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NEW APPLICATION OF ADRENALIN OXIDATION REACTION FOR ESTIMATION OF PRO-ANTI-OXIDANT PROPERTIES OF BIOACTIVE SUBSTANCES

TATJANA V. SIROTA, NATALIA V. LANGE, NELLY I. KOSJAKOVA, ALEKSEJ V. VANICHKIN, MARIE N. KONDRASHOVA

Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow region, 142290, Russia

INTRODUCTION

The existence of relationship between many diseases and processes of free radical oxidation has been revealed (Gutteridge, 1993). When control of antioxidant defence is exhausted free-radical oxidation can cause different diseases such as inflammation processes, hypoxic and reperfusion injury of tissues, bronchopulmonary diseases, senility, carcinogenesis and other (Menshchikova & Senkov, 1993).

Information on the state of antioxidant defence system is important for diagnosis, control of treatment and development of diseases (acute phase and remission) (Zhu, Zhang & Weng, 1994; Saito, 1987; Zima, Spicka, Stiper, Crkovska, Platenik, Metra, Nemecek & Tesar, 1996). The most important enzyme of this system is superoxide dismutase (SOD) – EC.1.15.1.1., which catalyses transformation of the highly reactive anion radical of oxygen (superoxide anion, $O_2^{\cdot}$) into a relatively less active hydrogen peroxide and molecular oxygen:

$$2O_2^{\cdot} + 2H^{+} \rightarrow H_2O_2 + O_2$$

Determination of antioxidnt activity (AOA) of entire blood in patients (antioxidant status) is of importance under different pathological and physiological conditions.

A number of methods of SOD activity measurements are described, however most of them demand expensive and hardly available reagents (Sirota, 1999a).

In this study a new approach for SOD activity and AOA determination is proposed.

In alkaline medium adrenalin is autooxidized and superoxide is an intermediate in this process. The inhibition of adrenochrome accumulation, measured at 480 nm, is used for SOD activity measurements (Misra & Fridovich, 1972).

Sub and Zigman (1978) reported estimation of SOD activity using adrenalin oxidation measured at 320 nm (UV-region).

One of us found that adrenaline autooxidation in alkaline medium can be observed under different wavelength (at 347 nm or absorption shoulder 320–380 nm) and for even lower concentrations (230 μM). It was stated that the appearance of this product of adrenalin oxidation (with absorption at 320–380 nm) is considerably more rapid than formation of adrenochrome and is inhibited by SOD (Sirota, 1999a, 1999b). This allows to monitor adrenalin oxidation immediately within 3 –5 min after addition.

The proposed test-system was effectively used for determination of pro-anti-oxidative activity of different redox-active substances. The possibility to use available not expensive chemicals (0.1% adrenalin solution from a drug store, 0.2 M carbonate buffer) serves as an additional advantage of the proposed method.

MATERIALS AND METHODS

0.1% pharmaceutical adrenalin solution (5.46×10^{-3} M) and 0.2 M Na_2CO_3-$NaHCO_3$ buffer, pH 10.65 were used throughout the work. Na_2CO_3 was from Sigma, USA; $NaHCO_3$ was from J. T. Baker, Holland. pH was adjusted by addition of dry $NaHCO_3$ to a 0.2 M solution of Na_2CO_3.

Solution of SOD (Serva, Germany) contained 0.1 mg enzyme protein/ml in 0.9% NaCl-Tris, pH 7.4.

All solutions were prepared with bidistilled water. The measurements were made in a thermostated cuvette at 23–25°C. UV-VIS spectra and OD were registered in an UV/VIS UNIKON-923 spectrophotometer (Italy). Kinetics of reactions were presented graphically using computer programs "Harvard Graphics" and "Sigma Plot".

For preparation of hemolysates as a source of SOD, washed erythrocytes were prepared as described earlier (Kosenko, Kaminsky, Stavrovskaja, Sirota & Kondrashova, 1997). For estimation of AOA 0.05 ml of entire blood was taken from the finger into 1 ml of heparinized saline and 0.33 ml of water.

RESULTS AND DISCUSSION

Protocol of adrenaline autooxidation reaction

0.1 ml adrenaline was added to 2 ml of 0.2M carbonate buffer with rapid and good mixing. The mixture was put in the spectrophotometer and after 30 s either whole UV-VIS spectrum was registered or only absorption at 347 nm was measured for 3–5 min.

Routinely, 0.01–0.05 ml hemolysates of whole blood or of washed erythrocytes or serum were added to a cuvette containing 2 ml of buffer, then 0.1 ml 0.1% adrenaline was added, solution was mixed and measurements started in 30 s intervals as indicated above. 0.01–0.1 ml of biological materials but without adrenalin was added in control sample for comparison. The same procedure was used for investigation of different chemicals.

Spectral measurement

The time-course of changes of adrenalin absorption spectrum in 0.2 M carbonate buffer, pH 10.65 are given in Fig. 1. The absorption at 320–350 nm appeared and increased in time. Investigation of adrenalin autooxidation in alkaline medium by continuous measurement of the whole spectrum for 10–15 min resulted in determination of kinetics of spectral changes at 347 nm. It was found that in the course of intensive autooxidation of adrenalin in alkaline medium a product with absorption at 347 nm is formed more rapidly than adrenochrome (absorption at 480 nm). As shown in Fig. 1, appearance of adrenochrome is not observed while absorption at 347 nm increases during initial 1–6 min at 15–20°C.

This effect was particularly pronounced when oxidation was induced by a strong oxidant $K_3[Fe(CN)_6]$. In the presence of the oxidant adrenaline oxidation occurred at pH 7.0 in K-phosphate buffer (Sirota, 1999a). The solution of yellow-lemon colour was changed into pink and then orange colour. The appearance of orange colour corresponds to the apperance of absorption at 480 nm, i.e. adrenochrome formation. It is important to notice that: (1) amplitude of the peak at

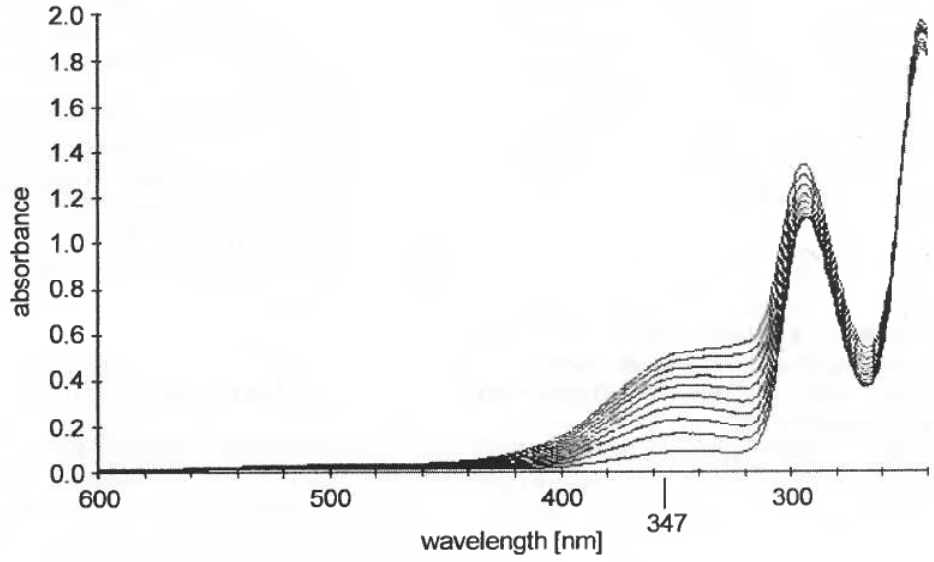

Fig. 1. The time-course of adrenaline absorption in 0.2 M carbonate buffer, pH 10.65. Concentration of adrenaline: 2.6×10^{-4} M. The time of registration: 6 min. Absorption shoulder at 320–380 nm

347 nm is not diminished after appearance of absorption at 480 nm and (2) amplitude of the peak at 347 nm is considerably higher than amplitude of the peak at 480 nm.

Finding the product of adrenalin oxidation absorbing at 347 which is formed more rapidly and absorbs more intensively than adrenochrome allow us to propose the measurement of absorbance at 347 nm as an indicator of adrenalin oxidation more sensitive than adrenochrome used in previous investigations (Misra & Fridovich, 1972; Fridoviv, 1979; Green, Mazur & Shorr, 1956; Simonjan & Nabaldjan, 1975; Mc Cord & Fridovich, 1969).

The effects of various substances on adrenalin autooxidation

The effects of various substances on adrenalin autooxidation was investigated (Table 1). It was shown that commercial SOD inhibited autooxidation of adrenalin as registered by absorption at 347 nm. The inhibitory effect of SOD was lost after 3-min boiling. Bovine serum albumin (BSA) used as protein control had no inhibitory effect.

Antioxidants as ascorbic acid and cysteine at low concentration inhibited autooxidation of adrenalin significantly (Table 1). These effects can be compared with influence of these substances on quercetin oxidation (Kostyuk, Potapovich & Kovaleva, 1990).

The quercetin oxidation was inhibited by higher concentrations of ascorbic acid (100% inhibition at 3 mM and 80% inhibition at 0.3 mM). 0.5, 1.0 and 2.0 mM sodium azide did not influence the adrenalin oxidation (Table 1).

Cu^{2+} ions and chelators such as EDTA and particularly EGTA inhibited adrenalin oxidation (Table 1). Ca^{2+} and serum of donor blood (from 7 donors) activated adrenalin autooxidation.

The value of prooxidant effect of serum of patients with cardiovascular diseases was dependent on the state of patient and was different than activity of healthy donors. The addition of investigated substances to 0.2 mM carbonate buffer did not alter its pH.

Table 1. Effects of various substances on adrenalin autooxidation

Substances	Concentration	Relative activity (%)*
None	–	100
SOD**	100 ng/ml	61
	600 ng/ml	32
SOD after boiling	0.001 mg/ml	90
BSA	0.001 mg/ml	100
Ascorbic acid	0.005 mM	26
	0.025 mM	13
Cysteine	0.0025 mM	36
	0.005 mM	18
	0.025 mM	7
$CuSO_4$	0.0 5 mM	61
	0.1 mM	21
$CaCl_2$	0.1 mM	160
	0.2 mM	180
EDTA	0.1 mM	61
	0.2 mM	38
EGTA	0.02 mM	76
	0.05 mM	45
Azide Na^+	0.5–2 mM	100
Hemolysates of whole blood (AOA)	0.001–0.002 ml of blood/ml	20–80***
Hemolysates of washed erythrocytes	0.0011 ml of blood/ml	20–80***
of patients	0.005 ml/ml	120
Serum of healthy donors	0.025 ml/ml	150
	0.05 ml/ml	145

* – relative activity (RA): intensity of adrenalin autooxidation in OD/min at 347 nm

** – SOD from Serva

*** – variations of RA is due to different states of patients

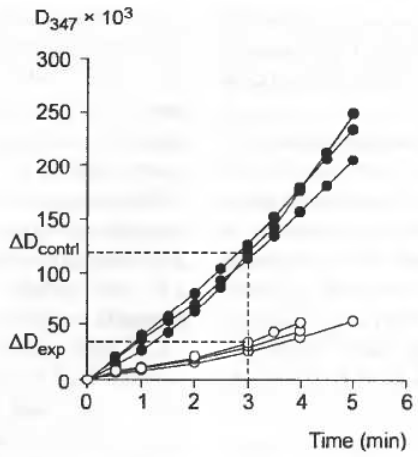

Fig. 2. Inhibition of adrenalin autooxidation by hemolysate of human blood erythrocytes. ● – control; O – in the presence of hemolysate. ΔOD_{contr}, change of OD value at 347 nm in 3 min since beginning of registration in the control sample; ΔOD_{exp}, the same plot for hemolysate. Data from three measurements are presented

Measurement of SOD activity in hemolysate of erythrocytes and AOA in whole blood hemolysates of patients

In order to test the possibility to using absorbance at 347 nm during autooxidation of adrenalin as an indicator of SOD activity or AOA of blood we used hemolysates of washed erythrocytes or hemolysates of whole blood of Pushchino Clinical Center "Moran" patients.

The example of inhibitory effect of hemolysate of washed erythrocytes on autooxidation of adrenalin is shown in Fig. 2. The intensity of autooxidation was calculated on the basis of graphs such as a value of optical density at 347 nm at 3 min since beginning of registration expressed per 1 min (ΔOD/min). ΔOD/min was measured for samples without hemolysate and with hemolysate, and % of inhibition by hemolysate was calculated:

$$[1 - (\Delta D_{exp}/\Delta D_{contr})] \times 100\% = \% \text{ of inhibition}$$

ΔD_{exp} and ΔD_{contr} are the rates of adrenalin antioxidation reaction with and without hemolysate.

Specific activity (relative unit) of the enzyme was calculated per 0.1 units OD at 280 nm (it is 1 mg of protein/ml) or 1 μl of whole blood.

The mean values of SOD activity for 18 people (n = 18) was within the range from 17 relative units to 42 relative units and depended on the individual state of a patient. AOA of blood was within the range from 22.2 relative unit/mg protein to 86.8 relative units/mg protein or from 4.17 to 14.8 relative units per 1 μl of blood. The SOD and AOA activities were dependent on the state of a patient.

SOD activity in hemolysate of washed erythrocytes of a patient suffering from hypochromic anemia was 37 relative units. After taking iron containing preparation SOD activity increased up to 72 relative units. This corresponds to data on decreased SOD activity in anemia (Kumerova, Petuchov, Shkesters, Silova & Lece, 1996; Zhu *et al.*, 1994). We also noticed that SOD activity is higher than control in hemolysates of patients with high erythrocyte sedimentation rate.

By this method SOD activity in erythrocytes of patients treated with air ions was investigated (Kondrashova, Grigorenko, Tichonov, Sirota, Temnov, Stavrovskaja, Kosyakova, Lange & Tikhonov, 2000).

Therefore our data on application of optical density measurement at 347 nm during adrenalin autooxidation to clinical investigations proved the possibility to use this approach which seems to be more sensitive than measuremnt of adrenochrome formation at 480 nm.

The proposed method has an additional advantage, particularly important for clinics, as it uses rather cheap and easily available reagents.

Adrenalin oxidation at 347 nm was shown to be detected also under physiological pH in the presence of a system generating superoxide. Such measurements were made in pigeon heart mitochondria in the presence of oxidation substrates, antimycin A, rotenone and an uncoupler (Sirota & Litvinova, 2000).

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REFERENCES

Fridovich I. (1979). Oxygen radicals, hydrogen peroxide and toxicity of oxygen, [In:] *Free Radicals in Biology*. Mir. Moscow, vol. 1, 273–308 (in Russian).

Green S., Mazur A. & Shorr E. (1956). Mechanism of the catalytic oxidation of adrenaline by ferritin. *J. Biol. Chem.*, **220**, 237–255.

Gutteridge J. M. C. (1993). Free radicals in disease processes: a compilation of cause and consequence. *Free Radic. Res.Commun.*, **19**, 141–158.

Kondrashova M. N., Grigorenko E. V., Tichonov A. N., Sirota T. V., Temnov A. V., Stavrovskaja I. G., Kosyakova N. I., Lange N. V. & Tikhonov V. P. (2000). The primary physico-chemical mechanism for the beneficial biological/medical effects of negative air ions. *Trans. Plasma Sci.*, **28**, 230–237

Kosenko E. A., Kaminsky Yu. G., Stavrovskaja I. G., Sirota T. V. & Kondrashova M. N. (1997). The stimulatory effect of negative air ions and hydrogen peroxide on the activity of superoxide dismutase. *FEBS Lett.*, **410**, 309–312.

Kostyuk V. A., Potapovich A. I. & Kovaleva Z. V. (1990). A simple, sensitive assay for determination of superoxide dismutase activity based on reaction of quercetin oxidation. *Vopr. Med. Khim.*, **36**, 88–91. (in Russian).

Kumerova A. O., Petuchov V. I., Shkesters A. P., Silova A. A. & Lece A. G. (1996). Possible use of erythrocyte antioxidant enzymes as markers of the course of disease in patients with hemoblastoses. *Vop. Med. Khim*, **42**, 144–147 (Russ.).

McCord J. M. & Fridovich I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.*, **244**, 6049–6055.

Menshchikova E. B. & Senkov N. K. (1993). Antioxidants and inhibitors of radical oxygenic processes. *Uspekhi Sovr. Biologiyi*, **113**, 442–455 (in Russian).

Misra H. P. & Fridovich I. J. (1972). The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.*, **247**, 3170–3175.

Saito T. (1987). Superoxide dismutase level in human erythrocytes and its clinical application to the patients with cancers and thyroidal dysfunctions. *Hokkaido Igaku Zasshi*, **62**, 257–268.

Simonjan M. A. & Nabaldjan R. M. (1975). Preparation of electrophoretically homogeneous erythrocuprein and investigation of the thermodenaturation. *Biochemistry*, **40**, 726–734 (in Russian).

Sirota T. V. (1999a). New approach in investigation of process of adrenaline autooxidation and its application to measuremant of superoxide dismutase of activity. *Vopr. Med. Khim.*, **45**, 263–272 (in Russian).

Sirota T. V. (1999b). Patent N 2144674 RU, 24. 02 1999 (in Russian).

Sirota T. V. & Litvinova E. G. (2000). Application of adrenaline oxidation for estimation of superoxide generation by pigeon heart mitochondria. *International symposium "Mitochondria, cells and reactive oxygen species"*, Pushchino, Moscow region. Reports, 138–140 (in Russian).

Sub M. & Zigman S. (1978). An improved spectrophotometric assay for superoxide dismutase based on epinephrine autoxidation. *Anal. Biochem.*, **90**, 81–89.

Zhu C. S., Zhang C. Y. & Weng W. Q. (1994). Study on correlation and clinical significance of superoxide dismutase (SOD) activity and content in 102 patients with leukemia. *Chung Hua Chung Liu Tsa Chin.*, **16**, 39–42.

Zima T., Spicka I., Stiper S., Crkovska J., Platenik J., Merta M., Nemecek K. & Tesar V. (1996). Lipid peroxidation and activity of antioxidation enzymes in patients with multiple myeloma. *Cas. Lek. Cesk.*, **135**, 14–17.