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Short communication

SHORT COMMUNICATION

Does cholesterol bound to haemoglobin affect the anti-oxidant enzyme defence system in human erythrocytes?

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Abstract: In a previous study, it was shown that the lipid fraction, which is occasionally observed in red blood cell hemolysates, represents cholesterol (Ch) associated with phospholipid firmly bound to haemoglobin (termed Hb-Ch). The current study was conducted to investigate whether Hb-Ch could affect the primary anti-oxidant enzyme defence system in human erythrocytes. Sixty healthy volunteers were used for the current study. Group 1 consisted of 28 subjects without or with a low level of Hb-Ch. Group 2 comprised 32 subjects with a considerably higher level of Hb-Ch. The activities of erythrocyte superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, as well as the content of methaemoglobin (metHb) were measured in both groups. The results indicated that the amount of Hb-Ch neither influenced the activities of the erythrocyte anti-oxidant enzymes nor altered the level of metHb. However, a higher amount of Hb-Ch changed the correlations in the part of the anti-oxidant defence system relating to glutathione, suggesting increased peroxidative pressure from plasma lipids. Group 2 also had significantly increased concentrations of total plasma Ch and triglycerides. Together, these facts are strong indications that the anti-oxidant defence system in human erythrocytes finely retunes its composition according to plasma oxidative demands.

Keywords: erythrocytes, haemoglobin, cholesterol, anti-oxidant defence enzymes.

INTRODUCTION

In normal aerobic cells, excessive production of reactive oxygen species (ROS) creates a situation known as cellular oxidative stress. This undesirable condition has been implicated in the aging of organisms and in various diseases, such

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as cancer.[1–4] A number of enzymes, termed anti-oxidant enzymes, form a defence system which has the capacity to eliminate ROS. Such a defence system is ubiquitously expressed in all cell types and organisms.

Due to both its structural and functional characteristics, the human erythrocyte is destined to be under continuous oxidative stress.[5] The main source of ROS in the erythrocyte is the auto-oxidation of oxyhaemoglobin (Hb) to methaemoglobin (metHb), whereby superoxide anions are generated.[6,7] Superoxide anions are dismutated by Cu-Zn superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2), which is decomposed by catalase (CAT) or reduced by a reduced glutathione (GSH)-dependent mechanism by way of glutathione peroxidase (GSH-Px). These enzymes, together with glutathione reductase (GR), maintain a high reduced/oxidised glutathione ratio in red blood cells. In addition, various dietary antioxidants constitute the major defence against oxidative damage in the erythrocyte.[8] Possessing an anti-oxidant activity, heamoglobin *per se* may be of importance in providing protection against oxidative damage to erythrocytes.[9] In addition, the cell membrane appears to protect intra-erythrocytic Hb against extracellular oxidative stress.[10]

For the first time, cholesterol (Ch) [associated with phospholipid (PL)] was recently detected bound to Hb in normal human erythrocytes (termed Hb-Ch), with a binding stoichiometry of about two molecules of both Ch and PL per one Hb molecule in Hb-Ch.[11] In the report, it was shown that Hb-Ch (up to 35 % of the total Hb) was formed in a healthy population as a consequence of a seasonally-related elevated plasma level of Ch (due to changes in plasma lipoprotein metabolism).[11] Generally, (phopho)lipids promote the oxidation of human haemoglobin and the formation of ROS.[12,13] Cholesterol, on the other hand, has a protective effect.[14]

The principal aim of this study was to evaluate the functional importance of this new lipid modification of Hb on the anti-oxidant system, which normally protects erythrocytes from the continuous production of ROS and, consequently, from cellular injury. The relationship between the content of Hb-Ch and the activities of the antioxidant enzymes in human erythrocytes was examined. The activities of SOD, GSH-Px, CAT and GR, as well as the content of metHb in erythrocytes were measured. In addition, the total plasma cholesterol (T-Ch), high density lipoprotein cholesterol (HDL-Ch), low density lipoprotein cholesterol (LDL-Ch) and triglycerides in healthy male volunteers with different levels of Hb-Ch were measured. The results demonstrate that the amount of Hb-Ch neither influences the activities of erythrocyte anti-oxidant enzymes nor alters the level of metHb. Only a higher amount of Hb-Ch changed the correlations in the part of the anti-oxidant defence system relating to glutathione.

EXPERIMENTAL

All the chemicals used in this study were purchased from Sigma (USA) and Merck (Germany), unless indicated otherwise.

Sixty volunteers, aged 21–44 years old, were recruited for this study. Most of them were students and workers in local institutes and universities. Only samples with normal haematological indices

were used throughout the experiments. The history of participants did not reveal diseases, medical conditions or the taking of drugs which could influence the activities of the anti-oxidant enzymes. Informed consent was obtained from all the subjects prior to initialising the study. The study took place in the spring of 2005. After an initial screening, the subjects were classified into two groups according to their level of Hb-Ch in the hemolysate. Group 1 (control group) consisted of 28 subjects without or with a low level of Hb-Ch (up to 6 % of the total Hb). Group 2 comprised 32 subjects with a higher level of Hb-Ch (from 7 % up to 20 % of the total Hb). No significant difference in smoking habits, alcohol intake, physical activity or in age difference was observed between the two groups.

Blood samples were obtained by venapuncture following an overnight fast. Citrate was used as an anticoagulant (3.8 %). The plasma and erythrocytes were separated from each other by centrifugation at 1500 x g for 10 min. The buffy coat was removed and the red blood cells were washed three times with isotonic (0.9 %) saline. All measurements described below were carried out using fresh plasma and erythrocyte lysates.

The T-Ch and triglyceride concentrations were determined based on enzymatic methods using Reanal (Hungary) kits. HDL-Ch was measured in the supernatant following the precipitation of ApoB-containing lipoproteins with dextran sulphate (Serva, Germany) and $MgCl_2$. The LDL-Ch was calculated using the Friedewald formula.

The total Hb contents of the erythrocyte suspensions and the hemolysates were measured as cyanmethaemoglobin using the Drabkin method. The metHb content was calculated as the percentage of the total Hb from the absorption spectrum from 500 to 700 nm.[15]

Hb-Ch was determined as described by Nikolić *et al.*[11] Briefly, aliquots of the hemolysates, from which the membranes had been carefully removed, were extracted according to Reed *et al*.[16] Cholesterol was enzymatically determined in the lipid extracts. The results are expressed as a percentage of the total Hb.

Anti-oxidant enzyme activities were determined using a Shimadzu UV-160 spectrophotometer. All enzyme measurements were carried out in duplicate and average data of the GSH-Px activities were normalised using the haemoglobin content (5 g %). Erythrocytes (0.5 ml) were hemolysed by adding 3 ml of ice-cold distilled water. Haemoglobin was removed from an aliquot of the hemolysate by the Tsuchihashi method.[17] The upper layer was used for the determination of the SOD activity, according to the method described by McCord and Fridovich.[18] One unit of activity is defined as the amount of enzyme necessary to decrease the rate of cytochrome *c* reduction to 50 % of the maximum at 25 °C and pH 7.8. CAT was determined according to Claiborne.[19] One unit of CAT activity is defined as the amount of enzyme which decomposes 1 mmol H_2O_2 per min at 25 °C and pH 7.0. The activity of selenium GSH-Px was determined using a modification of the assay described by Paglia and Valentine.[20] One unit of GSH-Px activity is defined as the amount required to oxidise 1 nmol NADPH per min at 25 °C and pH 7.0. The GR activity was determined using the method of Glatzle *et al*.[21] One unit of GR activity is defined as the oxidation of 1 nmol NADPH per min at 25 °C and pH 7.6.

The data are represented as mean ± standard deviation (*SD*). Differences among groups were assessed by analysis of the variance (ANOVA) followed by Tukey's post-hoc comparison test. Statistical significance was established by protocols as described in Hinkle *et al.*[22] and p values <0.05 were considered significant.

RESULTS AND DISCUSSION

The activities of the erythrocyte anti-oxidant enzymes are summarised in Table I. There was no statistically significant increase in erythrocyte SOD, CAT, GSH-Px or GR activity in subjects with a higher level of Hb-Ch (group 2) compared to the control group (group 1). In addition, no difference in the level of metHb between the two groups were detected (Table I).

TABLE I. The biochemical parameters of erythrocytes from patients classified into groups 1 and 2. The enzymatic activity of SOD, CAT, GSH-Px and GR were also measured

Parameter	Group 1 (n = 28)	Group 2 (n = 32)
Hb/g L^{-1}	142 ± 5	141 ± 6
Hb-Ch/%	4.3 ± 0.7	11.6 ± 1.5*
metHb/%	1.15 ± 0.14	1.19 ± 0.16
SOD	1.08 ± 0.09	1.09 ± 0.09
CAT	7.43 ± 1.26	7.53 ± 1.34
GSH-Px	11.46 ± 1.59	11.41 ± 1.57
GR	6.37 ± 0.97	6.19 ± 0.89

The values are mean ± *SD*. The units of activity are described in the Experimental section. The *n* values in parenthesis indicate the number of samples. Statistical significance was analysed by ANOVA; * $p < 0.001$.

A coordinated action of the components comprising the anti-oxidant defence system is necessary to limit ROS production and to preserve the redox state. Therefore, changes in the activity of some anti-oxidant components should be accompanied by correlative changes in one or more of the anti-oxidant defence enzymes. This phenomenon has been observed in the erythrocytes of Down's syndrome patients.[23] In erythrocytes, the anti-oxidant enzymes SOD, CAT and GSH-Px have complementary activities in the anti-oxidant defence system. H_2O_2, the product of the reaction catalysed by SOD, is also the substrate for CAT and GSH-Px.

It has been shown that this anti-oxidant system, at the level of coordinated expression, functions in the domain of positive correlation between SOD and CAT.[24] On the other hand, glutathione-dependent enzymes in the anti-oxidant system are separately regulated, probably *via* the concentration of (reduced) glutathione and the redox status of the cell.[25] The hypothesis made in this study is that by performing correlation analyses between the activities of the anti-oxidant component between study groups 1 and 2, an indication of the presence of coordinated actions of the anti-oxidant enzymes could be obtained. The results of such analyses are presented in Table II.

TABLE II. Correlation analysis of the activity of erythrocyte anti-oxidant defence enzymes between group 1 (horizontal categories) and group 2 (vertical categories)

	SOD	CAT	GSH-Px	GR
SOD		**0.636****	0.186	0.268
CAT	–0.147		0.246	0.405
GSH-Px	0.108	**0.539***		–0.213
GR	0.198	0.241	**0.525***	

Three significant correlations were observed (indicated in bold text). Statistical significance was analysed by ANOVA; * $p < 0.01$; ** $p < 0.001$.

Several points and conclusions can be drawn. A positive correlation between the activities of CAT and SOD in group 1 was found, implying that H_2O_2 is a molecular

species connecting this part of the anti-oxidant enzyme protection system in human erythrocytes, both without or with a low level of Hb-Ch (Fig. 1). This result is in accordance with that obtained from a group of 220 healthy Danish individuals. The authors reported that erythrocyte SOD activity was positively correlated with CAT but there were no significant correlations between GSH-Px and any of the other enzymes measured.[26] In group 2 there was positive correlation between the activities of GSH-Px and CAT, as well as between GR and GSH-Px. This reveals that lipid peroxides are the dominant chemical entity connecting this part of the anti-oxidant enzyme system in erythrocytes with a higher level of Hb-Ch (Fig. 1).

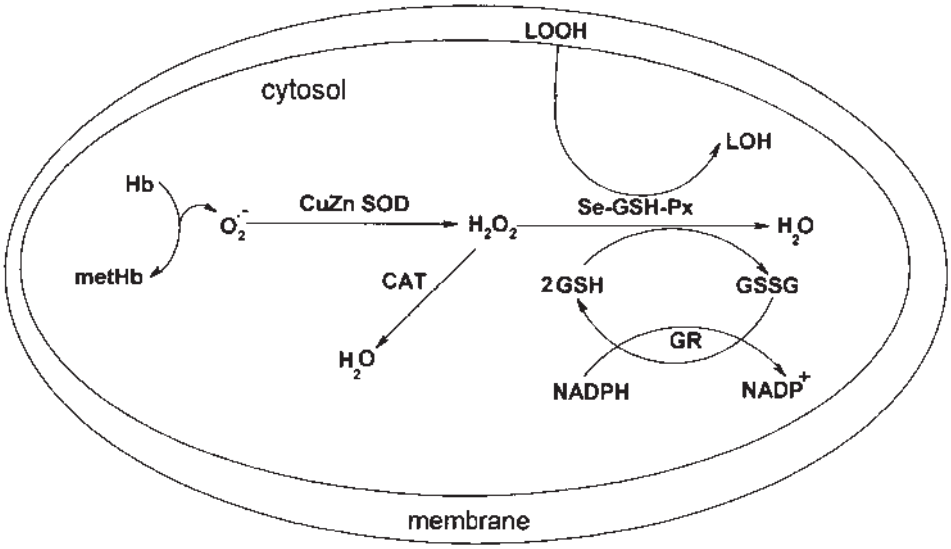

Fig. 1. A schematic representation of a part of the anti-oxidant enzyme system in erythrocytes. $O_2^{\bullet -}$: superoxide radical; H_2O: water; Cu-Zn SOD: copper-zinc superoxide dismutase; CAT: catalase; H_2O_2: hydrogen peroxide; GSH-Px: glutathione peroxidase; GSH: reduced glutathione; GSSG: oxidised glutathione; GR: glutathione reductase; $NADPH^+$: reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$: nicotinamide adenine dinucleotide phosphate; LOOH: lipid hydroperoxide; LOH: lipid alcohol.

The presented results lead to the conclusion that the presence of Hb-Ch does not favour the oxidation of Hb to metHb and that the level of Hb-Ch should correlate with the level of plasma lipids (as a possible exogenous source of erythrocyte peroxides). The content of plasma lipids in study groups 1 and 2 were determined and are presented in Table III. Statistically higher concentrations of T-Ch and triglycerides were found in group 2. It should be emphasised that all individual values for the plasma lipids were within the reference range (considering the age of the participants).

The presented results lead to three significant conclusions. Firstly, the amount of Hb-Ch has no direct influence on the production of superoxide radicals originating from the transformation of Hb to metHb. Secondly, a higher level of Hb-Ch

TABLE III. The biochemical parameters of plasma from patients classified into groups 1 and 2

Parameter	Group 1 (n = 28)	Group 2 (n = 32)
T-Ch	3.78 ± 0.73	4.46 ± 0.75*
LDL-Ch	2.43 ± 0.51	2.76 ± 0.70
HDL-Ch	1.01 ± 0.20	1.08 ± 0.14
Triglycerides	0.86 ± 0.30	1.35 ± 0.40**

The values are mean ± *SD* and the lipids are expressed in mmol/l. The values in parenthesis indicate the number of samples. Statistical significance was analysed by ANOVA; * p <0.01; ** p< 0.001.

correlates with alterations in the relationship between the activities of erythrocyte anti-oxidant enzymes, suggesting an increasing peroxidative pressure from the plasma. Thirdly, individuals with a higher level of Hb-Ch (group 2) have increased concentrations of T-Ch and triglycerides in their plasma. The last two points (when considered together) further underline the necessity to control diets, particularly of inhabitants in the western world where obesity and heart disease are on the increase. Caloric restriction leading to reduced body weight, lower T-Ch and reduced plasma lipid peroxidation is the preferred method to limit the above-described diseases. As a consequence, both the level of Hb-Ch and the peroxidative pressure on erythrocytes from the plasma would also decrease. Caloric restriction is, therefore, the preferred choice, compared with the intake of large quantities of anti-oxidants, such as vitamin E, to limit erythrocyte peroxidative pressure from plasma.[27] Studies which will hopefully lead to the recognition of the mechanisms and factors that govern the formation and accumulation of Hb-Ch in human erythrocytes are currently being pursued.

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ИЗВОД

ДА ЛИ ХОЛЕСТЕРОЛ ВЕЗАН ЗА ХЕМОГЛОБИН УТИЧЕ НА АНТИ-ОКСИДАТИВНИ ЕНЗИМСКИ СИСТЕМ У ХУМАНИМ ЕРИТРОЦИТИМА?

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У претходном раду показано је да липидна фракција која се јавља у хемолизату здравих људи представља холестерол (асосован са фосфолипидима) чврсто везан за хемоглобин (Hb-Ch). У овом раду испитиван је утицај Hb-Ch на анти-оксидативни ензимски систем у хуманим еритроцитима. Одређена је активност супероксид-дизмутазе, каталазе, глутатион-пероксидазе и глутатион-редуктазе, као и садржај мет-хемоглобина (metHb)

у еритроцитима 60 људи, подељених у две групе на основу количине Hb-Ch. Резултати показују да количина присутног Hb-Ch не мења активност мерених ензима, нити ниво metHb. Међутим, у групи испитаника са повећаним садржајем Hb-Ch запажене су корелативне промене у делу анти-оксидативног ензимског система повезаног са глутатионом. У истој групи детектоване су и веће концентрације укупног холестерола и триглицерида у плазми, што заједно указује на повећани пероксидативни притисак из плазме. Ови резултати указују да одбрамбени анти-оксидативни ензимски систем у хуманим еритроцитима прилагођава своју организацију према захтевима из свог окружења.

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