

Membrane Switch Hypothesis. 2. Domain Structure of Phagocytes in Horses with Recurrent Airway Obstruction

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The mechanism of recurrent airway obstruction (RAO) in horses was investigated by measuring the membrane domain structure and oxy–redox activity in phagocytes isolated from bronchoalveolar lavage fluid (BAL) and from the blood of healthy and RAO horses by electron paramagnetic resonance (EPR). Differences in the activity of intracellular antioxidant enzymes CAT, GPx, and SOD measured in phagocytes of RAO horses in comparison to healthy horses showed that the phagocytes were affected by oxidative stress. In comparison with polymorphonuclear leukocytes (phagocytes) from the blood of healthy horses the reduction mechanisms in BAL were faster and coincided with the merging of disordered membrane domains, while in horses with RAO the reduction and membrane domain structure remained unchanged. We assume that the merging of lipid domains observed in phagocytes from BAL of healthy horses could promote cluster formation of membrane proteins or ligands, which could trigger the activation process in phagocytes of healthy horses and consequently the physiological response that probably did not happen in phagocytes of RAO horses.

INTRODUCTION

Recurrent airway obstruction (RAO) in horses is associated with exercise intolerance, increased tracheobronchial exudates, mild to severely increased respiratory efforts, and occasionally a cough. Mature and older horses housed in stables for extended periods and fed hay are commonly affected.¹ RAO is recognized by pronounced airway obstruction, bronchial hyper-reactivity, and neutrophil inflammatory reaction in the distal airways.² It is said to be an organism's hyper-reactivity to inhaled fungi and endotoxins.³

As the phagocytes become activated in the distal airways a respiratory burst occurs and large quantities of reactive oxygen species (ROS) are released. These cause damage to the cells and dysfunction the cell membrane.^{4–6} Mammalian cells have both enzymatic and nonenzymatic antioxidant defense mechanisms to minimize the cellular damage caused by interaction between cellular constituents and ROS. Among intracellular antioxidant enzymes superoxide dismutase (SOD) catalyzes dismutation of the superoxide anion (O_2^-) into H_2O_2 ; catalase (CAT) and glutathion peroxidase (GPx) both detoxify H_2O_2 and convert lipid hydroperoxides to nontoxic alcohols.^{7–10}

Oxidative damages caused by free radicals released are harmful to the cell membrane and influences its fluidity.¹¹ One of the methods by which it is possible to follow the physical characteristics of the cell membrane and to get some data about its fluidity, domain structure, and the changes caused by different external influences is the electron paramagnetic resonance (EPR) method with spin probes.^{12–14}

EPR spectra line shape gives information about the properties of different membrane domains which can affect the membrane processes and activity of cells,¹⁵ while from the EPR spectra intensity decrease with time information about the activity of oxy–redox systems in the cell can be obtained.^{16,17}

The aim of the present study was to investigate plasma membrane related changes in connection to the response of phagocytes of horses with respiratory airway obstruction (RAO) and to establish changes in the antioxidant defense systems in blood and bronchoalveolar lavage fluid (BAL) of horses with RAO compared to healthy horses, which should be reflected in the activity of superoxide dismutase (SOD), catalase (CAT), glutathion peroxidase (GPx) and in the ascorbic acid level as well as in their overall activity of oxy–redox systems.

MATERIALS AND METHODS

Materials. The spin probe 5-doxylpalmitoyl methyl ester (MeFASL(10,3)) was synthesized by Slavko Pečar (Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia). Unless otherwise stated, all reagents for cell manipulation were obtained from Gibco – Invitrogen Corporation (Carlsbad, CA); sera was obtained from Sigma (Taufkirchen, Germany).

Animals. Twenty-five horses (RAO horses were on average 9.7 years old and healthy horses were on average 10.3 years old) were included in the study. In 14 cases RAO (recurrent airway obstruction) was diagnosed. The control group included 11 randomly chosen and clinically healthy horses—visiting the clinic for castration or shoeing. The study was approved by the Veterinary Administration of the Republic of Slovenia (License No. 323-84/2004/2).

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Sampling. *Bronchoalveolar Lavage Fluid – BAL.* Before bronchoscopy, horses were sedated with detomidine hydrochloride 10 $\mu\text{g}/\text{kg}$ (Domosedan, Pfizer GmbH, Germany), and some of them needed additional restraint in order to avoid sudden movements of the head—causing trauma with a bronchoscope. A bronchoscope, 3 m long, was used (VVS 13,7 \times 3 m, Olympus, Japan) and smeared with local anesthetic lidocain hydrochloride (Xylocaine gel 2%, Astra, Sweden) over the tip. Twenty milliliters of lidocain hydrochloride was applied at the furcation of trachea, and the bronchoscope was gently inserted in the trachea until it stopped (approximately 170 cm from the nostrils in a horse weighing 500 kg). Working channel of the fibroscope was used to apply 400 mL of buffered (pH 7.4) physiologic solution (PBS – phosphate buffered saline, GIBCO Invitrogen, GB) at 37 °C. The solution was immediately aspirated using 60 mL syringes. Specimens were brought on ice to the laboratory, where fluid was pooled and further processed.

Blood Samples. Venous blood was collected from v. jugularis into blood collection tubes with anticoagulant sodium citrate (Vacutainer, BD, U.S.A.) for isolation of polymorphonuclear leukocytes – PMNs (phagocytes). Tubes containing the anticoagulant EDTA (Vacutainer, BD, U.S.A.) were used for CBC and white blood cell differential count determination (H1, Bayer-Technicon, Germany), as well as for preparation of erythrocyte lysate, which was used for determination of SOD, GPx, and CAT activities. Venous blood was collected also into plain tubes (Vacutainer, BD, U.S.A.) in order to obtain serum for determination of ascorbic acid concentration. The samples were centrifuged at 1200 \times g for 10 min at 4 °C and serum frozen in liquid nitrogen and stored at –70 °C until analysis.

Preparation of Samples. *BAL Sample.* Pooled BAL was poured through coarse gauze to remove mucus. Thereafter, cell viability (>99%) was confirmed by trypan blue dye exclusion. Using Bürker–Türk's chamber number of cells in 1 mL of BAL was determined. BAL was then centrifuged at 500 \times g for 10 min at 4 °C, the supernatant was decanted, and the cell pellet was used for the preparation of slides for cytological differentiation.

For enzyme and ascorbic acid analysis, the volume of the BAL containing 10^{12} cells was centrifuged at 500 \times g for 10 min at 4 °C. Sediment was resuspended in 2 mL of HBSS (Hank's Balanced Salt Solution) without Ca^{2+} and Mg^{2+} and centrifuged again, the supernatant was decanted, and the sediment was resuspended in 2 mL of PBS (pH 7.4). Resuspended cells were lysed by sonication, 5 \times 10 s bursts, with sonicator Misonix (Misonix XL 2020, Lab Care Systems Limited, GB), and centrifuged at 15 000 \times g for 10 min at 4 °C to remove cellular debris. The supernatant was divided into four aliquots, frozen in liquid nitrogen, and stored at –70 °C until analysis.

For EPR measurements BAL was centrifuged at 500 \times g for 10 min at 4 °C, the supernatant was decanted, and the cells were washed with 3 mL of HBSS without Ca^{2+} and Mg^{2+} . Cells were centrifuged again and resuspended in 3 mL of HBSS.

Preparation of Erythrocyte Lysate for Enzyme Analysis. Five hundred microliters of EDTA whole blood was centrifuged at 2500 \times g for 5 min at 4 °C. The supernatant was decanted, and cells were washed three times with 2 mL of cold physiologic solution (0.9% NaCl). The erythrocyte pellet

was then resuspended in 4 packed-cell volumes of ice-cold deionized water and kept on ice for 10 min. Erythrocyte lysates were frozen in liquid nitrogen and stored at –70 °C until analysis.

Isolation of Polymorphonuclear Leukocytes (Phagocytes) from Whole Blood. Polymorphonuclear leukocytes (PMNs) were isolated from the whole blood with a one-step centrifugal technique using Polymorphprep (Axis Shield, Norway) according to the manufacturer's instructions. Briefly, 5 mL of EDTA blood was layered over 5 mL of Polymorphprep and centrifuged at 450 \times g for 30 min at 20 °C. After centrifugation, the mononuclear and the polymorphonuclear leukocytes were separated into two distinct bands free from red blood cells. The polymorphonuclear leukocyte band was harvested and diluted by the addition of an equal volume of 0.45% NaCl. PMNs were then collected by centrifugation at 450 \times g for 10 min at 20 °C and resuspended in 3 mL of lysis buffer (0.83% (w/v) NH_4Cl , 10 mM Hepes-NaOH, pH 7.0) to remove residual erythrocyte contamination. Lysis was performed in a water bath at 37 °C for 7 min. Cells were harvested by centrifugation at 450 \times g for 10 min at 20 °C and washed twice in 3 mL of HBSS (without Ca^{2+} and Mg^{2+}). The smear was made from the cell pellet, and the remaining cells were resuspended in HBSS containing Ca^{2+} and Mg^{2+} . The cells were counted using a hematological analyzer (Bayer-Technicon H1, Germany). The cell viability was confirmed by trypan blue dye exclusion.

Cytological Differentiation of Specimens Obtained from BAL. Dried specimens were stained according to the May-Grünwald-Giemsa protocol, and differential counting of 200 nonepithelial cells was performed under a light microscope (Nikon Eclipse 400, Japan) using 40 \times magnification. Cell integrity and eventual noncellular structures and microorganisms were described as well as contamination of the specimen with epithelial cells.

Enzyme Activity in BAL Cells and Erythrocyte Lysate Samples. *Superoxide Dismutase (SOD).* According to the method used for determining SOD activity (RANSOD, Crumlin, GB) the superoxide radicals were generated by the xanthine and xanthine oxidase reaction. The amount of superoxide radical produced was determined by 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrasodium chloride (INT) as an indicator, which reacts with a superoxide radical to form formazan dye. The SOD activity was determined by the grade of inhibition of the described reaction. The standard calibration curve of percentage of inhibition by standard solutions and log concentrations (U/mL) was used to determine the SOD activity in our specimens.

Glutathion Peroxidase (GPx). Activity of GPx (RANSEL, Crumlin, GB) was determined indirectly by measuring the rate of formation of oxidized glutation (GSSG). GPx catalyzes the reaction of GSH with synthetic cumen hydroperoxide to GSSG. In the presence of NADPH and glutathion reductase GSSG is transformed to glutathion, and NADPH is oxidized to NADP. The rate of oxidation of NADPH was measured spectrophotometrically as reduced absorbance at 340 nm and is proportional to the activity of GPx in the specimen.

Catalase (CAT). The method for determining CAT activity (BIOXYTECH Catalase-520, OxisResearch, U.S.A.) is based on a two-stage reaction. The rate of dismutation of hydrogen

peroxide (H_2O_2) to water and molecular oxygen is proportional to the concentration of CAT. A known amount of H_2O_2 was added to the specimen with CAT and incubated exactly 1 min. The reaction was quenched with sodium azide. The amount of H_2O_2 remaining in the reaction mixture was then determined by the oxidative coupling reaction of 4-aminophenase (PAP) and 3,5-dichlorohydroxybenzenesulfonic acid (DHBS), and the reaction was catalyzed by horseradish peroxidase. The resulting quinoneimine dye was measured at 520 nm with the biochemical analyzer Olympus AU600 (Olympus, Japan).

Ascorbic Acid Determinations in Serum and BAL. The levels of ascorbic acid in horse serum and in 10^{12} cells from BAL were determined using a modified isocratic high-performance liquid chromatographic (HPLC) method with UV detection.^{18,19} The HPLC system (Thermo Separation Products) that consisted of a Spectra Systems P2000 pump, an AS300 auto injector, and a Spectra Systems UV2000 detector was used. The separation was carried out on a Nucleosil 100 C₁₈ column (250 × 4.6 mm i.d.; 5 μm particle size) at room temperature. The mobile phase consisting of acetonitrile–0.06 M phosphoric acid (80:20, v/v) was pumped at 1.0 mL min⁻¹, and detection of ascorbic acid was performed at the wavelength of 248 nm. A mobile phase (1.0 mL) was added to 0.5 mL of sera and extracted on a vortex for 10 s. The solution was then centrifuged at 15 000 × g for 10 min at 0 °C. The supernatant was filtered through a 0.45 μm membrane filter, and 100 μL of the sample was then injected into the HPLC system. Ascorbic acid was determined by HPLC with UV detection at 248 nm in 0.5 mL of filtered BAL samples previously diluted to 1.0 mL with mobile phase.

EPR Measurements. The samples were spin labeled with the spin probe MeFASL (10,3). For this purpose 25 μL of 10^{-4} M ethanol solution of the spin probe was put into the test tube, and ethanol evaporated by rotary evaporation until a thin layer of the spin probe remained on the walls of the tube. Then the tube was filled in with 3 mL of the sample containing 5×10^7 cells in HBSS and agitated manually for 10 min enabling the spin probe to penetrate into the cell membranes. The sample was then centrifuged at 120 × g for 3 min at room temperature, and the supernatant was decanted. The spin labeled cells, from the sediment, were transmitted in a 1 mm diameter glass capillary tube for EPR measurement.

Measurements were performed on an EPR spectrometer Bruker ESP 300 (Karlsruhe, Germany) at microwave frequency 9.59 GHz, microwave power 20 mW, modulation frequency 100 kHz, and amplitude 2×10^{-4} T at the normal body temperature of a horse being 37.5 °C. For each sample the change in the EPR spectra intensity was measured with time after a spin labeling procedure. Fifteen sequenced spectra were recorded; 22 min on average was needed for each recording.

Computer Simulation of EPR Spectra and GHOST Condensation Procedure. The procedures were described in more detail in part 1. Briefly, to describe the EPR spectra of spin probes, the stochastic Liouville equation is used.^{20–22} The model takes into account that the local rotational motion is fast with respect to the EPR time scale and that the spectrum is composed of several spectral components reflecting different modes of restricted rotational motion of

spin probe molecules in different environments of the membrane. They are described with different sets of spectral parameters: order parameter (S), rotational correlation time (τ_c), polarity correction factors of hyperfine and g tensors (p_A and p_g), and broadening constant (W). Besides, the relative proportion of each spectral component (d) is determined. It describes the relative amount of the spin probes with a particular motional mode and depends on the distribution of the spin probe between the domain types as well as on the distribution and position of the spin probe within the domain. It should be stressed that the lateral motion of the spin probe is slow on the time scale of the EPR spectra.^{23,24} Therefore an EPR spectrum describes only the properties of a spin probe's nearest surrounding on the nanometer scale. The computer simulation procedure is implemented in the software package EPRSIM (<http://www.ijs.si/ijs/dept/epr/>).

To obtain the best fit of the calculated EPR spectrum to the experimental the stochastic and population-based genetic algorithm is used. It is good at finding promising regions in complex search space. When combined with Simplex Downhill and knowledge-based operators into an evolutionary optimization method (HEO)²⁵ it is also capable of fine-tuning. According to this procedure one still has to define the number of spectral components before applying the optimization. To resolve this problem a multirun HEO optimization is used together with a newly developed GHOST condensation procedure. According to this method 200 independent HEO simulation runs for each EPR spectrum were applied, taking into account 4 different motional modes of spin probe. The parameters of the best fits were presented by three two-dimensional cross-section plots: S - τ_c , S - W , and S - p_A diagrams; the two other parameters of each diagram are defined by the intensity of the colors: red, green, and blue for τ_c , W , and p_A , respectively (GHOST diagrams).^{26,27} From these plots information about the membrane domain types, dynamic of motion, and ordering within the domain types as well as about the polarity of spin probe surrounding can be obtained.

Statistics. Program SPSS 12.0 for Windows was used to statistically assess the results. A paired t -test was used to determine statistically significant differences among measured parameters in RAO and a control group of horses. RAO and a control group of horses were determined comparable in age, sex, and breed by using the chi-square test. Interdependence among variables was analyzed with the Pearson's correlation coefficient.

RESULTS

Cytological Differentiation of the BAL Specimens. The results of cytological differentiation of BAL specimens obtained from horses with RAO showed a significantly higher proportion on neutrophils and a significantly lower proportion of alveolar macrophages compared to the healthy horses. There was no significant difference in proportions of lymphocytes, eosinophils, basophiles, and epithelial cells (Figure 1).

Enzyme Activities and Ascorbic Acid. The activity of superoxide dismutase (SOD), catalase (CAT), and glutathion peroxidase (GPx) in phagocytes isolated from bronchoalveolar lavage fluid (BAL) and from the blood of healthy

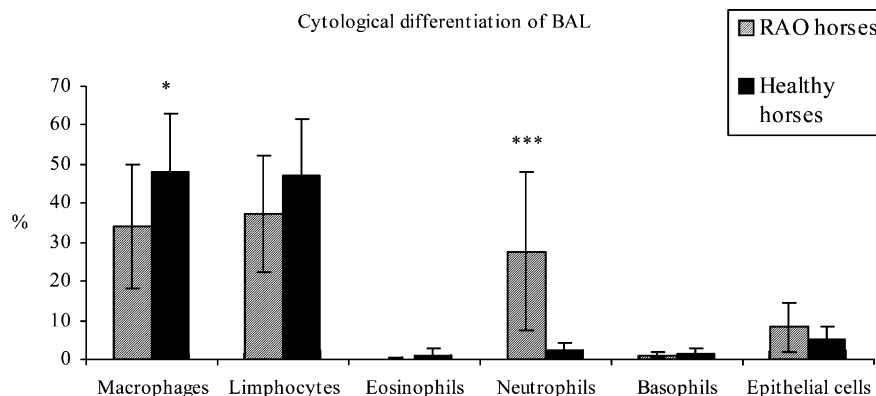


Figure 1. Cytological differentiation of the BAL specimens in healthy and RAO horses. (Mean \pm standard deviations, *t*-test, * – $p < 0.05$, *** – $p < 0.001$).

Table 1. Enzyme Activities in Erythrocyte Lysates (in U/g protein) and Bronchoalveolar Lavage Fluid (BAL) (in U/10¹² cells), Ascorbic Acid Concentration in Serum, the Reduction Rate (k_r) of the Nitroxide Group of the Spin Probe MeFASL(10,3), and the Proportion of the Most Disordered Membrane Domain (d_{dis}) in the Plasma Membrane of Phagocytes Taken from the Blood and BAL of Healthy Horses and Horses with Respiratory Airway Obstruction (RAO Horses)^a

	blood			BAL		
	erythrocyte lysate			phagocytes		
	RAO	healthy	<i>p</i>	RAO	healthy	<i>p</i>
enzyme ^c						
SOD	1300 \pm 300	1500 \pm 300	0.03	0.2 \pm 0.2	0.1 \pm 0.0	0.55
CAT	240 \pm 200	160 \pm 50	0.18	5 \pm 1	3 \pm 1	0.01
GPx	140 \pm 70	110 \pm 60	0.16	13 \pm 7	6 \pm 3	0.02
ascorbic acid (mg/dl)	1.1 \pm 0.3 ^b	1.0 \pm 0.2 ^b	0.25	ND ^d	ND ^d	/
EPR		phagocytes			phagocytes	
k_r (%/min)	1.9 \pm 0.8	2.3 \pm 0.8	0.30	1.9 \pm 1.1	3.4 \pm 0.9	0.004
d_{dis}	0.20 \pm 0.04	0.22 \pm 0.03	0.09	0.25 \pm 0.02	0.30 \pm 0.02	0.000

^a All values are given as mean \pm standard deviation, p = level of significance. Abbreviations: SOD – superoxide dismutase; CAT – catalase; GPx – glutathion peroxidase; k_r – reduction rate constant; d_{dis} – relative proportion of the most disordered mode of spin label motion (average membrane fluidity). ^b Concentration of ascorbic acid was determined in serum. ^c Enzyme activity is given in U/g protein for erythrocyte lysate and in U/10¹² cells for BAL. ^d Not detectable, ND.

horses and horses with RAO are presented in Table 1. In blood, SOD activity was significantly ($P < 0.05$) lower in RAO horses, comparing to the healthy horses, whereas in the blood of RAO and healthy horses there were no significant differences in CAT and GPx activities. In cells isolated from BAL of RAO horses activities of CAT and GPx were significantly higher ($P < 0.05$) compared to the cells from BAL of the healthy horses. It should be stressed that in blood the enzyme activity of the erythrocyte lysates was measured and not only phagocytes therefore the activities from BAL and from blood cannot be directly compared.

There was no significant difference in the ascorbic acid concentration in the serum of healthy horses compared to the serum of RAO horses, while in BAL samples ascorbic acid was not detectable (Table 1).

Activity of Oxy–Redox Systems. The information about the activity of oxy–redox systems in phagocytes was obtained from the intensity decrease of the EPR spectra with time after the incubation of cells with the spin probe (Table 1).

Due to the oxy–redox systems in cells the nitroxide group of the spin probe is reduced to hydroxylamine, which is not detectable by EPR.^{17,28} The main sources of nitroxide reduction are oxy–redox enzymes of respiratory chain^{29,30} ascorbic acid and free radicals.³¹ From the intensity decrease of the EPR spectra the data about the rate of nitroxide

reduction and therefore the data about the activity of the oxy–redox systems in cells can be obtained. The rate of nitroxide reduction, which is proportional to the rate of the EPR spectra intensity decrease, in the phagocytes of healthy and RAO horses is presented in Table 1.

The most significant difference was observed between the phagocytes from BAL of RAO and of healthy horses. The average rate of nitroxide reduction in phagocytes from BAL of healthy horses was approximately 75% faster than for RAO horses ($P < 0.005$), while the reduction rates of phagocytes isolated from the blood of healthy and RAO horses were comparable. When the active phagocytes from BAL were compared to the dormant phagocytes from the blood, one can see that the nitroxide reduction rate increased significantly ($P < 0.001$), upon the activation of phagocytes of healthy horses, whereas for the RAO horses the reduction rate remained in the range of experimental error.

Properties of Plasma Membranes. Dynamic properties of plasma membrane lipids can be obtained from the EPR spectra line shape, since only the spin probes in the plasma membrane contribute to the EPR spectrum. As already discussed the spin probes, which were transported to inner cell membranes, get reduced by oxy–redox systems inside the cells. Figure 2 shows the EPR spectra of the spin probe in plasma membranes of phagocytes isolated from BAL and the blood of healthy and RAO horses.

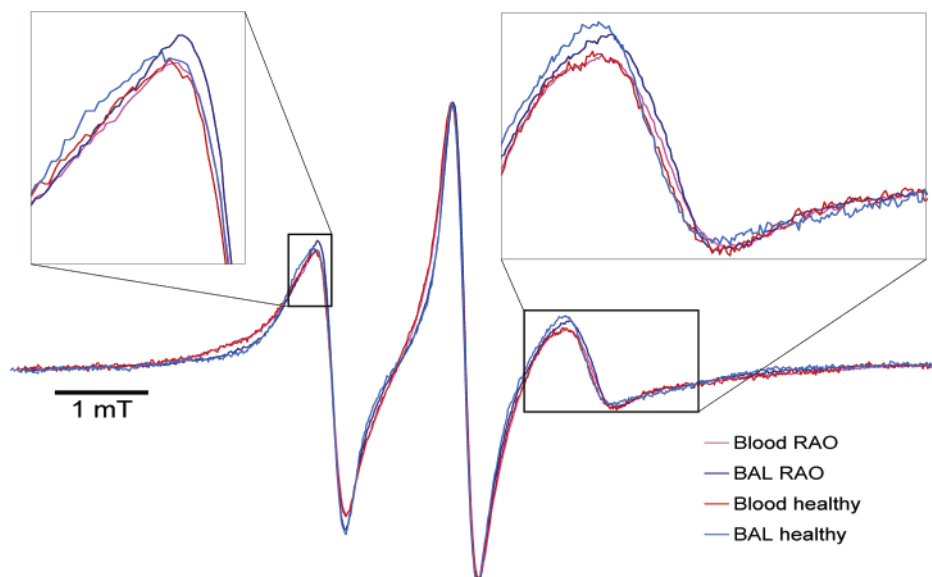


Figure 2. EPR spectra of lipophilic spin-probe methyl ester of 5-doxyipalmitate (MeFASL(10,3)) in the plasma membrane of horse phagocytes taken from the: (blood RAO) blood of horses with respiratory airway obstruction (RAO); (BAL RAO) bronchoalveolar liquid (BAL) of horses with RAO; (blood healthy) blood of healthy horses; and (BAL healthy) BAL of healthy horses.

The EPR spectra of the spin probe from the phagocytes isolated from the blood of healthy and RAO horses are overlapping, which indicates that dynamic properties of plasma membrane lipids of dormant phagocytes isolated from blood are similar for both population of horses (Figure 2 blood healthy – red and blood RAO – dark red).

On the other side, the spectra from BAL of healthy horses differ slightly, but significantly, from the spectra from BAL of RAO horses (Figure 2 BAL healthy – light blue and BAL RAO – dark blue). More quantitative information about the overall plasma membrane fluidity was obtained by decomposition of each EPR spectrum to three spectral components and calculation of EPR parameters (S , τ_c , W , p_A , and d) by which the best fit of calculated to the experimental spectra were obtained. The membrane is more fluid when the relative portion of the less ordered domain increases and/or when the order parameter of domains decreases. Since according to the simulation of the experimental spectra the main changes are observed in the proportions of the membrane domains, and only minor changes are observed in order parameter, we have taken the relative proportion (d_{dis}) of the spectral component with the lowest order parameter (S) as a measure for overall membrane fluidity (Table 1). To gain, more detailed insight into the difference of plasma membrane properties of phagocytes from BAL of healthy horses and of RAO horses a newly developed GHOST condensation routine was applied to the corresponding EPR spectra.

Domain Structure of Plasma Membranes. The results of the GHOST condensation routine, which was applied to the EPR spectra, are shown in Figure 3. In GHOST diagrams the results of several runs of computer simulation for one and the same spectrum are joined into the groups of solutions, each group represents a mode of spin label motion in a part of the plasma membrane with defined dynamic characteristics of constituent lipids. By this procedure the number of different modes of motion was not defined before applying computer simulation of an EPR spectrum. From Figure 3 roughly 3 modes of spin label motion could be identified in plasma membranes of phagocytes isolated from the blood of healthy

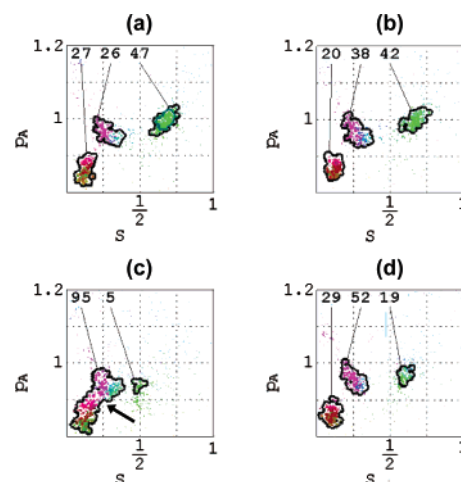


Figure 3. $S - p_A$ (order parameter – polarity correction) GHOST diagrams, which define motional modes of the lipophilic spin-probe MeFASL(10,3) in the plasma membrane of horse phagocytes taken from the (a) blood of healthy horses; (b) blood of horses with respiratory airway obstruction (RAO); (c) BAL of healthy horses; and (d) BAL of horses with RAO (spectral parameters τ_c , W , and p_A are defined by intensity of colors red, green, and blue, respectively). The arrow in diagram (c) indicates an additional mode of spin label motion that appears after the activation of phagocytes of healthy horses and connects two modes of motion, which are separated in plasma membrane of dormant phagocytes.

and RAO horses (Figure 3a,b). Upon activation of phagocytes of RAO horses the 3 modes of the spin label motion remained clearly present, only the relative proportion of the two less ordered modes of spin probe motion increased, mostly on the account of the most ordered mode of motion (Figure 3d). However, plasma membrane properties of activated phagocytes isolated from BAL of healthy horses underwent a substantial restructuring (Figure 3c). Two previously distinct modes of spin probe motion were merged together (Figure 3c). Besides two modes present in the dormant phagocytes from the blood (Figure 3a), new modes of spin probe motion appeared, which connect previously distinct modes of motion (denoted with an arrow in Figure 3c).

DISCUSSION

Cytological differentiation of the bronchoalveolar lavage (BAL) fluid is crucial for confirmation of recurrent airway obstruction (RAO) in horses.^{11,32} It is characterized by a high proportion of neutrophils in BAL fluid.³² That was also confirmed by our study, as cytological differentiation of BAL showed a significantly higher proportion of neutrophils in BAL obtained from horses with recurrent airways obstruction (RAO) compared to healthy horses.

Reactive oxygen species (ROS) have been involved in a wide variety of cellular functions, but they can be both essential and highly toxic to cellular homeostasis.³³ Oxygen free radicals and lipid peroxides have been implicated in the pathogenesis of a large number of diseases such as diabetes mellitus, cancer, infectious diseases, atherosclerosis, and in aging.^{34–38} It is expressed in changed activity of antioxidant enzymes. For example, in the leukocytes of patients with type I diabetes mellitus SOD and GPx activities were found to be decreased.³⁹ Whereas in patients with type II diabetes mellitus no significant difference was found in the activity of SOD and GPx, but lipid peroxidation in leukocytes of diabetics was significantly increased and was accompanied with a decreased level of vitamin C. This results showed that leukocytes of diabetics are affected by oxidative stress.⁷ Similarly in our study differences in CAT, GPx, and SOD activities were found in phagocytes of horses with recurrent airway obstruction (RAO) in comparison to healthy horses, proving that the phagocytes are affected by oxidative stress (Table 1). Increased activity of CAT and GPx in phagocytes from BAL of RAO horses in comparison to the healthy ones ($P < 0.05$) indicates a higher production of H_2O_2 . On the contrary in the blood the activity of SOD in RAO horses slightly decreased ($P < 0.05$), due to high systemic oxidative stress, while the activity of two other enzymes remained in the range of the experimental error.

The other parameter, which reflects the activity of oxy–redox systems in phagocytes, is the rate of nitroxide reduction (k_r , Table 1). In RAO horses the rate of nitroxide reduction in BAL was significantly lower compared to healthy horses ($P < 0.005$). Upon activation of phagocytes, when transferred from blood to the BAL it remained the same as it was in dormant phagocytes from blood. On the contrary, in healthy horses the reduction rate significantly increased after transformation of phagocytes from dormant to the active form. It seems that after the activation of phagocytes, when they were transferred from blood to BAL, oxy–redox systems were activated in healthy horses, which was not the case in RAO horses.

Nitroxides experience different reduction pathways in biological systems. The main mechanisms for reduction are enzymatic and originate mainly from the respiratory chain enzymes^{29,30} but could be also nonenzymatic due to different reducing agents in cells such as ascorbic acid, glutathione, free radicals, etc.; the ascorbic acid is one of the most reactive biomolecules, which was proven to reduce nitroxides in tissues.⁴⁰ As there was no detectable amount of ascorbic acid in BAL (Table 1), we suppose that the respiratory chain enzymes are the main source of nitroxide reduction. We speculate that in the phagocytes of RAO horses these enzymes are not able to activate, when they are transferred from blood to BAL, as the rate of nitroxide reduction in

phagocytes of RAO horses did not change. However, in healthy horses the respiratory enzymes in phagocytes from BAL do activate, as the rate of reduction increases significantly (Table 1).

The changes in the activity of oxy–redox enzymes as they were transferred from dormant to active form in healthy horses was reflected also in the membrane domain structure of phagocytes as is resolved from GHOST diagrams in Figure 3. Generally three distinct modes of spin probe motion are observed, which could correspond to three types of membrane domains. They are about the same in the blood of healthy and in the blood of RAO horses (Figure 3a,b). Also the domain structure of phagocytes from the blood and from BAL of RAO horses is similar (Figure 3b,d), although the cell population in the sample from BAL is not the same as in the sample of phagocytes (polymorphonuclear cells, mainly neutrophils) from blood. In the BAL there is about 70% macrophages and lymphocytes and only 30% neutrophils (Figure 1), which indicates that the domain structure in the samples of cells from blood and from BAL of RAO horses is similar irrespective of a partial difference in the cell population. However, there is a pronounced difference in the GHOST diagram of phagocytes from BAL of healthy horses (Figure 3c) in comparison to other cells. Additional modes of motion appeared here, between the two domains with low order parameter. Since in the BAL of healthy horses there were less neutrophils as in BAL of RAO horses (Figure 1), these additional modes of motion could not correspond to the additional cell populations. Comparing GHOST diagrams and cell population in different samples we can conclude that the reason for a different pattern in the GHOST diagram of phagocytes from BAL of healthy horses is not due to a different cell population but rather due to a different domain structure of the phagocyte membranes.

Based on the GHOST diagrams, which indicate that in healthy horses properties of two distinct plasma membrane domains in dormant phagocytes merge together upon the activation in BAL, which is reflected also in higher enzyme activity, we speculate that these two events are interconnected which means that the merging of membrane domains triggers an activation of reducing mechanisms in cells.

A new paradigm of cell membranes treats the membrane as a compartmentalized mosaic fluid in which the membrane constituents undergo free lateral diffusion limited to the size of compartments (typically from 30 to 230 nm²) and long-term hop diffusion between the compartments.^{41,42} It was proposed that large, stabilized rafts may be formed inside the compartments, after ligation or cross-linking, from small/unstable “reserve” rafts.⁴³ In their working hypothesis, Kusumi et al. suggested that a loose saturated-alkyl-chain cluster (induced by ligands, lectins, ...) of four GPI-anchored receptor molecules would recruit cholesterol, glycosphingolipids, and raft-preferring molecules into the cluster. This argument is analogous to that of the effect of transmembrane protein clustering on movement of the lipids that directly contact the transmembrane protein (boundary lipids).⁴⁴ Upon transmembrane protein oligomerization, the lipids become sandwiched between two transmembrane proteins or trapped in the protein-rich domain, and the rate of their exchanges with lipids in the bulk domain drops.^{45,46} Since conventional EPR is sensitive for molecular movement on a time scale of 10^{–12} to 10^{–9} seconds⁴⁷ and the diffusion rate within an average

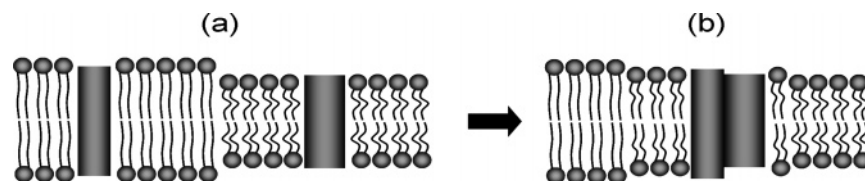


Figure 4. Membrane switch hypothesis. A schematic presentation of ligand cluster formation promoted by an appearance of a third membrane domain with intermediate membrane properties. (a) Part of a plasma membrane with two coexisting membrane domains and with membrane proteins separated each in its own membrane domain. (b) Part of a plasma membrane with a third membrane domain, which connects two previously distinguished domains and triggers clustering of membrane proteins.

sized cell membrane compartment is almost as large as that of lipid molecules in liposomes, a few $\mu\text{m}^2/\text{s}$,⁴³ an EPR spectrum describes only the properties of the nearest surrounding of a spin probe, which is in the range of several 10^{-2} nm. This means that by EPR we observe different modes of rotational movement of an ensemble of molecules, which could correspond to different types of coexisting domains with different motional characteristics inside the membrane compartments. The membrane compartments cannot be resolved by conventional EPR, but some motional modes could correspond to the restricted motion of lipids nearby the immobilized transmembrane proteins (boundary lipids).

Arachidonic acid, which is kept at low concentration in resting peripheral blood neutrophils by the constant reacylation of the fatty acid released in deacylation processes, can be liberated through the action of phospholipase A₂.⁴⁸ Taking into account that upon stimulation the plasma membrane of neutrophils substantially changes and a concurring change in motional modes of plasma membrane constituents of activated neutrophils of healthy horses we propose a membrane switch hypothesis: the plasma membrane might act as a switch to promote the clustering of membrane proteins by modifying the membrane domain structure, which could be limited to some membrane compartments. This hypothesis is schematically shown in Figure 4. In the plasma membrane with two distinct domain types, membrane constituents, proteins, and ligands might be separated in the preferred membrane domains. After the activation of phagocytes modifications of the plasma membrane lipids might cause additional membrane domain to merge, connecting both previously present domains. If properties of this domain are intermediate to the two previously present domains, then such a domain could become a connecting domain, allowing previously segregated constituents to more easily cluster in the new domain and to trigger subsequent physiological processes in phagocytes.

It should be noted that membrane domains are very dynamic structures with different lifetimes and are continuously forming and disappearing, and their constituent molecules can frequently enter or exit from the domains and can diffuse freely inside a compartment. The smallest might be cholesterol-rich domains, perhaps consisting of only several cholesterol molecules, and are short-lived, on the order of 1–100 ns.⁴⁹ It was speculated that these cholesterol enriched domains may be stabilized by the presence of saturated alkyl chains of sphingomyelin or glycosphingolipids and also by clustered raft proteins.⁴⁹ We speculate that the activation of macrophages, which results in the appearance of additional domains with intrinsic motional characteristics, enables formation of larger domains in which the interexchange and clustering of proteins is facilitated, similarly as

it was described for formation of larger and more stable rafts by Kusumi et al.⁴³

Phagocyte activity could be the result of the continuously varying dynamic properties of the two connected domains, which is normally not present in inactive phagocytes. Lateral lipid domains could in such a way act as a “switch” in activation of physiological processes in the phagocytes of healthy horses and that probably could not happen in the phagocytes of RAO horses.

In our work we wanted to pay attention to the possible correlation between the changes in membrane domain structure and activation of oxy–redox enzymes and not to explain complicated metabolic processes, which are connected with RAO disease. Similar correlation between membrane domain structure alterations and physiological changes was also observed during the cell growth of malignant cells, which are more amenable to the changes from environment as the nonmalignant cells,⁵⁰ and in the process of fusion of liposomes with cells.⁵¹

CONCLUSION

Phagocyte activity could be the result of the continuously varying dynamic properties of the two connected domains, which is normally not present in inactive phagocytes. Lateral lipid domains could in such a way act as a “switch” in the activation of physiological processes in the phagocytes of healthy horses and that probably could not happen in the phagocytes of RAO horses.

Only with the newly developed GHOST optimization method in the simulation of the line shape of the EPR spectra more detailed information about the membrane domain structure on the nanometer scale can be given, which could be useful in the investigation of the role of the membrane and its domain structure in different physiological processes.

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