Carbonylation of Plasma Proteins and Blood Hemolysis Induced by the Action of External Electrostatic Field In Vitro

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Abstract

The external electrostatic field (ESF) leads to an increase of oxidative modification of proteins and lipids in blood and in other tissues, as well as to changes in activity of pro- and anti-oxidative enzymes. The mechanism of such a response to the exposure of this physical factor is not well understood. In this paper the data on the influence of external ESF of 200 kV/m on carbonylation of plasma proteins and hemolysis of blood in vitro are presented. In order to clarify the role of blood cells in the development of oxidative processes in the plasma, ESF exposures were applied both to the whole blood and to the prior obtained plasma. In addition, samples were placed in closed and in open tubes, which allow to determine the role of air oxygen and air ions in the development of expected processes. It was shown that under the influence of external ESF plasma proteins’ carbonylation increased only when the whole blood was exposed to ESF and not the plasma alone. This indicates that the pro-oxidative processes, observed in plasma after ESF exposure, are mediated by the blood cells. Further, lysis and deformation of red blood cells were observed. Moreover, the deformation of red blood cells was most pronounced around monocytes and polymorph nuclear leucocytes, indicating the importance of these cells in the observed processes. The free air exchange had no significant effect on the results of experiments. Thus, in vitro experiments have shown that the effects of an ESF to oxidative processes in blood have been likely mediated by the activation of phagocytes independently from air ionization.

Keywords: Electrostatic field; Protein carboinylation; Red blood cells; Hemolysis; Polymorphnuclear leucocytes

Introduction

Studies on the biological effects of external electrostatic fields (ESF) are performed at different levels and in different directions. The importance of such research is dictated by the fact that the external ESF is one of the several environmental factors under constant influence of which all biological objects exist and develop. ESF of natural origin is stipulated by the charge redistribution between the upper layers of the atmosphere and the Earth surface. The tension of natural ESF near the Earth surface in a fair weather is about 150 V/m [1]. Nowadays, the background level of the external ESF is increased due to the widespread use of electrical appliances (both at home and in industry), high-voltage DC transmission lines and production of synthetic fabrics by the textile industry. In certain cases the technogenic background of the external ESF can reach up to 500 kV/m value [2].

The elucidation of the nature of ESF interactions with and mechanisms of action on biological systems is important not only in terms of purely scientific interest, but may also have applied value. In particular, the electrostatic shower or franklisation has been accepted as a therapeutic method, in which the patient has been exposed to a particular, the electrostatic shower or franklisation has been accepted as a therapeutic method, in which the patient has been exposed to a

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effects of ESF mentioned above were based on in vivo experiments. The goal of the current study was to clarify in vitro influence of the external ESF of 200 kV/m tension on plasma proteins carbonylation and blood hemolysis.

Methods

The study object and experimental design

The experimental animals were noninbred male rats weighing initially 250 to 300 g (2 months old); they were fed a standard diet (ration of food value from natural foods). Blood collection was initiated by the heart puncture using syringes washed with heparin. All manipulations with animals were done in accordance with necessary requirements of bioethics and were approved by the local Ethical Authority of Yerevan State Medical University. Blood, collected from 6 animals were combined into one. Total volume of whole blood was 16 ml. With the goal to clarify the role of cellular factor in the development of oxidative processes in the plasma, ESF exposure was applied both to the whole blood and to the plasma free of cells. Thus, blood cells were removed before (Part 1) or after (Part 2) the exposure of ESF (centrifugation, 2800 × g, 10 min). In order to elucidate the impact of the air ions in ESF-dependent modification of oxidative processes samples of the whole blood and the plasma were placed in the open tubes (free air exchange) or in the closed tubes (no air exchange). Pilot samples were subjected to the exposure of ESF (E=200 kV/m) during an hour. ESF was generated in the special capacitive type device, which was designed on the basis of X-ray apparatus "RUM-17" (former USSR) [14]. Control samples were treated in the same manner with the exception of ESF exposure. Design of the experiment is detailed in the Table 1.

Measurements

Carbonylation of proteins was measured in the samples of blood plasma using the reaction with 2, 4-dinitrophenylhydrazine (DNPH) by the method of Levine [15]. Results were expressed in terms of nmol of carbonyl groups × mg-1 of protein. Total protein content in plasma was determined by biuret reaction. The protein concentration was expressed in terms of mg × ml-1 of plasma. CAT activity in plasma was measured spectrophotometricaly by determination of the rate of H2O2 specific absorbance (l=240 nm) decrease in accordance to the method of Beers and Sizer [16]. The enzyme activity was expressed by means of nmol H2O2 min-1 mg-1 of protein. The rate of blood hemolysis was determined spectrophotometricaly by measurement of plasma absorbance at l=550 nm [17]. Results were expressed by means of units of the optical density (OD). All spectrophotometrical measurements were done using spectrophotometer Specord M40 (Analytik Jena, Germany). The RBC content in blood was determined by visual counting of cells in the Goryaev's counting chamber using the light microscope "Mikros" (Austria) at magnification 400×. Blood smears were prepared by the standard method and stained with azur-2-eosin by Romanovski-Giemsa. Statistical analysis of the results was done using the statistical functions of the GrafPad InStat software (GraphPad Software, Inc., San Diego California USA, www.graphpad.com). Two independent groups were compared using Mann-Whitney U test. All data were expressed as mean ± standard error of mean (M ± m). P-value<0.05 was considered statistically significant. n=5.

Results and Discussion

The degree of protein carbonylation is a sensitive indicator reflecting the intensity of oxidative processes in tissues [18]. Therefore, in order to identify the effect of an external ESF on the system of pro-/antioxidant homeostasis of blood we have been mainly based on the data of plasma protein carbonylation. Blood, being a liquid tissue, contacting to almost all organs and tissues, is also a convenient object for research, since the state of the blood to some extent reflects the state of the whole organism. This was the reason for the choice of the object of study. Antioxidant protection of blood is represented by enzymes localized inside the cell (SOD, CAT, peroxidase, etc.) – specifically neutralizing ROS and compounds represented both inside the cell and in blood plasma (albumin, vitamin C) – non-specifically neutralizing the ROS excess [15,19-20]. The starting compound responsible for the formation of ROS is the oxygen (O2), which is gradually being reduced and converted to superoxide anion radical (O2−), hydrogen peroxide (H2O2), hydroxyl radical (HO•), and other compounds having a high reactivity and therefore can damage the various molecular and supramolecular structures of the body [21]. Thus, in experiments in vitro, air oxygen might have a significant effect on oxidative processes in the blood. However, we did not find any significant differences in the carbonylation of plasma proteins between samples placed in open and closed tubes (Figure 1). This fact is apparently due to the buffer capacity of the antioxidant defense of blood. At the same time, external ESP differently reflected on the carbonylation of plasma proteins, depending on the whole blood (Part 2) or the plasma (Part 1) was exposed.

The effect of ESF on the level of plasma protein carbonylation

After ESF exposure the protein carbonylation in plasma had a tendency to decrease (-15%; P>0.05), compared with similar control samples (Figure 1). Furthermore, in the control samples (without exposure of ESF) alterations of plasma protein carbonylation also observed. So, carbonylation of the plasma proteins at the sham exposed (during an hour) plasma samples (Part 1) was higher by 33-52% (P

| Part 1 | Part 2 |
|--------|--------|
| (blood cells were removed before ESF exposure) | (blood cells were removed after ESF exposure) |
| Open | Closed |
| (samples were placed in the open tubes) | (samples were placed in the closed tubes) |
| Control | ESF | Control | ESF |
| Control | ESF | Control | ESF |

Table 1: Experimental design.
In contrast to the data obtained after the plasma exposure in ESF, the whole blood exposure in ESF during an hour (Part 2) was reflected with significantly enhanced carbonylation of plasma proteins (+42-50%; P <0.05) compared to similar control samples (Figure 1). Furthermore, there was a slight increase of the content of total protein - by 4-6% (P> 0.05) (Figure 2), and the increase of CAT activity - by 16% (P>0.05) - in samples placed in open test tubes; and by 77% (P <0.05) – in samples placed in closed tubes (Figure 3). This fact indicates that the air oxygen makes some changes in the specific pro-/anti-oxidant system. In our studies exposure to air expressed only on the values of CAT activity in plasma and only in the control samples of the part 2 (whole blood exposure). ESF also leads to an increase in CAT activity in plasma, placed in both open and closed tubes - thus overlapping the effect of air oxygen. In other words the impact of ESF on the investigated parameters apparently was independent of the air ions.

Thus, data obtained in samples of part 2 demonstrated the importance of the cellular factor in modification of the oxidative processes in plasma. In this particular case we are talking about intensification of prooxidant processes in blood in response to the action of an external ESF. As it is known, the main ROS-generating enzymes in the blood cells are NAD(P)H oxidases [22]. Earlier, in experiments in vitro, we have shown the increase of the activity of NADPH oxidase in polymorphonuclear leukocytes (PMNL) [23] and the increase of the activity of NADH oxidase in RBC of the peripheral blood of rats [9]. It is natural to assume that similar processes may also take place at the influence of ESF on isolated blood samples - in the experiments in vitro. It was found that the generation of ROS by leukocytes, especially PMNL, increased significantly at their activation [24]. Generated ROS can damage different supramolecular structures. In particular plasma membrane damage can lead to cell death. In our experiments this was manifested by an increase of CAT activity in plasma after the initial whole blood exposure in the ESF. It is well known that CAT is an intracellular enzyme [25]. Therefore, increased CAT activity in plasma can be interpreted as a consequence of enhanced permeability of plasma membrane or cell lysis/death - in particular RBC. We did not observe any statistically significant difference between samples placed in open and closed tubes which mean negligible effect of the air oxygen to mentioned parameters. Thus, based on the data obtained in the part 2, it can be assumed that the ESF apparently leads to activation of leukocytes, and consequently intensifying the generation of ROS. This causes an increase of carbonylation of plasma proteins and the blood hemolysis, which was manifested in CAT activity increase in plasma. In order to verify the assumptions made, we investigated parameters characterizing blood hemolysis: the content of RBC in blood and hemoglobin in the plasma. Also we made an analysis of stained blood smears to identify damaged RBCs.

**The effect of ESF on blood hemolysis**

RBC count in blood samples exposed in ESF for an hour did not differ significantly from that in the control samples (Psc. 4). Only a tendency to decrease of RBC count in blood was noted (-3%; P>0.05). The high inaccuracy of the data neither allows to state the fact of ESF induced hemolysis, nor determines the exact number of lysed RBCs. It is supposed that the number of lysed RBCs in blood samples exposed to ESF has the order of 10⁶ cells x ml⁻¹ (Figure 4). Data on CAT activity in plasma also indicate that the hemolysis occurred in blood samples exposed to ESF. Hemoglobin content in plasma was measured to clarify the degree of hemolysis as the backup "reverse" test.

Measurement of the optical density of plasma at λ=550 nm showed a significant level of hemolysis induced by an external ESF. Thus, the optical density of the plasma in ESF exposed samples exceeded that of the sham sample of part 1. n =5.

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**Figure 2:** Effect of the ESF (E= 200kV/m, 1 hour) to the total protein content in blood plasma in the experiments in vitro. Part 1 - The blood cells were removed prior to the exposure; Part 2 - the blood cells were removed after the exposure. *P value versus appropriate control sample; #P value against appropriate sample of part 1. n =5.

**Figure 3:** Effect of the ESF (E= 200kV/m, 1 hour) to the CAT activity in blood plasma in the experiments in vitro. Part 1 - the blood cells were removed prior to the exposure; Part 2 - the blood cells were removed after the exposure. *P value versus appropriate control sample; #P value against appropriate sample of part 1. n =5.
parameter in the similar control samples by 115% (P<0.05) - in the open tubes; and by 215% (P<0.05) - in the closed tubes (Figure 5). Smaller difference in the samples placed in the open tubes was stipulated by the fact that a certain degree of hemolysis occurred also in the appropriate control samples. The optical density of the plasma at λ=550 nm allowed us to calculate the number of lysed RBCs by the following equation:

\[ C_{RBC} = \left( \frac{\Delta OD}{e} \times M \right) / b \]

Where CRBC - the concentration of lysed RBCs (× 10^8 × ml⁻¹); ΔOD - the difference in absorbance between the control and test samples (0.139 and 0.189 – for the samples in open and closed tubes respectively); e - molar extinction coefficient of oxyhemoglobin (HbO₂) at λ=550 nm (14 × 10^6 × mol⁻¹ l⁻¹ cm⁻¹) [26]; M - molecular weight of hemoglobin (6.45 × 10⁸ pg mol⁻¹); b - the average content of hemoglobin in one RBC (20 pg). After substitution of the appropriate values of the variables the following results were obtained: 0.32 × 10^8 lysed RBCs × ml⁻¹ - for the ESF exposed samples placed in the open tubes; 0.44 × 10^8 lysed RBCs × ml⁻¹ - for the ESF exposed samples placed in the closed tubes. The calculated data agree well with the data obtained by direct counting of RBCs in the blood and determining the activity of CAT in the plasma.

Lysis of RBCs in the samples exposed to ESF apparently is stipulated by the damage of their plasma membranes which may be caused by the exposure of ROS. Furthermore, ROS may be generated by both RBCs and other blood cells. It should be noted that hemolysis was the extreme consequence of the impact of ROS to the plasma membrane of RBCs. The intermediate stage of RBCs’ damage appears evident.
in their deformation. Thus, oxidative damage of membranes leads to various forms of abnormal RBCs - ellipocytes, echinocytes, knizocytes, codocytes etc [27]. In order to identify the RBCs' deformation we analyzed the blood smears. Deformed RBCs - mainly echinocytes with a plurality of evenly sharp edges really were detected in the samples exposed to ESE. Clusters of echinocytes were mainly observed around monocytes, neutrophils and eosinophils, i.e. the cells capable of phagocytosis (Figure 6). The deformation of red blood cells was most pronounced around the neutrophils of blood samples exposed to ESE. Slight deformation of red blood cells was also observed around the neutrophils of the control blood samples. Around lymphocytes the deformed RBCs were not observed. The data provided above suggest the assumption that the external ESF ($E=200 \text{ kV/m}$) leads to the activation of phagocytes, which in turn leads to increased generation of ROS and all subsequent pro-oxidant processes in the blood. It has been described above - in the experiments in vitro, and in the experiments in vivo observed earlier.

Conclusion

A significant increase of the carbonylation degree of plasma proteins was shown after exposure of whole blood of rats in the ESF ($E=200 \text{ kV/m}$, 1 h) to the in vitro experiments. At the same time no significant effect was detected if only the plasma was exposed to the ESF. Similar results were observed for CAT activity in the plasma, indicating a key role of the blood cells in ESF-induced modification of oxidative processes in the plasma. Contact of the samples with the air had no significant influence on the effects induced by an external ESF. Nevertheless, some fluctuations of data were observed in the control samples which led to a certain distortion of results of investigations. Exposure of whole blood in the ESF also causes a deformation and the lysis of RBCs. The deformation of RBCs was most pronounced around the phagocytic leucocytes (neutrophils, monocytes, eosinophils), which indirectly indicated the ESF-induced activation of blood leucocytes. The last was manifested in the damaging of the neighboring RBCs, which likely was a result of increased generation of ROS.

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