THE NEW ARMENIAN MEDICAL JOURNAL
Vol.7 (2013), No 1, p.22-32

MODIFICATION OF OXIDATIVE PROCESSES IN BLOOD UNDER THE EXTERNAL STATIC ELECTRIC FIELD

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Received 7/21/2012; accepted in final form 12/10/2012

ABSTRACT

Rats were exposed to 200 kV/m external static electric field to study its effect on blood pro- and antioxidant systems and on targets of reactive oxygen species. We found inhibition of the NADPH-dependent superoxide radical ($O_2^{\cdot-}$) generation in blood of static electric field-exposed animals. This might be ascribed to the myeloperoxidase activation that is also responsible for increased level of the advanced oxidation protein products. Superoxide dismutase activity was found to increase with the simultaneous decrease of catalase activity after the exposure to static electric field. Under these conditions, H_2O_2 accumulation is anticipated. The latter is most probably followed with enhanced malondialdehyde content inside the red blood cell, as well as the increased tyrosine and tryptophan oxidation in plasma of experimental group animals. Nevertheless, we did not observe the effect of static electric field on carbonyl content of total plasma proteins. On the other hand, the carbonylation of fibrinogen and albumin was found highly increased after exposure to the static electric field. Taking into consideration the static electric field-dependent decrease of plasma fructosamine, we suggest the hypothesis of masking the mentioned effect on plasma proteins carbonylation by glycoxidation products.

Keywords: *protein carbonylation, superoxide dismutase, catalase, malondialdehyde, free radicals.*

INTRODUCTION

Oxidative stress, an imbalance toward the prooxidant side of the prooxidant/antioxidant homeostasis, occurs in several human diseases. Among these diseases are Alzheimer's disease, rheumatoid arthritis, diabetes, sepsis, chronic renal failure, and respiratory distress syndrome [*Dalle-Donne I. et al., 2003*]. Such etiopathogenic significance of oxidative processes makes important the identification of influencing factors. There is increasing experimental evidence that externally applied static electric field (SEF) exerts various effects on oxidative processes and antioxidative defense systems. Earlier provided data [*Sauer H. et al., 1999*] speak in favor of intracellular reactive oxygen species (ROS) enhancing under the action of SEF. It was shown that strong SEF causes transient inhibition of antioxidant enzymes activity in erythrocytes with subsequent adaptative stimulation of this activity [*Cieślar G. et al., 2003*]. The increase in superoxide dismutase (SOD) activity and thiobarbituric acid reactive substances (TBARS) levels of different tissues after the exposure to SEF was also shown [*Güller G. et al., 2006; Hardalaç F., Güller G., 2008*]. The enhanced activity of phospholipase A_2 in erythrocyte membrane of rats exposed to SEF might indicate the activation of lipid peroxidation scavenging system [*Artsruni G. et al., 1999*].

In spite of the provided facts evidencing SEF effects on biologic systems, knowledge of the mechanisms, by which the external SEF might exert an imbalance in the prooxidant/antioxidant homeostasis, is lacking.

The enhanced oxygen consumption by tissues after SEF exposure was described [*Kossler F., Gelbrich W., 1973*]. The increased supply of oxygen, if not properly utilized, might exert an oxidative damage along with reactive oxygen species (ROS) formation and/or after conversion to ROS [*Shacter E., 2000*]. Higher tissue oxygenation also supposed acceleration of mitochondrial respiratory chain and oxidative phosphorylation of adenosine diphos-

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phate (ADP) accompanied by enhanced outlet of superoxide radical from incomplete oxygen reduction, which normally has a ratio about 2% of the total oxygen consumed by mitochondria [*Boveris A., Chance B., 1973*]. Indeed, as it was demonstrated, the exposure of reconstituted H^+-adenosine triphosphatase (ATPase) liposomes to an external electric field results in a net formation of adenosine triphosphate (ATP) [*Rögner M. et al., 1979*]. Such ATP synthesis was demonstrated also with mitochondria and submitochondrial particles [*Hamamoto T. et al., 1982*]. On the other hand, there are data with reversed results, that is inhibition of ATP synthesis [*Artsruni G., Ter-Markosyan* A., *1987*].

The next possible source of SEF-dependent ROS generation may be based on Ca^{2+} releasing from mitochondria. It is known that Ca^{2+} efflux from mitochondria is accompanied by or depends on burst of ROS. In several studies, applying a few nanosecond SEF pulses on the suspensions of cells showed an increase of cytoplasmic Ca^{2+} due to its release from the intracellular calcium store [*Vernier P. et al., 2003*]. Similar effect was observed in liver cells of rats after SEF application [*Artsruni G., 2001*]. Although SEF effects on ROS generation, lipid peroxidaton, and antioxidative enzymes activity were shown earlier and the possible sources of superoxide leakage following SEF exposure were also suggested, the exact nature of oxidative processes development and the influence on oxidative modification of proteins at SEF-exposed animals is still unclear. Taking into account the sensitivity of proteins to oxidative attack and their importance in physiologic processes, one should give a special attention to the effect of SEF on oxidative modification of proteins. Inasmuch, we attempted to analyze and explain some of the oxidation relevant biochemical effects that occur in blood of rats exposed to SEF. We attached the particular emphasis to carbonylation of plasma proteins.

Methods

Animals and Experimental Design: The experiments were carried out with a total of 60 adult albino male rats weighing 160 *g* on average. The work was approved by the Ethical Committee of the Yerevan State Medical University after Mkhitar Heratsi. The rats were housed in plastic boxes under similar conditions of temperature, light, acoustic noise, and ventilation and received the same diet in the course of experiments. Animals were separated into two equal groups: the first group served as a control; rats of the second group were exposed to SEF of 200 *kV/m* strength during 1 hour. The SEF was generated in a special capacitor-type laboratory device [*Artsruni G. et al., 1987*].

Blood Sampling and Processing: Blood was drawn immediately after SEF action by cardiac puncture by a two-syringe technique: one of which was anticoagulated with 3.8% buffered citrate in a 1:9 citrate/blood proportion (for total blood, plasma, and red blood cells); another syringe was non-anticoagulated (for studying superoxide generation during blood coagulation). Blood was centrifuged for 10 *min* at 2800 ´ *g* for plasma separation. The red blood cells were washed thrice in phosphate buffered saline (PBS), 0.1M K/K phosphate buffer of pH 7.4 in 0.9% saline, to remove the buffy coat and plasma. Superoxide generation assay was initiated immediately, and the rest analyses were done during the same day.

Assay of Superoxide Radical Generation by Whole Blood: Superoxide radical ($O_2^{\cdot-}$) generation was measured by the nitroblue tetrazolium (NBT) reduction in the total blood according to a previously described procedure [*Demehin A. et al., 2001*] with modifications [*Bliznetsova G. et al., 2004*]. The data were represented as *umol* $O_2^{\cdot-}$ min^{-1} L^{-1}.

Antioxidant Enzyme Activity Assay: Superoxide dismutase (SOD) activity was measured according to the available method [*Kostyuk V., Potapovich A., 1989*]. SOD activity was expressed as *U/mg* of protein. Catalase (CAT) activity was measured by the appropriate method [*Beers R., Sizer I., 1952*]. The enzyme activity was expressed as *umol* H_2O_2 min^{-1} mg^{-1} protein.

Lipid Peroxidation: Lipid peroxidation in the blood plasma and hemolysate was determined by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction as described [*Uchiyama M., Mihara M., 1978*]. The results were expressed as MDA *nmol/mg* of protein.

Fructosamine Assay: Plasma and hemolysate fructosamine content was determined by the adequate method [*Johnson R. et al., 1982*] with modifications [*Ohkawara E. et al., 2002*]. The results were

expressed as fructosamine *nmol/mg* of protein.

Determination of Advanced Oxidation Protein Products (AOPPs): Spectrophotometric determination of AOPPs levels was performed by the corresponding method [*Witko V. et al., 1992*] with modifications. The results were expressed as AOPPs *nmol/mg* of protein.

Assay of Aromatic Amino Acids Oxidation (Fluorescence Measurements): Tyrosine and tryptophan oxidation was measured in both plasma and fibrinogen samples. Fibrinogen was isolated from citrated plasma by the sodium sulfate precipitation (Na_2SO_4, 10.6% final concentration) [*Cohen S., 1956*]. Tryptophan destruction and dityrosine production were measured with a "Hitachi-MPF-4" spectrofluorometer. Dityrosine production was assessed at 325 *nm* excitation and 415 *nm* emission [*Giulivi C., Davies K., 1994*]. Tryptophan oxidation was monitored by loss of protein fluorescence at 295 *nm* excitation and 340 *nm* emission [*Teale F., 1960*]. Results were expressed in relative fluorescence units per milligram (RFU/mg) of protein.

Quantification of Protein Carbonyls: Carbonylation of whole plasma/hemolysate proteins and fractions enriched with fibrinogen, prothrombin, immunoglobulin G (IgG), and albumin was assayed. Fibrinogen was purified as described above. Prothrombin was isolated from 0.2 *ml* of plasma by $BaSO_4$ precipitation in accordance to [*Esnouf M. et al., 1973*]. IgG was isolated from 0.025 *ml* of plasma by ammonium sulfate precipitation – $(NH_4)_2SO_4$ half saturation [*Page M., Thore R., 2002*]. Albumin was separated from supernatant of IgG purification by isoelectric precipitation at pH 4.7 [*Hao Y., 1979*]. The protein carbonyl content was measured by the method [*Levine R. et al., 1990*] with some modifications. The carbonyl content was expressed as *nmol/mg* of protein.

Statistical Analysis: 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). The Independent Group *t*-test was performed. The results were expressed as mean ± standard error of mean (SEM). A *p*-value of <0.05; <0.01; and <0.001 was considered as statistically significant (*), highly significant (**) and extremely significant (***), respectively.

RESULTS

SEF effect on superoxide generation: At intact animals blood coagulation process nicotinamide adenine dinucleotide phosphate (NADPH) addition brought to about 60-70% increase of superoxide anion radical ($O_2^{\cdot-}$) generation (Figure 1). Blood treatment with nicotinamide adenine dinucleotide (NADH) resulted in more than duplicated $O_2^{\cdot-}$ production. We observed diminished production of $O_2^{\cdot-}$ after SEF exposure in all samples compared to appropriate controls. Moreover, SEF almost completely abolished NADPH- and coagulation-dependent superoxide generation processes. At the same time, NADH derived stimulation of $O_2^{\cdot-}$ generation was not affected by SEF and retained the rate value twice higher than the appropriate non-stimulated sample in the control group.

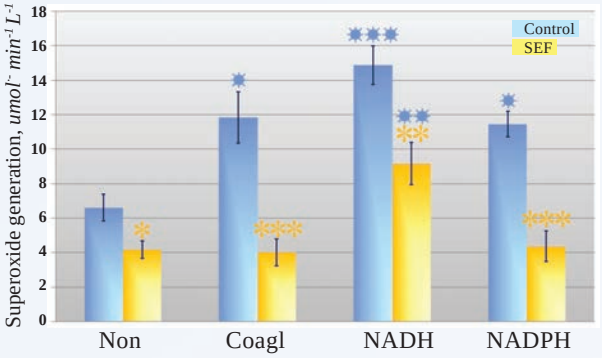

FIGURE 1. *SEF influence on superoxide radical generation by whole blood. "Non" – non-stimulated/spontaneous generation; "Coagl" – $O_2^{\cdot-}$ generation stimulated by blood coagulation process; "NADH" and "NADPH" – generation of $O_2^{\cdot-}$ stimulated by the addition of NADH/NADPH 0.15% for the final concentration. * – p value vs. control; ☼ – p value vs. "Non" sample; n = 10.*

SEF effect on antioxidative enzymes activity: SEF caused different effects on antioxidative enzymes activities (Figure 2). SOD activity remained unchanged in plasma and highly increased in hemolysate (65%) (Figure 2 A, B). On the other hand, we observed diminishing of CAT activity in plasma (-35%) (Figure 2 C, D). Effect of SEF on CAT activity in hemolysate had a decreasing tendency as well. At the same time, the SOD/CAT ratio (Figure 2 E, F) increased significantly both in plasma and hemolysate of SEF-exposed animals.

SEF effect on MDA content: SEF expressed opposite effects on MDA levels in plasma and hemo-

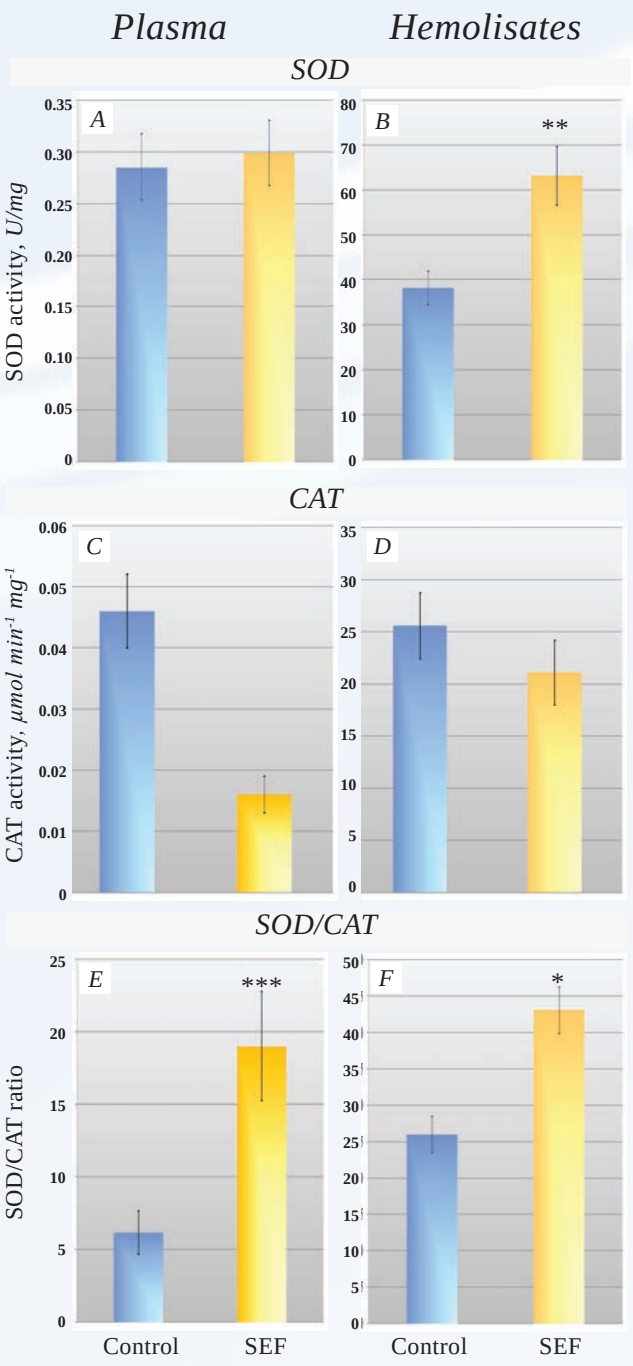

FIGURE 2. *The effect of SEF on the activity of antioxidant enzymes.* ***A, B*** *– superoxide dismutase and* ***C, D*** *– catalase activity,* ***E, F*** *– SOD/CAT ratio in plasma and hemolysate samples, respectively. * – p value vs. control; n = 10.*

lysate (Figure 3 A, B). We detected a decrease (-30 %) of MDA content in blood plasma, and at the same time significantly increased (65%) MDA levels were observed in hemolysate samples.

SEF effect on fructosamine content: We observed the substantially decreased (-74%) fructosamine content in plasma after SEF exposure (Figure 4 A). At the same time, SEF effect on the fructosamine level in hemolysate was negligible.

SEF effect on advanced oxidation protein products (AOPPs) content: AOPPs being alternative markers of proteins' oxidative damage were also increased after SEF exposure both in plasma (21%) and hemolysate (28%) (Figure 5 A, B).

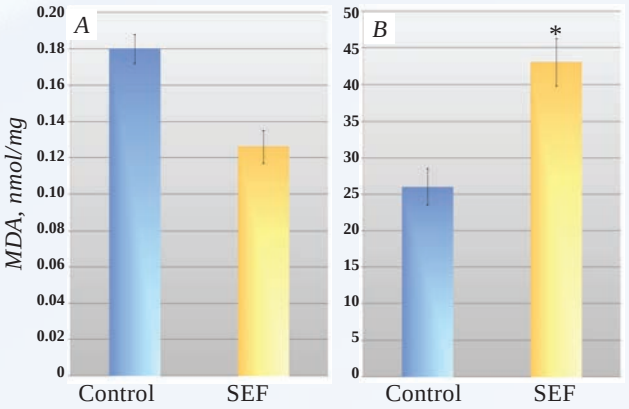

FIGURE 3. *The effect of SEF on the lipid peroxidation process. A, B – malondialdehyde content in plasma and hemolysate samples, respectively. * – p value vs. control; n = 10.*

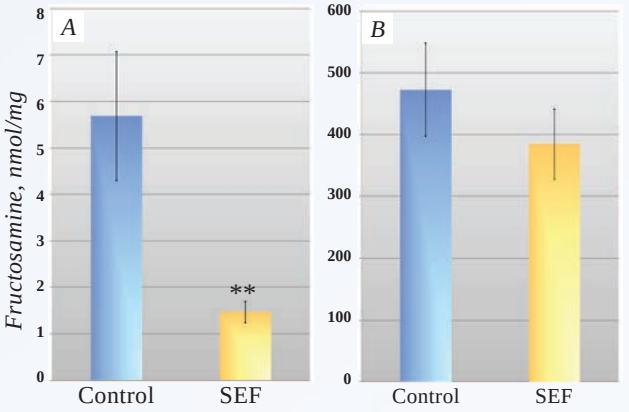

FIGURE 4. *SEF effect on the protein glycation level. A, B – fructosamine content in plasma and hemolysate samples, respectively. * – p value vs. control; n = 10.*

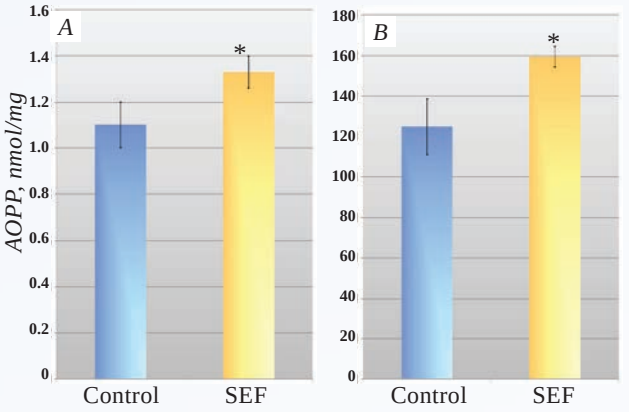

FIGURE 5. *The effect of SEF on the advanced oxidation protein products content in plasma (A) and hemolysate (B) samples. * – p value vs. control; n = 10.*

SEF effect on the level of aromatic amino acids oxidation: SEF brought to the pronounced increase (55%) of dityrosine content of total plasma protein (Figure 6). We also observed a moderate effect of SEF on rising dityrosine level in fibrinogen (35%). Unlike dityrosine formation, SEF did not affect tryptophan oxidation in fibrinogen; nevertheless, we observed marked decreasing (-46%) of tryptophan characteristic fluorescence in whole plasma samples of SEF-exposed animals.

SEF effect on protein carbonylation: SEF did not cause a significant alteration of total protein carbonylation in blood plasma and hemolysate (Figure 7 A, B). Nevertheless, the tendency to diminishing mentioned parameters was detected in both samples. On the other hand, the analyses of separate plasma proteins carbonylation revealed high susceptibility of fibrinogen and albumin to SEF (Figure 7 C). Carbonylation of both mentioned plasma proteins was almost duplicated at SEF-exposed animals. At the same time, carbonylation of prothrombin and IgG did not significantly alter after SEF short-term exposure.

DISCUSSION

We suggest a mechanism of external SEF impact on blood oxidative processes (Figure 8). First of all, we observed diminished production of $O_2^{\cdot-}$ by blood cells at SEF-exposed animals. Decreased $O_2^{\cdot-}$ generation should be followed by inhibited oxidation processes; however, this phenomenon seems controversial to the literature data evidencing in favor of pro-oxidative activity of environmental electrostatic fields [*Lupke M. et al., 2004; Güller G. et al., 2006*]. In spite of diminished $O_2^{\cdot-}$ production, the potential of NADH to stimulate $O_2^{\cdot-}$ generation after SEF exposure was retained. This probably means unaffected $O_2^{\cdot-}$ leakage from the NADH susceptible mitochondrial respiratory chain, on the one hand, and/or the

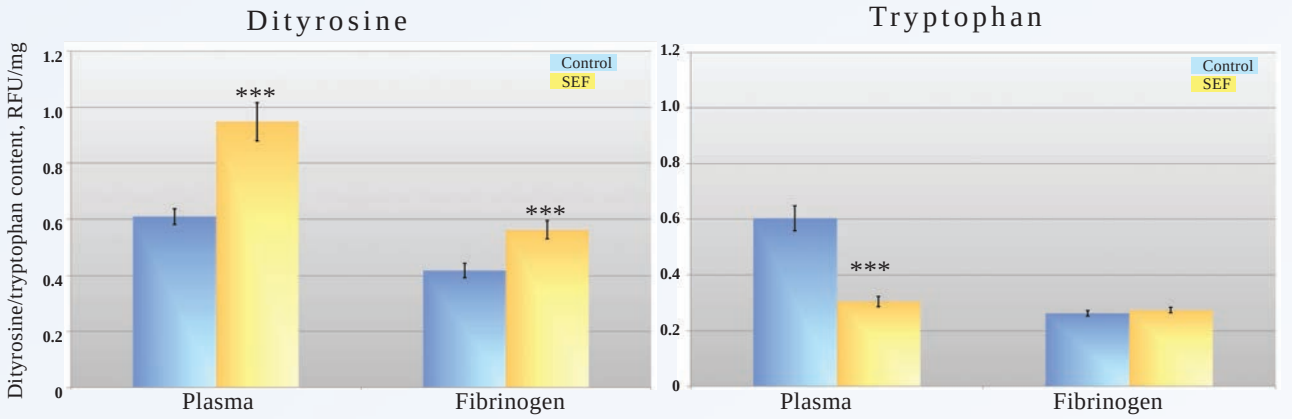

FIGURE 6. *Modification of dityrosine formation and tryptophan loss by SEF in plasma and fibrinogen samples. * – p value vs. control; n = 10.*

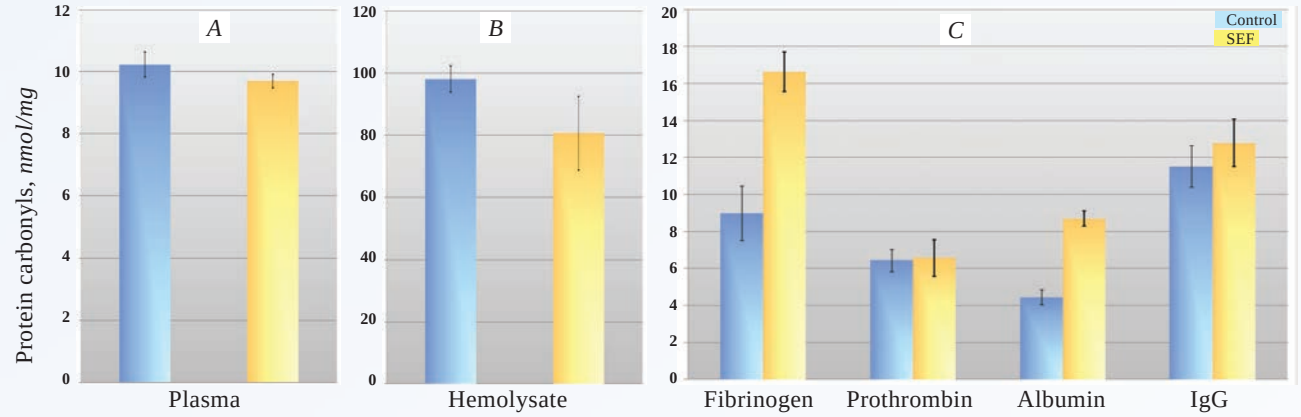

FIGURE 7. *The effect of SEF on the protein carbonyls content. A, B – carbonylation level of plasma and hemolysate total proteins, respectively. C – carbonylation level of the fractions enriched with fibrinogen, prothrombin, albumin, and immunoglobulin G. * – p value vs. control; n = 30*

activation of $O_2^{\cdot-}$ neutralization system, on the other hand. The SEF-driven activation of $O_2^{\cdot-}$ scavenging SOD activity was shown earlier [*Cieślar G. et al., 2003; Güller G. et al., 2006*]. Here we provide evidence on increased activity of intracellular (intraerythrocytic) SOD after SEF exposure, which might be responsible for less superoxide generation by blood cells. Complete neutralization of ROS anticipates simultaneous activation of CAT for scavenging the dangerous product of SOD activity: H_2O_2. We did not observe CAT activation after SEF exposure; furthermore, SEF caused an inhibition of CAT activity more expressed in plasma. Hence, at a condition of increased ratio of SOD/CAT activities the accumulation of H_2O_2 is expected. The earlier provided evidence of magnetic field-dependent H_2O_2 production by human fibrosarcoma cancer cells and bovine pulmonary artery endothelial cells [*Martino C., Cas-*

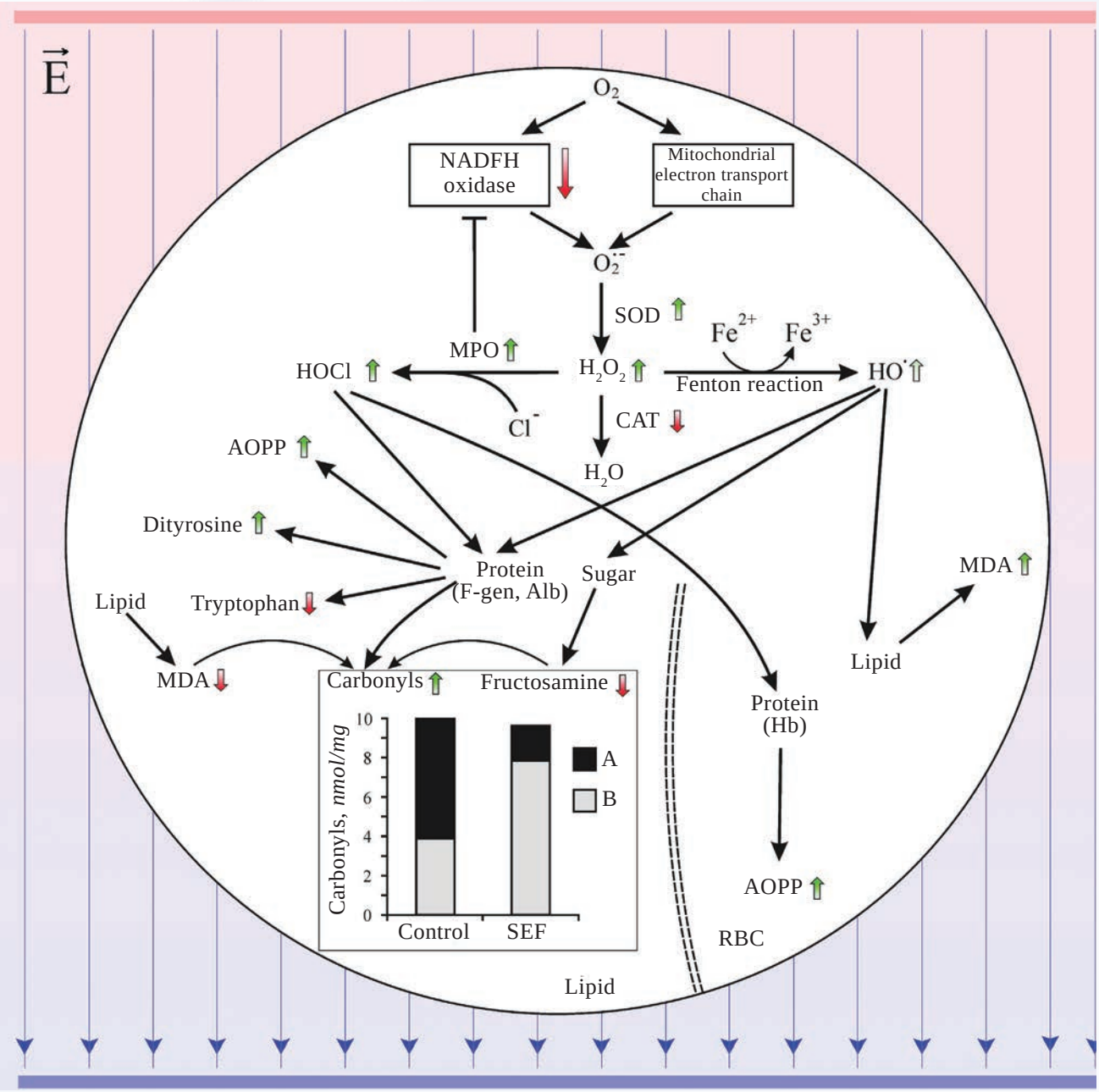

Figure 8. *A proposed concept of the oxidative processes alterations in blood by the externally applied SEF. "F-gen" – fibrinogen; "Alb" – albumin; "Hb" – hemoglobin; "A" – glycation/glycoxidation impact to carbonyl content of protein; "B" – carbonyl groups produced directly on protein side chains; RBC – red blood cell. ⇧ – direction of the SEF effect.*

tello P., 2011] also support this assumption. Analogous data for SEF effects was not found in the available literature. Metal catalyzed oxidation of H_2O_2 generates highly reactive hydroxyl radical ($HO^{\cdot}$) and can inflict the oxidative damage of lipids, sugars, proteins and nucleic acids [*Jana A. et al., 1990; Stadtman E., 1990*]. The alternative fate of H_2O_2 is oxidation of halide ions to hypohalous acids catalyzed by myeloperoxidase (MPO)(EC 1.11.2.2) [*Harrison J., Schultz J., 1976*]. Oxidative modification of proteins by hypochloric acid (HClO) generates products known as advanced oxidation protein products (AOPPs) [*Witko-Sarsat V. et al., 1998*]. Increased AOPPs levels both in plasma and hemolysate after SEF action was shown in our experiments, which might indicate MPO activation. Possible increase of MPO activity also explains the abolished $O_2^{\cdot-}$ generation during blood coagulation and after NADPH addition, bearing in mind data evidencing the contribution of MPO to NADPH-oxidase (EC 1.6.3.1) inhibition followed with termination of $O_2^{\cdot-}$ generation [*Jandl R. et al., 1978*]. Respiratory burst in neutrophils initiated by blood coagulation was shown earlier [*Gerasimov I., Ignatov D., 2005; Bakdash N., Williams M., 2008*]. According to our results, ROS generation by clotting activated neutrophils/platelets is based on NADPH-oxidase activity inhibited by SEF exposure: probably, by adaptive activation of MPO.

As it was mentioned above, oxidation of proteins, lipids, and sugars is anticipated at the stage of H_2O_2 accumulation. We observed rather ambiguous effects of SEF on these processes. First, SEF reduced MDA content in plasma with simultaneous increase of MDA in hemolysate. One can assume SEF-dependent inhibition of extracellular oxidative processes and the intracellular stimulation. Earlier A. Savchenkova and associates showed the predominant oxidation of fibrinogen and its competition with plasma lipids during Cu^{2+}-induced oxidation [*Savchenkova A. et al., 2003*]. These data are concordant with our results. Particularly, we observed increased carbonylation of fibrinogen and albumin and diminished MDA levels at SEF-exposed animals. Probably, albumin-like fibrinogen competes with plasma lipids during oxidative stress. Moreover, albumin and fibrinogen along with ceruloplasmin and transferrin are already recognized as natural antioxidants [*Halliwell B., 1988; Olinescu R., Kummerow F., 2001*]. At the same time, we did not observe SEF effect on proteins carbonylation in hemolysate; this latter coincided with the increased intracellular MDA level. It might mean that proteins (albumin and fibrinogen) are primary targets of free radicals attack in plasma, and lipids are damaged first in intracellular area of red blood cells. Hence, our data signify to H_2O_2, HClO, and $HO^{\cdot}$ driven oxidative processes stimulation by external SEF.

The second ambiguous result relates to plasma proteins oxidative modification. Especially, as it was stated above, we detected highly increased carbonylation of fibrinogen and albumin purified from SEF-exposed animals. Taking into account the abundance of albumin in blood plasma, its increased carbonylation should be reflected on plasma total protein carbonyl content, which we did not observe; moreover, total protein carbonylation had a decreasing tendency after SEF exposure and this latter was also reported earlier [*Güller G. et al., 2009*]. It seems even more peculiar having in view raised plasma dityrosine content and loss of tryptophan residues at SEF-exposed animals, thus signifying to SEF dependent oxidative attack on plasma proteins detectable on raw plasma level. With the goal to supply an explanation to this discrepancy, we are inclined to refer to contribution of different compounds to protein carbonylation. As known, besides carbonyl groups (aldehydes and ketones) production directly on protein side chains (especially of Pro, Arg, Lys, and Thr) they may be introduced into proteins by secondary reaction of the nucleophilic side chains of Cys, His, and Lys residues, with aldehydes produced during lipid peroxidation or with reactive carbonyl derivatives generated as a consequence of the reaction of reducing sugars or their oxidation products with lysine residues of proteins (glycation and/or glycoxidation reactions), with the eventual formation of the advanced glycation/lipoxidation end products (AGEs/ALEs) [*Stadtman E., Berlett B., 1997*]. ALEs adducts might contribute to increased carbonylation of fibrinogen and albumin and decreased MDA level observed in plasma after

SEF action. AGEs generation based on sharp decreasing of fructosamine level in blood plasma might be also supposed due to our research findings.

Nonenzymatic glycation of proteins results in the formation of an early glycated and stable Amadori adduct, fructoselysine, which undergo further irreversible chemical reactions to form Maillard's AGEs [*Miyata T. et al., 1998*]. A. Lapolla and coworkers reported about determination of amino group glycation by colorimetric reaction of NBT, which is specific for Amadori products only (early glycation product) and not for late AGEs [*Lapolla A. et al., 2005*]. This signifies to SEF stimulated generation of NBT-undetectable AGEs from early glycated and stable Amadori protein adducts in plasma proteins. On the other hand, we did not observe SEF effect on fructosamine content in red blood cells. As mentioned, this might be explained by predominant oxidation of lipids in the cell.

Returning to the discussed discrepancy in unchanged plasma total protein carbonylation and SEF-induced increase of oxidative modification of separated fibrinogen and albumin, we refer to slow and reversible (at the first Schiff base formation stage) character of Millard's reaction. This process also strongly depends on sugar concentration in the media [*Monnier V., 1990*]. During protein purification by the method of salting out and several washings of protein precipitate, unstable glycation adducts might segregate from purified protein resulting in diminishing of dinitrophenyl hydrazine (DNPH) respondent carbonyls on protein molecule. This hypothesis is supported by lower level of carbonylation of purified albumin referred to total plasma value in both control and SEF-exposed groups. Disappearing of carbonyls upon incubation of fructated bovine serum albumin (BSA) under physiological conditions in the absence of excess sugar was also reported earlier [*Liggins J., Furth A., 1997*]. According to Namiki pathway of AGEs formation [*Glomb M., Monnier V., 1995*], Schiff base adducts of the amine with glycolaldehyde could hydrolyze to release glycolaldehyde, which might contribute to diminishing DNPH reactive carbonyls in protein. Besides, the main final product of Maillard's reaction – carboxymethyllysine (CML) is not a carbonyl (CO) compound but carboxyl (COOH), the reaction of which with DNPH is not expected. Obviously, different stages of glycation and then glycoxidation processes are characterized by more or less expressed carbonylation of the target protein.

Thus, we propose the masking of SEF-induced protein direct carbonylation by counterpoising with diminished contribution of glycoxidation through DNPH non-respondent AGEs formation or segregation of carbonyls from proteins upon SEF dependent oxidation. Proteins carbonylation assay after purification probably diminishes the impact of glycation/glycoxidation on carbonyl groups content and reveals SEF inducible "real" carbonylation of plasma proteins.

Finally, our results showed diminished oxidation of aromatic amino acids (tyrosine and tryptophan) in fibrinogen as referred to total plasma levels. Particularly, dityrosine formation at SEF-exposed animals was almost twice less expressed, and we did not observe SEF effect on tryptophan destruction in fibrinogen. Simultaneously, at the level of total plasma proteins both amino acids were highly affected by SEF. Having in view high-level of SEF induced carbonylation of fibrinogen, we suppose the ROS capture by amino acids inclined to carbonyls formation (primarily Pro, Arg, Lys, and Thr), which in this context act as intramolecular antioxidants. Besides, tryptophan residues are not expected to be oxidized by metal-catalyzed oxidation, because tryptophan is not typically a site for metal iron binding [*Stadtman E., 1993*].

Conclusion

We described the effect of external SEF on oxidative processes in blood of rats. The SEF induced disbalance between SOD and CAT activities leading to H_2O_2 accumulation is considered a triggering and key point responsible for stimulation of oxidative modification of proteins, lipoxidation, and glycoxidation. Predominant oxidation of proteins is shown in blood plasma, and lipids are described as the primary targets of ROS inside the red blood cells. We also showed the masking of the SEF effect on the plasma total protein carbonylation by diminished contribution of glycoxidation products, which were probably segregated from the protein molecule by oxidative attack or transformed to non-determined compounds.

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