Original Article

PHAGOCYTE MITOCHONDRIAL SUPEROXIDE PRODUCTION AND ERYTHROCYTE Cu-Zn SUPEROXIDE DISMUTASE ACTIVITY DECLINE IN VERY OLD AGE

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Summary

Reactive oxygen species (ROS), constantly generated during respiration, damage important cellular components, which may result in mitochondrial dysfunction, cellular energy production failure and accelerated ageing. We aimed to assess the age-related changes in mitochondrial superoxide production by zymosan-stimulated phagocytes in whole blood and study the relationship between this production and the activity of erythrocyte Cu-Zn superoxide dismutase (SOD), a key enzyme that eliminates the intracellularly generated superoxide. We found that "age" was a significant factor, affecting oxidative activity of stimulated phagocytes. The change, however, was not linear within the age range studied. The cellular oxidative activity initially increased with age and then decreased in people over 70 years of age. We also established that the changes in erythrocyte Cu-Zn SOD with age followed a similar pattern. The likely causes for the observed decline in phagocyte mitochondrial superoxide production and erythrocyte Cu-Zn SOD activity in very old age remain the subject of future research which may reveal the mechanisms that determine longevity and potential targets for therapeutic interventions.

Key words: superoxide, superoxide dismutase, mitochondria, peripheral phagocytes, lucigenin chemiluminescence

Introduction

Mechanisms of ageing have been the subject of extensive investigations. According to the frequently tested mitochondrial free radical theory of ageing, reactive oxygen species (ROS) that are constantly generated during respiration, damage important cellular macromolecules in postmitotic tissues. The relatively unprotected mitochondrial deoxyribonucleic acids (DNA) undergo significant oxidative injury, which may cause mitochondrial dysfunction, cellular energy production failure and accelerated ageing [1]. Recently, data have been reported that oxidative injury to mitochondrial enzymes and to the mitochondrial genome itself plays an important role in a number of age-related degenerative processes [2].

On the other hand, some data published have pointed out that ROS generation also plays

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important roles in maintaining homeostasis within certain limits. Thus, for instance, ROS production by phagocytes is an important defense mechanism in combating infections. The radical generation by vascular cells following stimulation with growth factors participates in the proliferative response regulation. Under conditions of metabolic stress, ROS of mitochondrial origin function as signaling molecules [3].

The activated phagocytes have enhanced metabolism and release more ROS in mitochondria when stimulated by different agents. At the same time, the control of intracellular redox status is essential for normal cell function [4]. The aim of the study was to assess the age-related changes in the mitochondrial superoxide production by zymosan-stimulated phagocytes in whole blood, and to search for a relationship between this production and the activity of erythrocyte Cu-Zn superoxide dismutase (Cu-Zn SOD), a key enzyme that eliminates the intracellular superoxide production.

Materials and methods

Study population

Forty-five healthy volunteers aged 40-80 years were included in the study. All individuals were in good health with no clinical evidence of acute or chronic infection. They did not receive vitamins, minerals or any other food additives with antioxidant properties. All experiments were conducted in accordance with the rules and regulations approved by the University research ethics committee.

Peripheral venous blood, anticoagulated with heparin (20 U/ml), was collected in a fasting state. The experiments were carried out within 30 min of blood collection.

Blood parameters

The following laboratory parameters were determined: total leukocyte count, differential blood count, hemoglobin concentration, erythrocyte and platelet count.

Lucigenin chemiluminescence

The activity of opsonised zymosan-stimulated phagocytes to generate superoxide was evaluated in whole blood by lucigenin chemiluminescence (LgCL) [5, 6]. The LgCL kinetics were recorded by a computer chemiluminometer [7].

The tested samples contained: 0.1 ml (1:10) whole blood, lucigenin (10^{-5} mol/l), zymosan (4 mg/ml) and Krebs Ringer phosphate buffer in a total volume of 2 ml. LgCL kinetic curves were analyzed in relation to the maximum phagocyte oxidative activity, represented by the maximum value of the chemiluminescent intensity $I_{max.}$ To compare chemiluminescent responses recorded from the blood of different individuals, the data were normalized with respect to the total phagocyte number and erythrocyte absorption in the blood samples [8].

Erytrocyte Cu-Zn superoxide dismutase activity

The erythrocyte Cu-Zn superoxide dismutase activity (Cu-Zn SOD) was measured spectrophotometrically according to the method of Maral et al. [9]. It is based on inhibiting the reduction of nitroblue tetrazolium by superoxide, generated via photoreduction of riboflavin. A blank in the absence of Cu-Zn SOD was irradiated for 2 min at λ=365 nm at a distance such as to provide an increase in optical density at λ=560 nm of 0.20 to 0.21. One unit of activity was defined as the amount of enzyme causing 50 % inhibition of the riboflavin reduction to formazan observed in the blank. The results, normalized with respect to the blood erythrocyte number, were expressed as U/10^9 RBC.

Statistical analysis

The statistical analysis was performed with the Statistical Package for Social Sciences 12.0 (*SPSS Inc., Chicago*). The interval variables were represented as mean (standard deviation) or median (minimum-maximum value) depending on the type of distribution. Normality of data was checked with the Shapiro-Wilk test. Depending on age, the study population was classified into 4 groups, as follows: group 1 (40-50 years), group 2 (51-60 years), group 3 (61-70 years) and group 4 (71-80 years). The Kruskal Wallis test was applied to study the effect of the "age" factor on the value of the oxidative markers. The analysis of the relationship between phagocyte oxidative activity and the erythrocyte Cu-Zn SOD was performed using the Spearman's correlations. The value of $p<0.05$ was taken to be the threshold of statistical significance.

Results

The blood parameters of the study population are given in Table 1.

Table 1. Blood parameters of the study population

Variable	Mean ± SD or median (min-max)
WBC [10^9/L]	6,3 (16,3-3,6)
GRA [10^9/L]	4,2 (9,7-1,8)
PLT [10^9/L]	252±58
Lym [10^9/L]	1,9 (8,2-1,00)
RBC [10^{12}/L]	3,52±0,45
Hb [g/L]	107±13

Lucigenin chemiluminescence

The "age" factor was found to be significant for the maximum oxidative phagocyte activity through opsonin dependent mechanism of stimulation (Kruskal Wallis test, χ^2 = 14.958, p=0.002). The effect, however, was not unidirectional. The activity initially increased with age, and then decreased in individuals aged over 70 (Fig. 1).

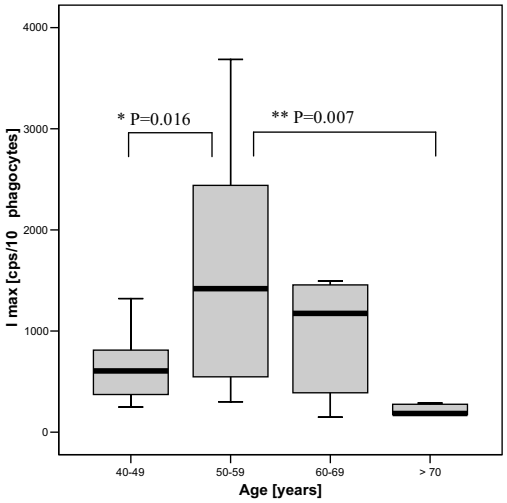

Figure 1. Maximum intensity I_{max} of zymosan-stimulated LgCL depending on age

Erytrocyte Cu-Zn superoxide dismutase

It was found that the erythrocyte Cu-Zn SOD activity changed with age (Kruskal Wallis test, χ^2 = 13.227, p=0.004). The change observed was not linear within the age range studied. After an initial increase, enzyme activity decreased in individuals from the oldest group (>70 years) (Fig. 2).

Spearman's Rho correlations confirmed the existence of similarity between the age-related changes in mitochondrial oxidative phagocyte activity and erythrocyte Cu-Zn SOD activity (r_s=0.345, p=0.020) (Fig. 3).

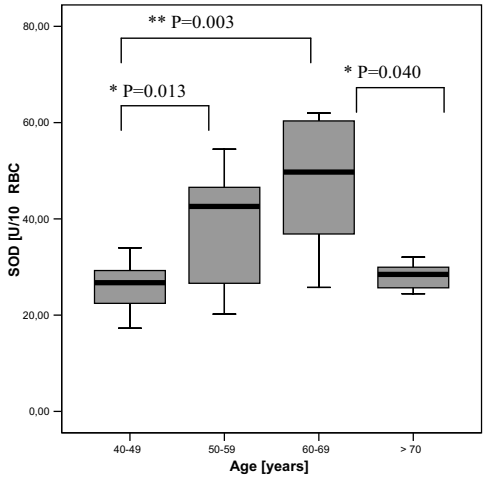

Figure 2. Erythrocyte Cu-Zn SOD activity depending on age

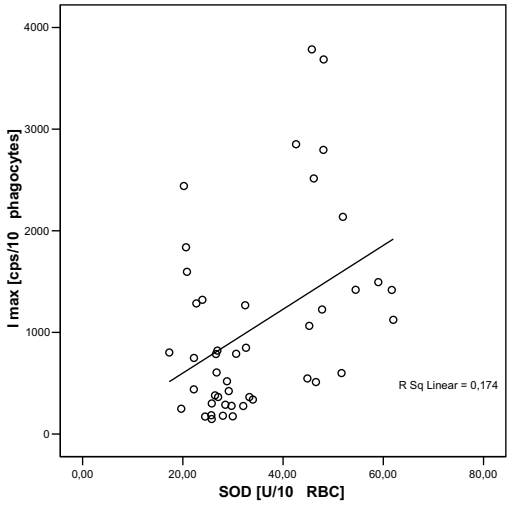

Figure 3. Correlation between the maximum intensity I_{max} of zymosan-stimulated LgCL and erythrocyte Cu-Zn SOD activity

Discussion

The majority of intracellular ROS production has been considered to originate from mitochondria [10]. Superoxide production in mitochondria occurs basically at two discreet points in the electron transport chain, namely at complex I (NADH dehydrogenase) and complex III (ubiquinone–cytochrome c reductase). Complex III is the main source of ROS under normal metabolic conditions and ageing [11]. Thus, ROS production in mitochondria may be considered as a function of metabolic rate. Some other by-products, such as glyoxal and methylglyoxal, can be produced during respiration, as well. They have been considered to contribute to the formation of glycated end products, a distinguishing feature of ageing phenotype. *In vitro* experimental data have shown that mitochondria convert 1-2% of consumed oxygen into superoxide. *In vivo* rate of ROS production is undoubtedly considerably less intensive but whatever it may be, defense mechanisms are required to reduce the potentially harmful effects from this reduction. Cells have elaborated an intricate antioxidant defense system to establish a proper balance between pro- and antioxidants.

The aim of the present study was to assess the age-related changes that occur in the mitochondrial superoxide generation by zymosan-stimulated phagocytes. Since Cu-Zn SOD is a key enzyme that eliminates superoxide produced during cellular oxidative metabolism, we aimed to evaluate the age-related changes in the activity of the enzyme in erythrocytes, as well.

Lucigenin chemiluminescence is a technique, widely used to evaluate the extracellular superoxide generation by phagocyte NADPH oxidase [5]. Besides, lucigenin can be used to measure quantitatively the phagocyte mitochondrial superoxide production [6]. Hence, the LgCL kinetic registered will visualize two different processes – the extracellular superoxide production during respiratory burst, and the superoxide production in mitochondria.

The results obtained showed that the maximum intensity of LgCL increased with age. In a previous study we reported that the extracellular superoxide production by stimulated phagocytes decreased with age [12, 13]. Therefore the enhanced LgCL recorded undoubtedly pointed out an increase in the mitochondrial cellular oxidative activity with age. Such a conclusion is supported by other authors who have established that the rate of mitochondrial superoxide generation increases with age due to the progressive oxidative modification of mitochondrial enzymes [14,15]. What is more, a negative correlation has been documented to exist between the intracellular oxidative damage and organism's maximum lifespan [16]. In the present study, an increase in the mitochondrial suproxide production was observed until the age of 70 years and thereafter a significant decline in that activity was registered.

The data published on the age-related changes in erythrocyte Cu-Zn SOD activity are inconsistent. Increased [17-19], decreased [20, 21] or even normal [22, 23] Cu-Zn SOD activity in erythrocytes with age has been reported. We found that the enzyme activity initially increased until 70 age and then decreased similarly to the mitochondrial superoxide production. Such a result is in accordance with the data of Mecocci et al. [24], who have also established an initial age-related increase in the erythrocyte Cu-Zn SOD activity with a subsequent decline in very old individuals (over 80 years of age). Andersen et al. [25] have also reported a decrease in CuZn-SOD activity in centenarians. The authors consider that the decrease in the enzyme activity is due to the lower metabolic rate and oxygen consumption in these individuals because of reduced calorie intake and decreased physical activity.

In the present work we found a statistically significant correlation between the maximum phagocyte oxidative activity and the erythrocyte Cu-Zn SOD activity. The result is not unexpected since a positive feedback has been reported to exist between the phagocyte oxidative activity and the expression of Cu-Zn SOD [4]. The overexpression of the enzyme may assist the cells to cope with the enhanced radical generation, thus preventing initiation of apoptosis and other cell-arrest producing signal pathways. In other words, the age-related increase or decrease in radical generation induces respective antioxidant enzyme expression necessary to maintain a proper cellular redox status.

Conclusion

Until recently, there was a general consensus that all physiological and biochemical functions decline with aging. Thus, predisposition of the elderly to infection has been considered to be due to a decline in phagocyte functions. Our previous reports, however, revealed an unchanged

intracellular myeloperoxidase-dependent radical generation by stimulated phagocytes through opsosnin-dependent and independent mechamism of stimulation [12, 13]. What is more, the potentially destructive extracellular ROS production reduces with age which fact does not support the notion of decline in phagocyte oxidative activity but rather modulation of that activity in relation to the existing redox environment.

Ageing is obviously a multifactorial process. Oxidative injury caused by mitochondrial dysfunction probably plays an important role in the mechanisms of ageing. It should be noted, however, that there is not enough evidence to support the relationship between oxidative stress and longevity. In experimental models, for instance, a direct relation between oxidative stress, mitochondrial DNA mutations and ageing has not been established [26]. Furthermore, there are data demonstrating that the organism may cope with the increased radical production without developing premature ageing. We believe that it is now time to revise the mitochondrial theory of ageing [27]. The elucidation of the likely reasons for the recorded decline in mitochondrial superoxide production and erythrocyte SOD activity in very old subjects remains a subject of future investigations, whose results may reveal the mechanisms that determine longevity and potential targets for therapeutic interventions.

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