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ABSTRACT

The filterability of human red and white blood cells has been studied parallel to the concentration of lipid peroxidation product malonyldialdehyde (MDA) in an in vitro model. The addition of a free radical generating system induced a significant increase of the MDA concentration in nmol/g hemoglobin (Hb) in the red blood cells (from 2.1±0.04 nmol/g Hb to 4.42±0.06 nmol/g Hb, $p<0.002$) and a decrease of red blood cell filterability (RFR) (from 48±5 ul/s to 11±1.8 ul/s, $p<0.007$) compared to the controls. These effects were prevented by addition of the antioxidant, Polbax®. A peroral four weeks administration of the same preparation to 20 healthy volunteers caused a similar amelioration of both variables namely the RFR values significantly increased (83±5 ul/s, $p<0.001$) compared to the pretreatment values (53±4 ul/s) and a significant decrease of MDA production (from 5.2±0.1 nmol/g Hb to 2.8±0.06 nmol/g Hb, $p<0.05$) after four weeks of treatment was also noted. Furthermore, the present study showed also a significant correlation between the production of MDA and the decrease in the RBC filterability ($r=0.46$; $p<0.05$).

INTRODUCTION

Oxygen free radicals (OFR) are known to cause lipid peroxidation in living cells (1). The human red blood cells (RBC's) are normally exposed to high oxygen tension. RBC's are rich in polyunsaturated lipids and iron, a potent catalyst for free radical formation. RBC's are therefore constantly exposed to the effects of free radicals. Intracellularly, the formation of methemoglobin from oxyhemoglobin produces superoxide anions and the extracelluar release of hydrogen peroxide and superoxide anions even in granulocytes and macrophages (2,3). OFR oxidize the polyunsaturated fatty acids and esterify cholesterol in the membranes of RBC's and leads to formation of lipid hydroperoxides (LHP) (4,5). A chain of reactions could be initiated, resulting in multiple LHP. Most LHP are unstable and decompose to secondary lipid peroxidation products such as malonyldialdehyde (MDA).

Keywords: Polbax®, Malonyldialdehyde, Blood Cell Filterability, Hypoxanthine and Xanthine Oxidase, Oxygen Free Radicals.

OFR can damage the RBC's membrane structures, resulting in a leakage of intracellular hemoglobin and potassium ions. The water permeability is altered and red cells can be lysed. Furthermore, lipid peroxidation induced by OFR, causes polymerization of membrane components and reduces cell deformability. MDA itself is probably engaged in the cross linking of RBC's membrane components containing amino groups (6,7,8). MDA is in fact known to increase membrane rigidity and to reduce red cell deformability (9,10). MDA can be detected by using its reaction with thiobarbituric acid, commonly used as indicator of lipid peroxidation (11).

The aim of this study was to find out if Polbax®, an OFR scavenger, could enhance the filterability and decrease lipid peroxidation in the presence of in vitro induced mechanical trauma and in a similar ex-vivo test set-up after four weeks of oral administration in healthy volunteers.

MATERIAL AND METHODS

In Vitro Experiment

Fresh red blood cells from 12 healthy blood donors were used in the study. The blood samples were separated into red blood cells (RBC) and plasma by centrifugation (five minutes at 2000 rpm; 800g) and the plasma was discarded.

The erythrocytes were washed twice for five minutes at a speed of 4000 rpm (2200g) in Krebs solution. The buffy coat was removed after each centrifugation and saved for white blood cell filtration analyses (WFR). A 20% erythrocyte solution was used for further incubation. The supernatant plasma was discarded and the buffy coat then diluted with isotonic saline giving a count of 4 x 10^9/L. OFR were generated by the addition of hypoxanthine (HX) (BDH, Biochemical Ltd, England) at the dose of 500 µl/mg and xanthine oxidase (XOD) (Sigma, USA) 0.7 U/mg $prot^{-1}$ at the dose of 30 µl/mg to the incubation medium. The incubation was performed in four different 15 ml glass beakers which contained: erythrocyte suspensions only (control, group I); erythrocytes with HX and XOD to produce OFR (group II); erythrocytes plus Polbax® (group III); erythrocytes with HX and XOD to produce OFR and Polbax® (group IV). Mechanical trauma was induced by constantly shaking the beakers in a gyratory shaking water bath at 37°C for 180 minutes.

The Polbax® preparation was added in a concentration of 54 mg/ml. The concentration of malonyldialdehyde (MDA/nmol/gm Hb) in RBC and the red and white blood cell filterability (RFR ul/s and WFR µl/s) were determined in all beakers after 30 and 180 minutes of incubation using the thiobarbituric acid method according to Yagi (11) in modification of Görög (12), and the standard microfiltration technique for the measurements of red and white blood cell filterability (13).

Ex-Vivo Experiment

Twenty healthy volunteers were recruited through an announcement in the local daily. All participants went through detailed medical examination and were found healthy. After a thorough description and explanation of the aims and all examination included in the study they gave their written consent for their participation. The study was accepted by the Ethical Committee at the University of Gothenburg.

The blood samples were taken before and after the treatment period of four weeks with Polbax® (2 tablets, 3 times daily). All participants were advised to maintain their normal living habits specially in terms of qualitative and quantitative food intake as well as physical activity. No side-effects were noted.

The concentration of malonyldialdehyde (MDA/nmol/gm Hb) in RBC and the white and red blood cell filterability (WFR and RFR/µl/s) were determined, as described above, before and after treatment.

Pollen-pistil extract with antioxidative activity

The preparation used in this investigation is unique as to its composition, method of production, source and high SOD activity. The source is freshly harvested pollen grains and pistils from the

family Gramineae spp. The pollen grains and the pistils are collected separately by machines specially designed for this purpose. After collection they are thouroughly analyzed for purity and specificity (14). The free radical scavenging base material is produced in a reactor, where pollen grains and pistils are allowed to react under very specific and well defined conditions. The reactant solution is partly evaporated to concentrate the solution and increase the activity. The hypothesis regarding what substances are obtained in this reaction is still under investigation (14). The "SOD mimics" i.e antioxidants of type polyphenols (pycnogenols-leucoanthocyanides) have been recognised in pistils and as low-molecular water soluble substances easily absorbed through the intestinal wall (15). This pollen-pistil extract exhibits a SOD-activity of approximately 30.000 units per gram of substances (15). When adsorbed and complexed to a defined mixture of proteins it gives a SOD-activity of 4.000-6.000 units per gram of extract (15). The test preparation (Polbax®, Allergon, Sweden), is manufactured from this extract (15) but the contribution of SOD as such, to the scavenging activity of the preparation is less probable as a relatively big protein molecule of SOD is hardly expected to be resorbable from the digestive tract in humans.

Statistical methods

The data were expressed as mean ± standard deviation (SD). A p value <0.05 was considered significant. Differences between means were evaluated using the non-parametric Mann-Whitney Test. Relationship between the MDA and filterability values were calculated using the linear regression analysis and the Pearson correlation coefficients. The intergroup comparisons were done by means of variation analysis ANOVA. The Statview II statistical program (Abacus Concepts Inc Berkley CA) was used for the analysis.

RESULTS

In vitro experiment

The mean RFR before trauma in all the groups was 48±5 ul/s, and was reduced significantly in all of them after 30 min. ($p<0.001$), With due increasing duration of trauma, the RFR values decreased significantly in all groups, Table I.

The addition of Polbax® ameliorated the deterioration of RBC filterability after 30 minutes, the filterability being significantly better in groug III than in group I ($p<0.05$), and in the group IV than in the corresponding group II ($p<0.01$). The same trend was still observed after 180 minutes of incubation, Table I.

TABLE I
Changes in red cell filterability (μl/s), means ± SD

Group	30 min.	180 min.	p
I (RBC suspension only)	21.5±1.6	14.0±1.9	0.001
II (RBC+HX+HOD)	17.6±1.7	11.2±1.8	0.01
III (RBC+Pbx)	25.9±2.0	16.0±1.8	0.002
IV (RBC+HX+HOD+Pbx)	20.8±1.6	13.2±1.9	0.002

RBC=Red blood cells; HX=Hypoxanthine; HOD=Hypoxanthine oxidase; Pbx=Polbax

The mean MDA concentration at the start of the incubation was 2.1 nmol/g Hb and increased significantly in all group after 30 minutes. Only a slight further increase of MDA was observed after 180 minutes, Table II.
The intergroup comparison after 30 minutes of trauma revealed that the addition of the OFR generating system (group II) was associated with a significant increase of MDA values ($p<0.05$) in comparison with group I, Table II.
The addition of Polbax® (group IV) prevented this increase ($p<0.05$) in comparison with group II), Table II.
The addition of Polbax® to the incubation medium in the absence of the OFR generating system tended also to ameliorate the increase of MDA, the difference versus group I, not being significant however. The difference between group I and group III was higher after 180 minutes ($p<0.05$). Again the addition of OFR (group II) was associated with the further increase of MDA values (in comparison with 30 minutes). The presence of Polbax® in the incubation medium prevented the increase of MDA, the difference between group II and group IV being significant ($p<0.001$).

TABLE II
Changes in malonyldialdehyde (nmol/g Hb), means±SD

Group	30 min.	180 min.	p
I (RBC suspension only)	3.75±0.13	3.91±0.13	0.08
II (RBC+HX+HOD)	4.15±0.08	4.42±0.06	0.03
III (RBC+Pbx)	3.58±0.13	3.74±0.12	0.1
IV (RBC+HX+HOD+Pbx)	3.71±0.14	3.82±0.12	0.2

RBC=Red blood cells; HX=Hypoxanthine; HOD=Hypoxanthine oxidase; Pbx=Polbax

Effect of Polbax® on healthy volunteers
Following 4 weeks of Polbax® treatment, the ex-vivo RFR changes showed a highly significant increase ($p<0.001$). The WFR showed a similar pattern, Table III. The mean MDA levels in nmol/g Hb, following incubation with OFR was also significantly decreased (5.2 to 2.8, $p<0.05$) after Polbax® treatment. Furthermore there was a significant correlation between MDA reductions and increases in RFR ($r=0.46$; $p<0.05$).

TABLE III
Filterability changes in µl/s before and after 4 weeks treatment with Polbax®. Means ± SD

	Before	After	p
RFR	54±3.6	83±4.8	0.001
WFR	16±2.4	23±3.2	0.001

DISCUSSION

The results of the present study shows that mechanical trauma and oxygenation associated with gyratory shaking of red blood cells as well as a free radical generating system containing hypoxanthine and xanthine oxidase influenced negatively the filterability of red cells. This deleterious effect on the filterability rate seems to be additive since the incubation with HX/XO system caused a significantly greater decrease in the filtration rate both after 30 and 180 minutes of incubation. The use of mechanical trauma in this model produces sufficient damage to the red cells and was limited to 180 minutes.

The addition of hypoxanthine and xanthine oxidase was intended to mimic the events that occurs in ischemic reperfusion injuries. This leads to an increased generation of purine degradation products (like hypoxanthine) and a conversion of xanthine dehydrogenase to xanthine oxidase, as a result of an increased concentration of cytosolic calcium. When combined with molecular oxygen the HX/XO-system produces superoxide anions, which in turn generate a variety of short-lived toxic OFR (H_2O_2, OH, HOCl). These OFR are known to induce lipid peroxidation of the polyunsaturated fatty acids (PUFA's) to form lipid hydroperoxides (16), which in turn cause a hemolytic decomposition. As mentioned before, MDA is a decomposition product of oxidized PUFA's. The significant increase of MDA in the in vitro experiment indicate most probably, that the generation of OFR was causing the direct peroxidation of PUFA's in RBC membranes. MDA itself is capable of cross linking membrane components containing amino groups and can increase membrane rigidity resulting in the reduction of RBC deformability (9,10). Even at micromolar concentrations, MDA can significantly reduce the whole cell deformability detected either by a counter rotating cone plate rheoscope (10) or by an ektacytometer (8). Furthermore, reduced deformability of MDA treated red blood cells is associated with increased IgG-binding (16) and with shortened Cr51 survival in vivo (9).

There are still some uncertainities regarding both the methodological adequacy and the directness of the relationship between MDA production and the generation of free radicals and therefore MDA may be considered to be an experimental "window" rather than a true measure of the OFR-production. Furthermore, it is not warranted to claim that the OFR and the lipid peroxide formation should be treated as the only mechanism responsible for the decreased RBC filterability. On the other hand the negative significant correlation between the MDA concentration and the RBC filterability for all measured values at various times during the incubation seem to indicate a possible causal relationship between the filterability of RBC and lipid peroxidation and/or production of MDA.

Polbax® was found to improve the RBC and WBC filtration rate in healthy volunteers after four weeks of oral administration.The filterability test depends on the deformability of red and white blood cells. Thus, the increase of filterability should be interpreted as a finding indicating a beneficial effect of a treatment most probably depending on the decreased production of lipidperoxides as indicated by the diminished concentration of MDA. The mechanism of the in vivo effect is still not entirely clarified, but is thought to be dependent on Polbax® antioxidative activity due to the presence of flavonoid-polyphenols (17-20). The possibility of active absorption of native SOD-enzymes (17) seems less probable as the SOD activity was not found to be increased in the erythrocytes of healthy volunteers after four weeks of Polbax® treatment (Halliwell, unpublished results). The flavonoides, on the other hand, show in vitro a high SOD-like activity and being water soluble are easily resorbed in the gastro-intestinal tract after oral intake (21). After four weeks oral administration, Polbax® was also found to decrease the production of MDA as well as the lactate in muscle tissue after heavy exercise (22). The association between the decrease of both MDA and lactate might indicate some other mechanisms involving specific metabolic effects and the production of NADPH (22-24). Whatever, the mechanism might be, the application of Polbax®, a preparation characterised by the antioxidative activity, was found to prevent the increase of MDA as well as the reduction in RFR filterability, both effects being correlated to each other. This is in harmony with the observations in vivo, in patients undergoing coronary bypass surgery or angioplasty where morbidity and mortality was related to the severity of blood cell rheologic damage and where the use of antioxidants can have some effect on the postoperative complications (25-27). Our in vitro experiment can be regarded to correspond to the conditions in these clinical studies, with the comparable addition of exogenous hypoxanthine and xanthine oxidase and the consequential

production of free radicals and lipid peroxidation. Further studies are needed to confirm the beneficial effect of Polbax® in different clinical situations where a high production of OFR is anticipated. Thus, our in vitro system may be suitable as a model to follow several in vivo effects where ischemia/reperfusion events are to be evaluated and prevented.

In summary, this study showed a likeness between the production of MDA and the changes in the RBC's filterability. The well known hypoxanthine/xanthine oxidase system was used to accelerate the generation of OFR ex-vivo during the incubation of RBC's subjected to mild mechanical trauma. Polbax®, an OFR scavenger, with the antioxidative activity had a positive protective effect on blood cell filterability and MDA production. The effect on blood cell filterability was even confirmed in the in vivo study, where four weeks with Polbax® administration was found to be associated with significant improvement of the red and white cell deformability. The findings may have the important clinical implications.

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