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Review article
Pregledni članak

COMPLEXITY OF FREE RADICAL METABOLISM IN HUMAN ERYTHROCYTES

KOMPLEKSNOST METABOLIZMA SLOBODNIH RADIKALA U HUMANIM ERITROCITIMA

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Summary: The auto-oxidation of oxyhaemoglobin to methaemoglobin generating superoxide anion radical ($O_2^{\cdot -}$) represents the main source of free radicals in the erythrocytes. Hydrogen peroxide is produced by $O_2^{\cdot -}$ dismutation or originates from the circulation. Human erythrocytes are also exposed to the prooxidative actions of nitric oxide (NO) from circulation. Free radicals that may induce reactions with direct dangerous consequences to erythrocytes are also preceded by the reaction of $O_2^{\cdot -}$ and NO producing peroxynitrite. In physiological settings, erythrocytes show a self-sustaining activity of antioxidative defence (AD) enzymes, such as: superoxide dismutase (SOD, EC 1.11.16), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSHPx, EC 1.11.1.9) and glutathione reductase (GR, EC 1.6.4.2), as well as low molecular weight antioxidants: glutathione and vitamins E and C. Their coordinate actions protect the erythrocyte's biomacromolecules from free radical-mediated damage. Since there is no *de novo* synthesis of AD enzymes in mature erythrocytes, their defence capacity is limited. Free radicals influence antioxidative enzymes capacities and relative share of particular components in the whole antioxidative system. Therefore, by measuring changes in the activity of individual AD components, as well as their interrelations by statistical canonical discriminant methods, valuable data about the complexity, overall relations and coordinated actions in the AD system in erythrocytes and its relevance for systemic effects can be acquired.

Keywords: free radicals, superoxide anion radical, nitric oxide, antioxidants

Kratak sadržaj: Produkcija slobodnih radikala u eritrocitima uglavnom se odnosi na nastajanje superoksid anjon radikala ($O_2^{\cdot -}$) putem autooksidacije oksihemoglobina u methemoglobin. Ljudski eritrociti izloženi su prooksidacionom delovanju vodonik-peroksida nastalog dismutacijom $O_2^{\cdot -}$ ili iz cirkulacije, kao i azot oksidu (NO) iz cirkulacije. Od direktnih reakcija slobodnih radikala, reakcija $O_2^{\cdot -}$ i NO uz nastajanje peroksinitrita je reakcija sa primarno štetnim posledicama po eritrocite. U eritrocitima se nalaze enzimi zaštite od oksidacionih oštećenja, kao što su superoksid dismutaza (SOD, EC 1.15.1.1), katalaza (CAT, EC 1.11.1.6), glutation peroksidaza (GSHPx, EC 1.11.1.9) i glutation reduktaza (GR, EC 1.6.4.2) kao i komponente male molekulske mase (glutation, vitamini E i C). Njihovim sadejstvom se kanališu reakcije slobodnih radikala tako da direktna oštećenja biomakromolekula budu što manja. Međutim, kako nema *de novo* sinteze enzima u maturiranim eritrocitima, kapacitet ovih sistema je ograničen, jer slobodnoradikalske vrste i direktno inhibiraju neke od enzima. Promene na enzimima i njihova inhibicija slobodnim radikalima utiču na kapacitet zaštite od oksidacionih oštećenja i relativni udeo pojedinih komponenti u ukupnom antioksidativnom potencijalu. To se može pratiti i preko promena aktivnosti pojedinačnih komponenti, ali i međusobnih odnosa između komponenti antioksidativne odbrane diskriminacionim statističkim metodama, koje ukazuju na sveukupnost i kompleksnost odnosa antioksidativnih komponenti u eritrocitima i njihov sistemski značaj.

Ključne reči: slobodni radikali, superoksid, azot-oksid, antioksidanti

Introduction

The concept of an antioxidant defence system (ADS), as a means of preventing oxidative cell damage and promoting erythrocyte antioxidative instead of prooxidative role in the circulation, implies balanced activities of the erythrocyte ADS. Erythrocytes are exposed to oxygen radicals that are continuously generated primarily due to the auto-oxidation of oxyhaemoglobin (Hb) to methaemoglobin (1, 2). Erythrocytes

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are also exposed to oxidative pressure from plasma (particularly hydrogen peroxide – H_2O_2 and nitric oxide – NO). Under normal conditions erythrocytes contain sufficient levels of scavenger enzymes such as Cu,Zn-SOD, CAT, and selenium-dependent GSH-Px to protect themselves from free radical injury. Cu,Zn-SOD catalyses the dismutation of superoxide ($O_2^{\cdot -}$) to H_2O_2, which is then independently converted to water by CAT or by GSH-Px (3). It is important to note that the activities of these enzymes in erythrocytes are higher than in most other tissues in the body (4). In humans, erythrocytes lack a nucleus, mitochondria and other organelles. Hence, there is no *de novo* synthesis of ADS components, making it difficult to maintain erythrocyte membranes intact for a long period – 120 days. A comprehensive understanding of the ADS should be based on the knowledge of activities and mutual interactions of the enzymes involved in free radical detoxication (5). Balanced action of antioxidant components is necessary for ROS homeostasis and appropriate redox state. Furthermore, if there is any coordinated action between the components, there might be a statistically significant correlation (positive and negative) between them. Therefore, changes in the activity of some antioxidant component should be accompanied by changes in other antioxidative enzymes in a correlated manner. Since correlation analysis calculates the relations between individual components, one can perform an alternative statistical test: canonical discriminant analysis, which calculates differences between groups taking into consideration the complete correlation matrix (i.e. composition of antioxidant defence) of separate groups. The ADS in human erythrocytes with preserved homeostasis finely retunes its composition according to plasma oxidative demands. An increase in the level of a specific plasma lipid component may potentiate membrane lipid peroxidation in erythrocytes and decrease intra-erythrocyte production of $O_2^{\cdot -}$, which could result in a negative correlation between SOD and GSH-Px activities found in some experiments (6–9). The discovery of the haemoglobin-cholesterol (Hb-Ch) complex implies the way in which cholesterol may influence the organisation of ADS in erythrocytes (7).

Physiologically active molecules (such as nitric oxide ($NO^{\cdot}$) can also react with ROS. The reaction between $O_2^{\cdot -}$ and NO naturally occurs in cells producing peroxynitrite ($ONOO^-$) which is considered to be a cytotoxic molecule. The production of so-called reactive nitrogen species (RNS) represents a consequence of $ONOO^-$ formation. RNS provokes nitrosative modifications of molecules and can induce nitrosative stress (10). The interactions of ROS and RNS with antioxidative enzymes leading to the inhibition of their activities, represent the primary cause of disturbed correlations between their activities in erythrocytes. If ROS production overwhelms the ADS, oxidative stress occurs leading to oxidative damages. Non-specific interactions of ROS with different classes of intracellular molecules induce oxidative damage leading to the impairment of cellular homeostasis. As accurate detection of *in vivo* ROS concentrations is still problematic, changes in the ADS may serve as a good indicator of processes within the organism, as it responds to ROS production, changes of the environment (as low environmental temperature) (11) and pathological conditions (9). It should be stressed that the ADS is species- and tissue-specific (12) and that antioxidative enzymes in erythrocytes and the dominant source of ROS in erythrocytes are in many aspects quite different in comparison with extracellular fluids and other tissues.

Antioxidative enzymes in erythrocytes

Human erythrocytes are the most abundant and one of the most specialized cells in the body. The main function of erythrocytes is the transport of oxygen (O_2) and mediation of carbon dioxide (CO_2) production (13). As the red blood cell emerges from the bone marrow, it loses its nucleus, ribosomes, and mitochondria, and therefore the capacity for cell division, protein synthesis, and mitochondrial-based oxidative reactions (13, 14). As a consequence of their physiologic role, erythrocytes are exposed to continuous oxidative stress. Although oxidative stress may damage the red cell itself, the mass effect of large quantities of ROS leaving the red cell could have a tremendous potential to damage other components of the circulation (15), and *vice versa*.

Normal erythrocytes have been shown to have a reducing capacity that is 250 times greater than their oxidizing potential (16). However, in some pathological conditions, the erythrocyte ADS seems to be insufficient (17, 18). Erythrocytes contain a significant quantity of CuZn SOD, which keeps intra-erythrocyte $O_2^{\cdot -}$ levels at concentrations as low as 10^{-13} mol/L. The GSH dependent enzymes (GSHPx and GR) are also normally present, enabling removal of H_2O_2 by oxidation/reduction of GSH. No manganase superoxide dismutase (MnSOD) is present, as there are no mitochondria in erythrocytes.

Erythrocytes can act as sinks for extracellular H_2O_2 (freely crosses membranes) and $O_2^{\cdot -}$ as it can enter the cells via anion channels. These channels have different functions: anchoring the cytoskeleton and exchanging HCO_3^- for Cl^-. However, $O_2^{\cdot -}$ and NO_2^-, as well as the very toxic $ONOO^-$ can also enter erythrocytes through these channels. Once inside the cell, $ONOO^-$ reacts with GSH and oxyhaemoglobin, yielding NO_3^-, methaemoglobin, some H_2O_2 (via $O_2^{\cdot -}$ dismutation), and some ferrylhaemoglobin.

Unfortunately, erythrocytes are particularly vulnerable to oxidative stress due to constant exposure to endogenosly (autooxidation of haemoglobine-Hb) and exogenously generated radicals (H_2O_2 and $NO^{\cdot}$ from plasma). As well as other erythrocyte components,

antioxidative enzymes are exposed to the prooxidative pressure of $O_2^{\cdot -}$, H_2O_2, $ONOO^-$. In the reaction with different radicals, antioxidant enzymes may lose their primary activity and gain prooxidative properties, which can lead to enhanced oxidative pressure and generalised oxidative stress in the whole body.

Inhibition of antioxidant enzymes

The examination of AD enzyme activities *in vitro* has shown that CuZn-SOD possesses a constant specific activity (activity per mg of purified enzyme) and may be inhibited irreversibly by CN- or reversibily by H_2O_2 or by copper chelators such as DDC (diethyldithiocarbamate) (19). Multiple electroforetic profiles of CuZn-SOD develop as a consequence of the enzyme aging (20). Hydrogen peroxide, or rather its conjugate base (OH_2^-), reacts with CuZnSOD, reducing Cu(II) to Cu(I), followed by the reaction of Cu(I) with a second hydrogen peroxide. This leads to the oxidation of the active site, putatively described as copper-bound hydroxyl radical (21). This, in turn, leads to the inactivation of enzyme through 2-oxohistidine formation (22) and to the oxidation of various substrates in the enzyme's behaviour known as the »peroxidative« activity of CuZn-SOD (21–24). DDC is known to be a potent Cu chelating agent and is one of the most widely used SOD inhibitors both *in vivo* and *in vitro* (25-28). The mechanism of DDC-mediated CuZn-SOD inhibition has been described (29). DDC-mediated SOD1 inhibition could serve as a screen for oxidatively damaged SOD 1 protein in the blood of acute myocardial infarction subjects. Oxidative inactivation of red cell SOD by its product H_2O_2 generates a modified protein which is recognised and selectively degraded by an intracellular proteolytic pathway (30). Both the loss of SOD activity and modified binding of copper by the active site appear to precede proteolytic recognition and degradation. The selective degradation of H_2O_2–modified SOD in red cell extracts is now seen to be catalysed by an ATP-independent proteolytic pathway. Oxidised histidine 118 to 2-oxo histidine is generated at the active site of CuZn-SOD exposed to H_2O_2 (22). An increased concentration of H_2O_2 allows the production of hydroxyl radicals, especially in the presence of catalytically-active metals (23, 31).

Oxidatively damaged SOD may cause further increase in free radicals due to more solvent exposed Cu^+. Such a situation is presented in *Figure 1*. Electron paramagnetic resonance spectra of SOD inhibited with H_2O_2 show that 60 minutes after the application of H_2O_2, oxidatively damaged SOD catalyse the Fenton reaction and promote the generation of hydroxyl radicals.

Similarly to CuZn-SOD, other antioxidative enzymes can also be changed or inactivated by reactive oxygen species. Pigeolet et al. (32) tested the effect of

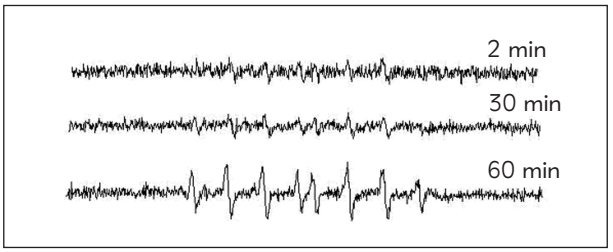

Figure 1 Electron paramagnetic resonance spectra of hydroxyl radicals 'trapped' with a DEPMPO spin-trap in the system composed of CuZn-SOD and hydrogen peroxide (5 mmol/L). In the min lane it is evident that an intensive Fenton reaction occurs.

H_2O_2, cumene hydroperoxide, t-butyl hydroperoxide and $OH^{\cdot}$ and $O_2^{\cdot -}$ on GSH-Px, CuZn SOD and CAT. The activity of GSH-Px was decreased by 50% inactivation in the presence of hydrogen peroxide (0.1 M), cumene hydroperoxide ($3x10^{-4}$ mmol/L), and t-butyl hydroperoxide ($5x10^{-5}$ mol/L). Unlike $OH^{\cdot}$, $O_2^{\cdot -}$ did not inactivate this enzyme. CAT was inactivated by NO, $OH^{\cdot}$ and by $O_2^{\cdot -}$ but organic peroxides had no effect. Similar to our results, Pigeolet showed a 50% SOD inhibition with 4×10^{-4} mmol/L H_2O_2 (32).

Clearly, the ADS is affected by an imbalance between the production of reactive oxygen species and their decomposition, which leads to new ADS settings. Our scientific research has been directed towards finding a cross-relation between the ADS profile of easily accessible human material such as erythrocytes, and the prooxidative changes at the primary site of radical generation in other tissues. In other words, how a changed ADS composition implicates the redox processes in pathological conditions. To answer the above questions, we applied the mathematical model of canonical discriminated analysis to ADS changes measured in the erythrocytes of patients with three different diseases: cardiovascular (AIM), neurological (ALS) and psychiatric (SCH).

Acute myocardial infarction (AMI)

A disturbed balance between the production of both reactive oxygen and nitrogen species and their elimination as a consequence of acute myocardial infarction (AMI) has been postulated to represent the molecular basis of oxidative stress and damage which are important factors in reperfusion injury (33). During myocardial reperfusion injury the production of reactive oxygen species arises via several routes including mitochondrial respiratory-chain enzymes, xanthine oxidase, and non-phagocytic and neutrophil NADPH oxidase (34). An additional source of reactive oxygen species during heart failure may be angiotensin II and catecholamines (more precisely, the auto-oxidation of catecholamines), whose increased levels represent a consequence of sympathetic activation. An excess of H_2O_2 and NO in extracellular medium is followed by

the rise of intracellular level due to the fact that these species can permeate through biological membranes. H_2O_2 and NO may affect the ADS by inhibiting CuZn-SOD (20) and CAT (35). This results in conditions that propagate reperfusion injury via oxidative changes in erythrocytes related to the shift of enzyme behavior from antioxidant to prooxidant. Tsao and colleagues have shown that endothelial cells lose their function 2.5 min after reperfusion injury by a mechanism implicating reactive oxygen species formation (36). Mitochondria in an ischemic heart may additionally increase the generation of hydrogen peroxide during reperfusion (37). Hydrogen peroxide may diffuse from myocytes into the bloodstream to affect erythrocyte function.

Presented settings imply strong correlation between the ADS of erythrocytes and oxidative conditions in the heart and vessels. However, in some pathophysiologies this may not be the case. During reperfusion induced by streptokinase treatment, no significant correlations between the antioxidant defence enzyme activities were apparent (38), while in the control population we found a significant positive correlation between the activities of CAT and GSH-Px in erythrocytes (38, 39). This indicated that ischaemia/reperfusion had disturbed the coordinated action of AD enzymes in the erythrocytes of AIM patients. Only after sustained carvedilol therapy (168 hours duration) a significant correlation between CuZnSOD and CAT was found, indicating that carvedilol therapy had a positive effect on re-establishing the normal relationship between antioxidant setup in the erythrocytes and heart. This is supported by the results of the two-way ANOVA that showed a significant effect of treatment (38).

Amyotrophic lateral sclerosis (ALS) – a fatal progressive disorder

Amyotrophic lateral sclerosis (ALS), often called motor neuron disease (MND), is an adult-onset neurodegenerative disease characterised by progressive injury and death of lower motor neurons in the spinal cord and brainstem, and upper motor neurons in the motor cortex (40). The primary cause of disease is unknown, and the mechanism of motor neuron injury is complex and incompletely understood. In 1993 Rosen and co-workers found that 20% of familial MND cases (5–10% of all cases are familial) are caused by mutations in copper/zinc superoxide dismutase (41). Since that time several hypothetical mechanisms have been predicted: oxidative stress, excitotoxicity caused by aberrant glutamate signaling, mitochondrial dysfunction, disruption of the neurofilament network and intracelular trafficking along neurofilament aggregation of proteins, and the involvement of non-neuronal cells in the vicinity of motor neurons. Events in these mechanisms culminate in a caspase-dependent programmed cell death pathway resembling apoptosis (42). Recent findings corroborated the hypothesis that glutamate-mediated NO overproduction plays an important role in the pathogenesis of ALS (43). Due to their ability to permeate through biological membranes, an excess of NO and H_2O_2 may be present in the surrounding media of motor neurones (44). Some data suggest that the ADS activity is altered in erythrocytes from ALS patients (45, 46) before and after adjuvant ALS therapy (47). Antioxidative defense enzymes in erythrocytes are capable of detoxifying reactive oxygen species (produced endogenously or exogenously), but as mentioned in the preceding sections, the enzymes may be structurally modified and inactivated by reactive oxygen and nitrogen species. Both balanced and coordinated ADS activities are of the utmost importance for their correct physiological function.

In the erythocytes of SALS patients a significant negative correlation between GSH-Px and CAT was found (39). However, in FALS patients (with mutated SOD) and healthy subjects with mutations in the SOD molecule, no significant correlations were found (unpublished data). These mean that the basic relationships between the ADS components in ALS are different and depend on systemic conditions, i.e. the amount of produced ROS and RNS.

Using the canonical discriminant analysis, it is possible to discriminate different categories (in this study, the analysed groups) according to the composition of the antioxidative defense components, and to determine which component significantly contributes to this difference (48, 49). In this way the antioxidative erythrocyte enzymes in SALS, FALS patients, asymptomatic carriers and controls were analyzed by two-way ANOVA and canonical discriminant analyses. The results obtained showed that all examined AOS enzymes significantly contributed to the difference in antioxidative defense composition observed between the groups (*Figure 2*).

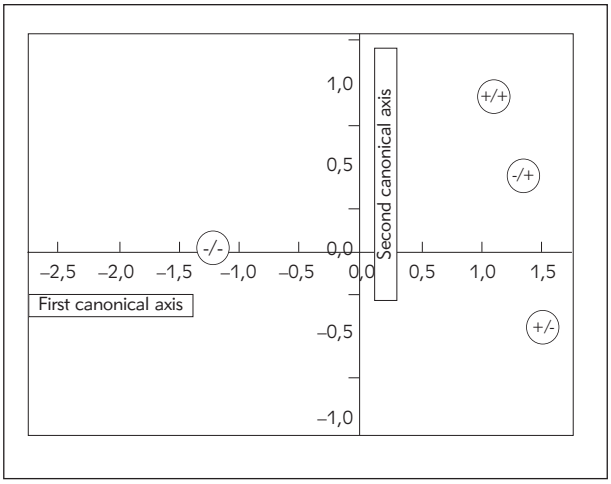

Figure 2 Two-dimensional discriminant analysis showing differences in the ADS activities in erythrocytes from SALS (–/+), from FALS (+/+), from asymptomatic carriers of SOD1 (Leu144Phe mutation) (+/–) and from the control group (–/–).

Schizophrenia

Recent findings suggest that multiple neurotransmitter systems may be faulty in schizophrenic patients (50). Metabolic products of such a non-functional system, such as H_2O_2 and RNS, may inhibit erythrocyte AD enzymes. From the initial studies of antioxidative defence in schizophrenia (Sch.) (51) to the most recent one (52), disturbed balance in the activity of antioxidant defence enzymes in the erythrocytes of schizophrenics was found. In never-medicated first-episode psychotic patients, lower levels of SOD activity were reported, but no change was observed in the activities of CAT and GSH-Px (53). According to our findings, it appears that schizophrenia creates conditions that increase the level of H_2O_2 affecting circulating cells.

Our idea in examining the ADS erythrocyte enzymes in Sch patients was to investigate whether systemic oxidative misbalance reflects on the composition of the ADS iof erythrocytes from Sch type I (Sch I) and type II (Sch II) patients. The correlation analysis of antioxidant defence enzymes showed a significant negative correlation between GSH-Px and CAT activities in patients with Sch I. In patients with Sch II, GSH-Px activity showed a statistically significant positive correlation with GR. Canonical discriminant analysis distinguished Sch I and Sch II patients from the controls, and among each other, with a high degree of certainty, according to the overall group composition of AD enzymes (*Figure 3*) (54). The results indicated a difference in the composition of ADS between controls and antipsychotic treated Sch I and Sch II patients, with a possible negative feedback influence on the pathological process, and may represent a rationale for applying antioxidants in Sch therapy.

According to our findings, it appears that schizophrenia creates conditions that increase the level of hydrogen peroxide, affecting circulating cells. The source of H_2O_2 in the circulation may be an increased monoamine oxidase activity, since an increased turnover rate of catecholamines was found in schizophrenic patients. Such changes in the circulation may shift erythrocyte role from antioxidative to prooxidative. Presumably the best way to address these negative effects is through controlling oxidative stress in a physiological manner, not aiming only at the level of antioxidants, but rather at their interactions. According to Crow (55), Sch I and Sch II show different pathogenetic mechanisms which result in differences in the therapeutic response to antipsychotics. Crow wrote that there were two syndromes, due to different pathophysiological processes: Sch I with changes in dopamine transmission and Sch II with encephalopathy (55). Our results on ADS changes seem to confirm such a statement.

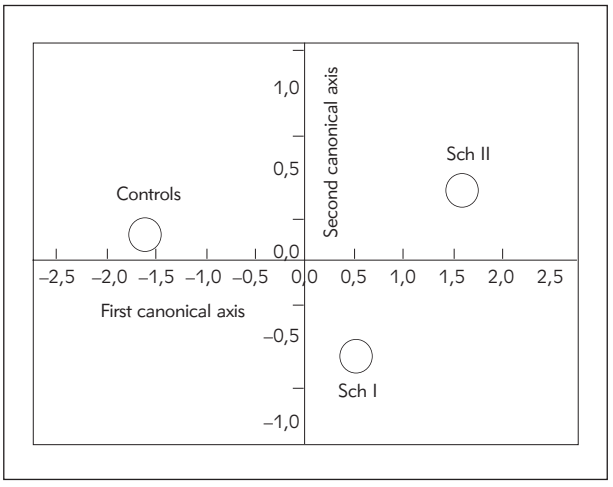

Figure 3 Two-dimensional discriminant analysis showing differences in the activities of antioxidant defense enzymes in the erythrocytes of controls, Sch I and Sch II.

Conclusion

We first used antioxidative defence enzyme relations as a possible bioindicator of the effects of ionizing radiation (56). Since then the concept that the oxidative status of various tissues may be determined by studying the antioxidant defence system has been verified in several papers. Erythrocytes are particularly vulnerable to oxidative stress because they are exposed to oxygen radicals, that are continuously generated primarily due to the auto-oxidation of haemoglobin (Hb). There is a defence system against oxidative stress in erythrocytes composed of CuZn-SOD, CAT, selenium-dependent GSH-Px, and GR. Erythrocytes are also exposed to oxidative pressure from plasma (particularly mediated by H_2O_2 and NO). The measurements of changes in the activities of individual antioxidative defence components as well as their interrelations, using statistical cannonical discriminant methods, are capable of providing a valuable insight into the complexity of overall relations, coordinated actions in the antioxidative defense system in erythrocytes and its relevance to systemic effects.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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