UDK 612/902. 622. 3147

The effect of Light on the Oxidase Activity of Human Ceruloplasmin in Complexes with Cationic Zn-porphyrins

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Key words: photosensitizers, cationic porphyrins, Zn-porphyrins, ceruloplasmin

Introduction

Photodynamic therapy (PDT) has been acknowledged as an alternative technique for cancer treatment. In PDT, tumor is destroyed by reactive oxygen species (ROS) such as hydroxyl radical (OH^-), singlet oxygen ($^1O_2$), and superoxide radical (O^{2-}), which are produced by the reaction of the excited photosensitizers (PSs) and oxygen (O_2) [17].

Porphyrins are ubiquitous in the fields of photodynamic therapy and are among the most prominent classes of photosensitizers [21]. Several porphyrin derivatives have been synthesized and tested for use in PDT. The molecular structure, in particular the number and position of the positively charged groups and the hydrophobic character of the porphyrin molecules, probably are important in their interactions with biological targets. The electrostatic interaction of positively charged porphyrins with the negative charges on the tumor cell membranes and bacterial surfaces possibly facilitates their penetration through membranes and increases their PDT efficacy [18]. Currently, scientists are looking at the development of novel photosensitizers (PSs) to improve the therapeutic outcome and reduce the nonspecific side-effects of these anticancer agents [18]. PSs were successfully bound to several classes of water soluble compounds such as proteins. The protein carriers are inherently biocompatible, they have the benefits of a nano-metric-size object, which improves bioavailability of the compound [15].

We have previously studied the complexes of several cationic porphyrins with some blood proteins (hemoglobin [12], albumin [13], transferrin [10], ceruloplasmin (CP) [11]). It was shown that these proteins can serve as carriers of cationic porphyrins in the blood for PDT. Since CP is actively used as an anticancer drug [2, 16], and its complexes with cationic porphyrins with

anticancer activities [12, 13] can exhibit a synergistic effect in anticancer therapy. In particular, the spectral and fluorescent characteristics of complexes of photosensitizers (PSs) with CP, desorption of PS with changes in the physiological conditions of the medium (pH and salt composition of the medium) [14] were investigated. We also studied the effect of light at various exposures (0 - 60 minutes) on the optical absorption of PS and their complexes with CP [1].

CP (132-kDa) is β2 glycoprotein that is synthesized and secreted by hepatocytes as a holoprotein. It is a member of a multicopper oxidase group, which contains more than 95% of the copper ions in serum [3]. Copper is a necessary element of angiogenesis (tumor vascularization), ensuring the tumor active growth, inducing to the surrounding tissues and weight gain [5]. Among the physiological functions of CP, besides participation in copper transport, its ferro-oxidase activity must be noted [16]. The oxidation of Fe^{2+} to Fe^{3+} by CP is necessary for apo-transferrin conversion to transferrin [16]. Due to increased need of iron for tumor development, the transferrin receptor (TfR) is more expressed in tumor cells compared with the surrounding normal tissue [6, 23]. After the binding of transferrin to TfR on the cell membrane, its internalization occurs by endocytosis [19].

In this connection, the study of influence on the oxidase activity of CP caused by its' involvement in the complexes with cationic porphyrins seems to be an important aspect for PDT of tumors.

This paper discusses the oxidase activity of CP separately and in complexes with porphyrins. Our aim was to reveal whether complexation or illumination change the oxidase activity of CP. Also, electrophoresis was performed to clarify the change in the protein structure due to illumination.

Material and Methods

Isolation of CP from human blood plasma. For the isolation and purification of human CP, the gel filtration chromatography on Sephadex G-150 (super fine) and G-25 (Pharmacia Biotech, Sweden) columns and ion exchange chromatography on a DE-52 (Whatman) were used according to works [4, 9]. The purification procedures were modified and briefly consisted of: ammonium sulfate precipitation of blood plasma proteins, ion exchange chromatography on DE-52 cellulose column, dialysis and after concentration on DE-52 column, the obtained blue-green CP fraction (in 0.05 M PBS + 0.2 M NaCl) was subjected to the gel filtration on Sephadex G-150. The collected light blue fractions were again concentrated on the DE-52 column. This column was washed consecutively with 0.01 M PBS (pH 7.2), 0.025 M PBS, 0.05 M PBS and 0.2 M PBS. The main fraction of CP was eluted with 0.2 M PBS (pH 7.2) and additionally purified on a Sephadex G-150 column. The obtained CP sample has a high purity index: $I = A_{610} / A_{280} \geq 0.049$ [22]. The CP concentration was

measured using the known extinction coefficient: $\varepsilon_{280нм}^{1\%}$ =14.6 [9]. As a result of realized purification procedures, 1.5 ml with concentration of 9 mg/ml (5.6 x 10^{-5} M) protein was received. The total amount of obtained CP was 13.5 mg. Fig. 1 shows the absorption spectrum of the purified human CP. The absorbance at 600 nm conditioned the characteristic blue color of the CP, due to content of Cu^{+2} ions.

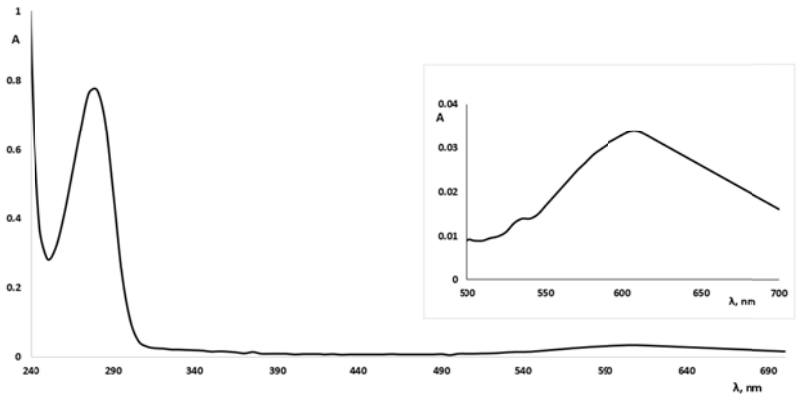

Fig. 1. The absorption spectrum of purified from human blood CP in 0.01 M PBS, pH 7.2

Oxidase activity of CP. The oxidases have two substrates, oxygen and reducing substrate. The blue oxidases - laccase, CP, and ascorbate oxidase, as well cytochrome c oxidase are the only enzymes known to reduce both atoms of molecular oxygen to water, that is to transfer four electrons. The net reaction is, thus (S is a reducing substrate) [20]:

$$O_2 + 4S + 4H^+ \rightarrow 2H_2O + 4S^{\cdot+}. \quad (1)$$

The activity of CP was determined spectrophotometrically using the method described by Curzon et al. (1960) [7]. CP oxidizes p-phenylenediamine and related substances to colored products. In our case, CP oxidizes N, N-Dimethyl-p-phenylenediamine (DPD):

$$\text{N, N-Dimethyl-p-phenylenediamine} + H_2O \xrightarrow{\text{Ceruloplazmin}} \text{Oxidized product}. \quad (2)$$

Conditions for measurement were as follows: T = 37°C, pH = 5.5, λ= 550 nm, light path = 1 cm. The absorption measurements were carried out by Shimadzu UV-VISIBLE Recording Spectrophotometer UV-2100 (Japan). Constant temperature was maintained during the measurements with the thermostat MLW UH 4 (Germany).

The reaction mixture in 2 ml contained Sodium Acetate Buffer, (200 mM, pH 5.5), 0.1 ml of Sodium Chloride Solution in case of control sample (100 mM, enzyme diluent) and 0.1 ml of CP or complex in case of test sample. The test samples contain 0.39 mg/ml CP in cold sodium chloride solution (Table).

Table

Reaction mixtures

Solutions	Test	Blank
Sodium Acetate Buffer	2 ml	2 ml
Deionized Water	0.80 ml	0.80 ml
Sodium Chloride Solution (Enzyme Diluent)	-	0.1 ml
CP or Complex of CP with porphyrin	0.1 ml	-

The solutions were mixed by inversion and equilibrated to 37°C and then 0.1 ml DPD (153 mM) was added to each reaction mixture (test and blank). After that, the increase in A_{550nm} for approximately in 5 minutes was recorded.

The calculation of CP oxidase activity was performed by following formula [27]:

$$\text{Unit}/_{\text{ml}} = \frac{\left(\frac{\Delta\ A_{550nm}}{\text{min}}\ \text{Test} - \frac{\Delta\ A_{550nm}}{\text{min}}\ \text{Blank}\right)(3)(df}{(0.01)(7)(0.1)} \quad (3)$$

In this formula:

(3) = Total volume (in milliliters) of assay

(df) = Dilution factor

(0.01) = Change in Absorbance at 550 nm (Unit Definition)

(7) = Conversion Factor to published Unit Definition of a 7 ml reaction volume

(0.1) = Volume (in milliliters) of enzyme used.

One unit will cause a ΔA_{550} of 0.01 per min using N, N-dimethyl-p-phenylenediamine as substrate at pH 6.4 and 37 °C, in a 7 mL reaction volume [25].

Electrophoresis. We carried out polyacrylamide gel electrophoresis and relied on the recommendations for the preparation of gel solutions in the basic buffer system [8]. We modified the method for our purpose. The electrophoresis was carried out by using the MUPID-EXU (Japan) horizontal electrophoresis system. To perform protein electrophoresis using a horizontal electrophoresis system, we modified the equipment, added a horizontal mica plate on the gel, thus inhibiting the entry of oxygen, and providing favorable conditions for the polymerization of the polyacrylamide gel. After the procedure of electropho-

resis, the gel was stained by Coomassie Brilliant Blue G-250 (5 mg in 3.5 % Perchloric acid).

Photosensitizers. The following cationic Zn-porphyrins were used in the work: Zn-TBut4PyP, Zn-TOEt3PyP, Zn-TOEt4PyP [11].

Statistical analysis. The statistical parameters (mean values, standard deviation) used in the experiments were calculated using Microsoft Excel and Origin 7.0 (Origin Lab Corporation).

Results and Discussion

The oxidase activity of CP was assessed, which was isolated from donor blood plasma using ion exchange chromatography on cellulose DE-52 [9] and additionally purified gel filtration on a column with Sephadex G-150 (see in Materials and Methods). After purification on a column with Sephadex G-150, CP had an oxidase activity of 33.2 U/mg (unit/mg). The oxidase activity of BIOCERULINUM® produced by ©biopharma (Kiev, Ukraine, register number № UA/0763/01/01) is not less than 9 U/mg [26]. Hence, the oxidase activity of purified CP by described method demonstrates 3.7 times more than the oxidase activity of BIOCERULINUM®.

Complexes of CP with the following Zn-porphyrins were obtained: Zn-TBut4PyP, Zn-TOEt3PyP, Zn-TOEt4PyP according to the previously described method [1, 11, 24]. The unbound metalloporphyrin was removed by gel filtration on a Sephadex G-25 column. CP obtained after Sephadex G-25 column was used as a control. In this sample, the oxidase activity was 2.1 times lower compared to CP after G-150 (Fig. 2). CP and its complexes with Zn-porphyrins were illuminated for 30 minutes with a tungsten lamp with a power of 50 W (30 mW /cm^2) with the illumination range of 380 - 1100 nm [1]. The oxidase activity of all studied samples was calculated using the formula (3). The results are shown in Fig. 2.

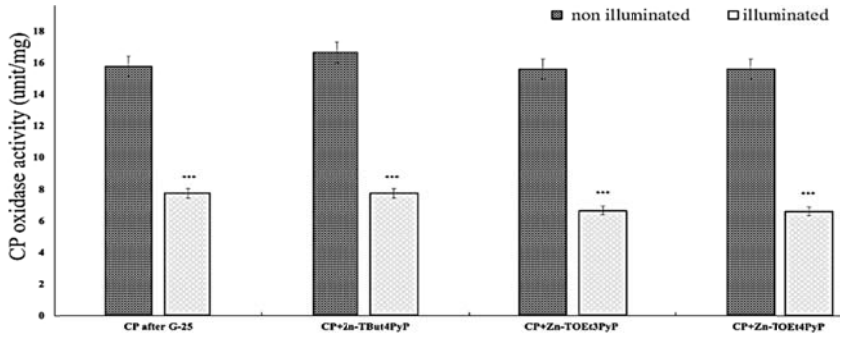

Fig. 2. The CP oxidase activity. The data represent the mean ± SD (standard deviation) of three experiments each in triplicate. P<0.05, n=3

As follows from Fig. 2, 30-min illumination with a tungsten lamp of all solutions: of CP without any additions and of its complexes with three Zn-porphyrins resulted in a nearly identical twofold decrease in the oxidase activity of the protein.

For a more detailed characterization, we applied the electrophoresis method for the following samples:

1. CP (additionally purified by gel filtration on a column with Sephadex G-150).
2. CP+Zn-TBut4PyP (non illuminated).
3. CP+Zn-TOEt3PyP (non illuminated).
4. CP+Zn-TOEt3PyP (illuminated).
5. CP+Zn-TOEt4PyP (non illuminated).
6. CP+Zn-TOEt4PyP (illuminated).

Each sample (12 μl) contained 2 mg/ml of CP.

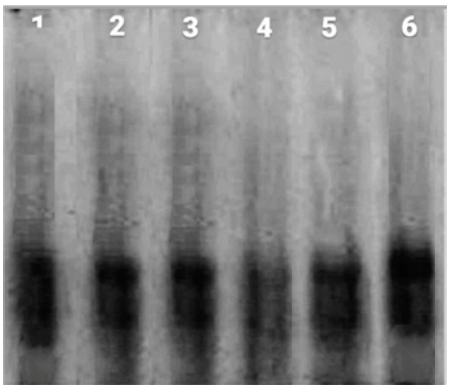

Fig. 3. Polyacrylamide gel electrophoresis

The results of electrophoresis are shown in Fig. 3. where CP is presented in the form of two bands of its isozymes. As follows from Fig. 3, no significant changes occur in the peptide molecule upon the binding of cationic porphyrins to CP.

The obtained data manifested that the type of the peripheral group (butyl, hydroxyethyl) and their position (3rd or 4th) in the pyridyl ring of Zn-porphyrins did not significantly affect the oxidase activity of CP. In the presence of [CP + Zn-porphyrin] complexes in the blood, CP perhaps retains oxidase activity, which is one of the most important physiological functions of this protein. The illumination of CP complexes with porphyrins did not destroy the protein structure but decreased the oxidase activity of the protein nearly twofold (the same effect as for CP without porphyrins). This observation suggests that at application at PDT, the illumination of [CP + Zn-porphyrin]

complexes in the microenvironment of cancer cells, in addition to the porphyrins' photodynamic effect, will bring to decrease of the oxidase activity of CP, resulting in the hindering of angiogenesis in the malignant tissue.

Conclusions

- Neither the types of peripheral group (butyl, hydroxyethyl) nor their positions (3rd or 4th) in the pyridyl ring of Zn-porphyrins have significantly influence on the oxidase activity of CP.
- The illumination of the CP complexes with porphyrins did not destroy the structure of protein.
- The illumination for 30-minute of the solutions of CP and its complexes with Zn-porphyrins with a tungsten lamp decreased the oxidase activity of the protein nearly twofold.

Accepted 09,06,21

Влияние света на оксидазную активность церулоплазмина человека в комплексах с катионными Zn-порфиринами

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В исследовании представлено влияние облучения светом видимой области на оксидазную активность церулоплазмина (ЦП) человека в комплексах с катионными металлопорфиринами. Исследованы комплексы ЦП с Zn-порфиринами с высоким квантовым выходом генерации синглетного кислорода: Zn-TBut4PyP, Zn-TOEt3PyP, Zn-TOEt4PyP.

Целью исследования было определить, влияет ли образование комплекса и освещение на оксидазную активность ЦП. Показано, что образование комплексов с исследуемыми порфиринами существенно не влияет на оксидазную активность ЦП. Облучение вольфрамовой лампой в течение 30 минут растворов ЦП и комплексов [ЦП+Zn-порфирины] снижало оксидазную активность белка почти вдвое. Согласно полученным результатам, для активности ЦП не важны ни тип периферической группы (бутил, гидроксиэтил), ни их положение в пиридильном кольце (3-е или 4-е) Zn-порфирина. Освещение ЦП и его комплексов с порфиринами не расщепляло белковую молекулу.

Լույսի ազդեցությունը կատիոնային Zn-պորֆիրինների հետ կոմպլեքսներում մարդու ցերուլոպլազմինի օքսիդազային ակտիվության վրա

Ա.Ա. Զաքոյան, Լ.Վ. Մկրտչյան, Գ.Վ. Գյուլխանդանյան

Աշխատանքում ներկայացվել է լուսային ճառագայթման ազդեցությունը մարդու ցերուլոպլազմինի (ՑՊ) օքսիդազային ակտիվության վրա կատիոնային Zn-պորֆիրինների հետ կոմպլեքսներում: Ուսումնասիրվել են ՑՊ-ի կոմպլեքսները սինգլետային թթվածնի բարձր քվանտային ելք ունեցող հետևյալ Zn-պորֆիրինների հետ՝ Zn-TBut4PyP, Zn-TOEt3PyP, Zn-TOEt4PyP:

Այս ուսումնասիրության նպատակն է պարզել՝ ազդո՞ւմ են արդյոք ՑՊ-ի օքսիդազային ակտիվության վրա կոմպլեքսագոյացումը և լուսավորումը: Ցույց է տրվել, որ ուսումնասիրված Zn-պորֆիրինների հետ ՑՊ-ի կոմպլեքսագոյացումն էականորեն չի ազդում սպիտակուցի օքսիդազային ակտիվության վրա: ՑՊ-ի և [ՑՊ + Zn-պորֆիրին] կոմպլեքսների լուծույթների՝ 30 րոպե տևողությամբ վոլֆրամի լամպով լուսավորումը բերում է սպիտակուցի օքսիդազային ակտիվության կիսով չափ նվազման: Համաձայն ստացված արդյունքների՝ Zn-պորֆիրինների ծայրային ֆունկցիոնալ խմբերը (բութիլ, օքսիէթիլ) և դրանց գտնվելու դիրքը պիրիդիլային օղակում (3-րդ կամ 4-րդ դիրք) ՑՊ-ի օքսիդազային ակտիվության համար էական չեն: ՑՊ-ի և պորֆիրինների հետ դրա կոմպլեքսների լուսավորումը չի բերում սպիտակուցի քայքայման:

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