O_2 and high doses of radiation. At presence of 14.4 μM of pPIX, the 0.54 μM of H_2O_2 generated from 1 Gy high energy X-ray irradiation [10], was converted into $^1O_2$, reflected by reduction of DMA fluorescence by 41.5%±20.2% (P = 0.0036, n = 6, **Figure 6**). Compared to those reactions at 37°C for 3 hours, irradiation had higher yield of $^1O_2$ in a dose dependent manner. It is known that X-ray ionization of water can produce hydroxyl radical (* OH), hydrated electrons and H_2O_2 [10]. In presence of 50 mM of DMSO (hydroxyl radical scavenger) or N_2O (hydrated electron scavenger), irradiation of the reaction mixture containing 14.4 μM pPIX and 1.05 μM did not block yield of $^1O_2$, indicating that $^1O_2$ was generated from H_2O_2, instead of hydroxyl radical or hydrated electrons.

Specificity of the catalytic activity of pPIX

There are many porphyrin species. To determine the specificity of the catalytic activity of pPIX, we compared pPIX with the structurally similar porphyrin, haematoporphyrin (HP), in terms of their catalytic activities of converting H_2O_2 into $^1O_2$. With 50 MV X-ray, specimens containing 1.05 μM of DMA, and 14.4 μM of pPIX or HP, were irradiated with 0, 0.65, 1.25, 2.50 and 5.00 Gy, respectively (**Figure 7**). In presence of pPIX, the decrease of DMA fluorescence was proportional to the irradiation doses. While in presence of HP, DMA fluorescence value was slightly decreased at 1.25 Gy, which was inversely proportional to the radiation dose. These results suggested pPIX but not HP is the specific catalytic player in the reaction generating $^1O_2$.

Discussion

Photosensitizers are a class of substances sensitive to visible light. After absorbing visible light with specific wavelengths, they are excited to the excited state then to the baseline state,

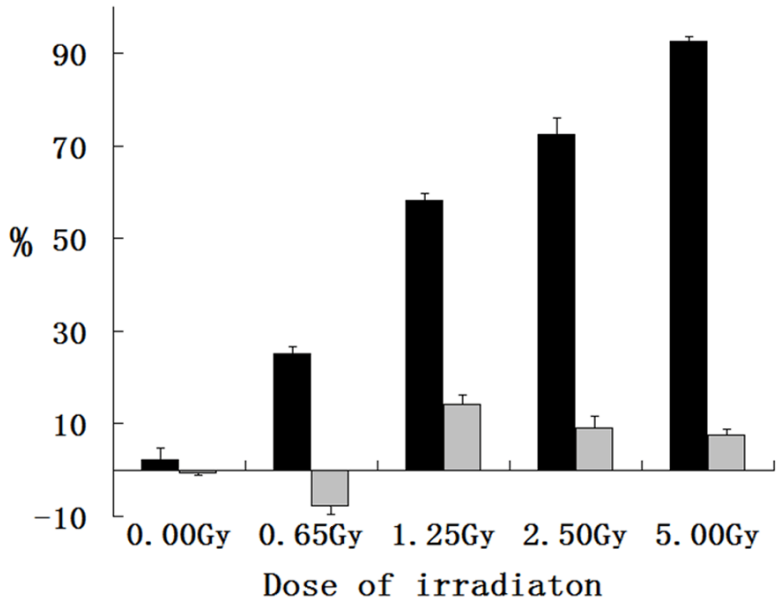

Figure 7. Specificity of pPIX in catalyzing H_2O_2 to $^1O_2$. In presence of 8.80 μM of pPIX (black bars) or HP (grey bars), the conversion of H_2O_2 to $^1O_2$ was treated different doses of irradiation. The production of $^1O_2$ was monitored with 2.12 μM of DMA.

converting non-toxic oxygen into toxic $^1O_2$. Due to their low toxicity and tendency of accumulation in lesions, they are used clinically to treat human diseases. This modality is called photodynamic therapy. The biggest flaw of this modality is that visible light has poor tissue penetration capability, and therefore not suitable for treatment of large lesions, deep lesions and systemic diseases. Novel strategies or new photosensitizers need to be developed to enhance this H_2O_2 to $^1O_2$ conversion. Our data showed that pPIX specifically catalyzes conversion of H_2O_2 into $^1O_2$.

The pPIX can be converted into heme with the catalysis of FECH. Heme exerts a lot of important unique physiological functions, including carrying oxygen [11], binding and sensing biatom small molecules [12], transferring electron [13], conducting extracellular signals [14], regulating gene translation and translation [15], modulating miRNA process [16], detoxification [17] and metabolizing [11]. Initial and final steps of heme synthesis reactions are carried out in mitochondria [18], with two rate-limiting enzymes, 5-aminolevulinic acid (5-ALA) synthase and FECH. The 5-ALA synthase condenses glycine and succinyl into 5-ALA, while FECH chelates iron into pPIX. It is believed that an excess of 5-ALA can make pPIX concentration in mitochondria. This study argued that pPIX accumulation in mitochondria is harmful to the cells because pPIX catalyzed within mitochondria production of $^1O_2$, a toxic oxygen species.

We found that the catalytic activity of pPIX was structurally specific. Results from other labs have shown that FECH or FECH antibody can twist pPIX's structure [9, 19], which might be the reason for the observed promotion of catalytic efficiency of pPIX in this study, when FECH was present. Mechanism of enhanced yield of $^1O^2$ by X-ray irradiation in this study is not clear yet. It could be due to the red radioluminescence, red radiochemiluminescence [20], simply more H_2O_2 being generated, or combination of any of these factors.

Our finding of catalytic activity of pPIX converting H_2O_2 into $^1O_2$ carries great potential value for photodynamic therapy for human diseases. Taking advantage of this reaction, we can enhance photodynamic therapy from many aspects. First, we can greatly increased mitochondrial pPIX in lesions by giving excess 5-ALA [9, 21]. Second, we can increase H_2O_2 in lesions by heating, irradiation or giving exogenous H_2O_2. Third, to improve the catalytic activity of pPIX by X-ray irradiation to develop so called "Radio-dynamic therapy", and we can locally give patients FECH or FECH antibody. To explore these potentials, it is worthwhile to collect clinical and experimental data to justify the efficacy of combination of these strategies.

Disclosure of conflict of interest

None.

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