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Membrane Oxidation Assay—A Novel Lipoxygenase-based Evaluation of Membrane Oxidizability

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A membrane oxidation assay is presented which uses isolated erythrocyte membranes ("ghosts") and lipoxygenase as a selective catalyst for the transfer of oxygen to *cis–cis*-1,4-pentadiene-moieties. The latter are, for instance, present in linoleic and arachidonic acids, both of which are integral parts of membranes. These non-conjugated double bonds represent energetically favorable sites for oxidative attack and therefore, may be rearranged and partially consumed during oxidative stress. Consequently, the measurement of oxygen consumption in the course of the lipoxygenase-mediated oxidation provides a tool for the quick and reliable determination of such double bonds. Significant inter-individual differences have been noted in 11 subjects, which also correlate to the total radical antioxidant parameter (TRAP) values obtained. This assay will be helpful in the assessment of oxidizable structures in erythrocyte membranes that may be diminished as a consequence of oxidative damage suffered by an individual. In conclusion, a simple and rapid assay for the assessment of the oxidizability of erythrocyte membranes is presented complementing the TRAP assay for plasma antioxidative status.

Keywords: Lipoxygenase; Erythrocyte; Oxidative stress; Total radical antioxidant parameter

INTRODUCTION

The antioxidative capacity of blood plasma can be easily evaluated by measuring its resistance to oxidative attack using the total radical antioxidant parameter (TRAP).[1] In contrast, the oxidative vulnerability of membranes is more difficult to determine. However, it could provide insights into the overall stress resistance of an organism *in vivo*. Erythrocytes are degraded after approximately 120 d in circulation and the antioxidative potential of their membranes measured *in vitro* reflects the cumulative oxidative stress of the past months.[2] Therefore, a quick and reliable measurement of the oxidizability of erythrocyte membranes should be valuable. Red blood cells are especially prone to oxidative stress due to their function as oxygen carriers, and they have a delicate antioxidative system which consists of antioxidative enzymes like superoxide dismutase, catalase and peroxidase as well as considerable amounts of glutathione. While their membranes are protected mainly by α-tocopherol and small amounts of ubiquinol, no *de novo* synthesis of protective proteins, such as catalase and peroxidase, occurs in these cells.[3]

For measuring the resistance of erythrocyte membranes against radicals, frequently a hemolysis test is employed which is based on a steady flow of free radicals generated *in vitro*.[4] However, these conditions are not physiological and therefore, can only provide limited insight into radical-mediated damage occurring *in vivo*. A further disadvantage of the hemolysis test is the fact that outflow of intracellular material will influence the results. Therefore, we have developed a method in which isolated erythrocyte membranes ("ghosts") and the enzyme lipoxygenase (E.C. 1.13.11.12) are used for the selective oxidation of unsaturated fatty acids present in membranes. The results of this membrane oxidation assay (MOX) allow the direct comparison of oxidative stress from different

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individuals as reflected in the composition of their cell membranes.

MATERIALS AND METHODS

General

Lipoxygenase with an activity of 360,000 U/mg was purchased from Sigma (Deisenhofen, Germany). All other reagents and chemicals were purchased from Aldrich (Taufkirchen, Germany). TRAP and membrane oxidation measurements were carried out on an oxygen electrode by Yellow Springs Instruments Co. (Yellow Springs, OH, USA).

Blood Samples

Five milliliters of venous blood from 11 non-fasting adult volunteers working in our department were drawn into EDTA-containing tubes.

Cholesterol Determination

Samples were analyzed with an enzyme assay from Roche Diagnostics (Mannheim, Germany). The resulting absorbance at $\lambda = 546$ nm was measured on a Lange photometer (Berlin, Germany).

Hematocrit Determination

Hematocrit was determined by centrifugation of blood capillaries and comparison to a graded scale.

Protein Determination

Protein was determined with an assay from Pierce (Rockford, IL, USA) which uses a modified method according to Lowry *et al.*[5]

Total Radical Antioxidant Parameter

A TRAP assay has been adapted from Wayner *et al.*[1] Briefly, 3 ml of a phosphate-buffered saline (PBS) solution were filled in a reaction cuvette at 37°C. The oxygen saturation was adjusted to 100%. A mixture of plasma and linoleic acid was added and the assay was started with an injection of 2,2′-azobis-(2-amidinopropanehydrochloride) [ABAP]. ABAP is a thermolabile azo compound which decomposes at 37°C generating free radicals at a constant rate. Lipid peroxidation is inhibited by antioxidants which are contained in plasma. Oxygen consumption is measured and after all antioxidants are used up an acceleration of the peroxidation process can be observed. After reaching an oxygen saturation of 50% 25 μl of Trolox (0.4 mM, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a water-soluble vitamine E analog, are added for calibration purposes and lipid peroxidation is inhibited. TRAP values are calculated as μmol lipidperoxyl-radicals trapped by 1 l of plasma.

Hemolysis

A hemolysis test according to Boda *et al.* was employed.[4] Briefly, an erythrocyte suspension (0.5%) was treated with ABAP (74 mM) and incubated at 37°C. The progress of cell lysis was recorded by measurement ($\lambda = 540$ nm) of an aliquot on an ATI Unicam photometer (Offenbach, Germany). A blank containing PBS without erythrocytes and a cuvette with completely lysed cells were measured accordingly. Hemolysis was plotted over time and the time elapsed to obtain 50% cell lysis was noted.

Isolation of Erythrocyte Membranes

One milliliter of EDTA-blood was centrifuged and the resulting plasma was used for cholesterol and TRAP determinations. The erythrocyte pellet was washed with 0.9% NaCl and lysed in cold demineralized water (2 ×). After centrifugation at 13,000 rpm for 10 min the resulting pellet was washed twice with 0.9% NaCl. The supernatant was discarded and the membranes were suspended in 200 μl of a PBS solution which contained 10 mM deoxycholate. The suspension was briefly homogenized with ultrasound and 10 μl were removed for protein determination.

Membrane Oxidation Assay

The reaction cuvette of the oxygen electrode contained 3 ml of the PBS/deoxycholate buffer (10 mM). The oxygen saturation was adjusted to 100%. One hundred and ninety microliters of the membrane suspension was added to the buffer and immediately afterwards the reaction was started by injection of 50 μl of the lipoxygenase solution. The decrease in oxygen saturation was recorded for 30 min. A blank measurement was performed using the *same* electrode in PBS/deoxycholate buffer without the addition of membranes. The results are given as the fraction of the putative oxygen content of 3.2 ml saline medium (approximately 650 nmol) in nmol which has been transferred to double bonds. These values, calculated for 100 ml, were adjusted for blood hematocrit and electrode blank.

RESULTS AND DISCUSSION

Lipoxygenases (linoleate:oxygen oxidoreductase, E.C. 1.13.11.12) are non-heme iron-containing

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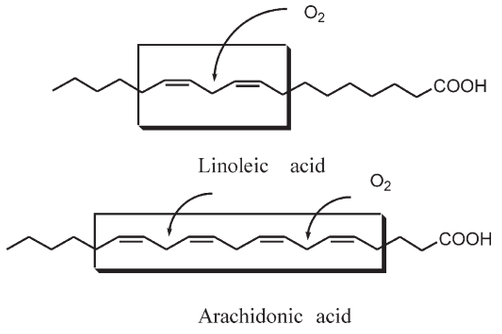

FIGURE 1 Erythrocyte membranes contain high amounts of linoleic and arachidonic acid. Lipoxygenase catalyzes the oxidation of *cis–cis* pentadiene moieties present in these fatty acids. Hydrogen abstraction from bis-allylic methylene groups is energetically favored due to mesomerical stabilization of the radical intermediate.

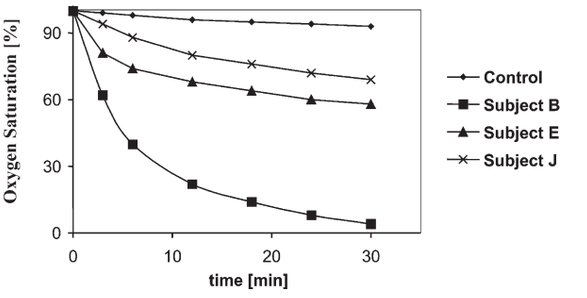

FIGURE 2 Oxidation of isolated erythrocyte membranes ("ghosts") of subjects B, E and J using lipoxygenase. For each electrode a control has been recorded without the addition of membranes to accommodate electrode deterioration. The oxygen consumption during the course of the reaction has been used to monitor the catalytic conversion of fatty acid substrates which contain one or more pentadiene moieties.

dioxygenases, which enantio- and regioselectively transfer oxygen to fatty acids containing at least one 1,4-*Z*,*Z*-pentadiene system to yield *Z*,*E*-conjugated hydroperoxides (see Fig. 1).[6–9] The attack of oxygen is energetically favored at those positions, as the radical intermediate can be mesomerically stabilized. It is known that membrane lipids can be oxidized enzymatically by, e.g. lipoxygenase and non-enzymatically yielding biologically active compounds such as leukotrienes.[10–13] Therefore, prolonged and elevated oxidative stress will preferably consume unsaturated fatty acids to yield their corresponding oxidation products. Thus, the selective reaction of lipoxygenase with these fatty acids which form an integral part of erythrocyte membranes gives important information on the composition of and potential oxidative damage to such membranes. Linoleic and arachidonic acid, both of which contain pentadienyl sytems, constitute each about 10–15% of the total amount of fatty acids in erythrocyte membranes.[14] We have developed an assay using commercially available lipoxygenase and isolated erythrocyte membranes to elucidate the amount of native *cis–cis* pentadiene systems present therein whereby, each of these may be oxidized by LOX under consumption of oxygen. As the enzymatic reaction does not necessarily proceed quantitatively, the results do not provide an exact count of pentadiene moieties but yield comparable data which is proportional to polyunsaturated fatty acid content and thereby, allows the quick evaluation of the oxidizability of biomembranes.

The reaction of lipoxygenase can be easily monitored with an oxygen electrode by measuring the consumption of oxygen present in the reaction cuvette (see Fig. 2).

Considerable differences between erythrocyte membrane preparations have been observed (Patients B, E and J; Fig. 3B). Results for the MOX ranged from 379 to 1202 nmol/dl (adjusted for blood hematocrit and electrode blank). These large inter-individual differences point to variations in membrane composition and oxidative status of the subjects. In addition to the MOX, each blood sample

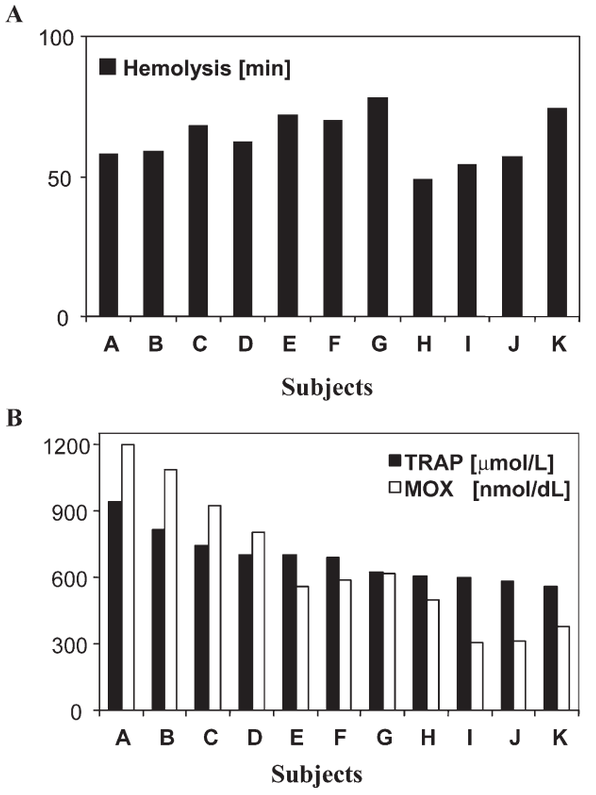

FIGURE 3 Blood samples from volunteers A to K were subjected to (A) Hemolysis and to (B) TRAP [μmol lipidperoxyl-radicals trapped per liter of plasma] and MOX [nmol oxygen transferred per 100 ml whole blood] assays for the evaluation of their oxidative status. Hemolysis, given as the amount of time to reach 50% cell lysis, does not correlate to MOX assay results. Remarkable inter-individual differences in MOX values can be observed depending on membrane composition and correlating to plasma antioxidative status.

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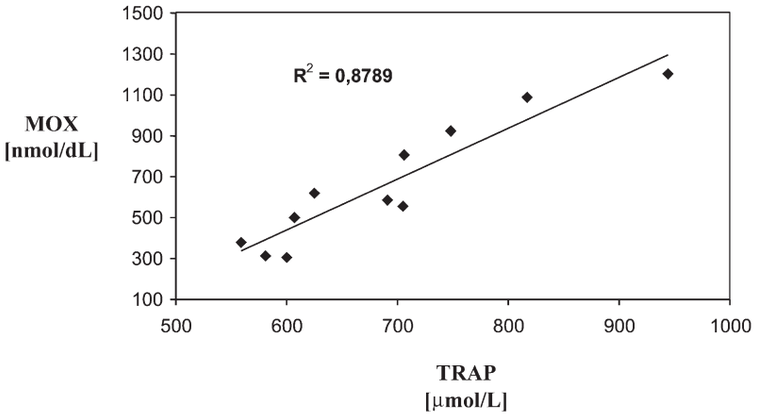

FIGURE 4 Correlation between plasma TRAP and MOX.

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has been analyzed for plasma cholesterol concentration, hematocrit and membrane protein content. Furthermore, erythrocytes were subjected to a radical-induced hemolysis test, and the TRAP of the corresponding plasma sample was determined. The amount of erythrocytes in the sample did not correlate to membrane protein content (data not shown). Inter-individual variations of this parameter do not allow its use as reference value. Alternatively, the method has been strictly standardized regarding the amount of blood used and its work-up procedure. Additionally, results are referred to blood hematocrit to accommodate inter-individual differences. Similar results were obtained for different samples from the same batch of blood (data not shown).

No correlation between the erythrocyte hemolysis assay and MOX was observed (cf. Fig. 3). Therefore, it appears that radical-induced hemolysis does not reflect the same factors of cell membrane integrity as does the MOX, like for instance lipophilic antioxidants vs. membrane fatty-acid composition.

Strikingly, the results obtained for plasma TRAP and MOX correlate strongly (see Fig. 4). In both samples, sample A and B of figure 3, high TRAP levels of 944 and 817 μmol/l and also high MOX values which exceed 1000 nmol/dl were found. In the samples J and K of Fig. 3 low TRAP levels (581 and 559 μmol/l) also correspond to the lowest MOX results measured in this study (300 nmol/dl on average). At first glance, a connection between TRAP and MOX is unexpected as they employ completely different systems (plasma vs. ghosts). However, it is likely that healthy dietary habits, i.e. a diet rich in vitamins and essential fatty acids, directly increase plasma antioxidant levels and at the same time contribute to the essential fatty acid and lipophilic antioxidant content of membranes.

In summary, our method offers a quick and reliable way to evaluate the amount of non-conjugated, vicinal double bonds in erythrocyte membranes. These polyunsaturated fatty acids contribute to the radical scavenging ability of cells and thereby, provide insight into the assessment of the membrane antioxidative status. However, the concentration of these polyunsaturated fatty acids may also indicate a higher risk for possible lipid peroxidation depending on duration and intensity of radical-inflicted damage. Further investigations assisted by our method are necessary to elucidate which of these contradictory effects has a greater biological impact under pathophysiological conditions.

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