Turkish Journal of Medical Sciences

Volume 31 | Number 2 Article 1

1-1-2001

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ÖZAYDIN, AHMET; ONARAN, İLHAN; AVŞAR, KADİR; TEZCAN, VECDET; and SULTUYBEK, GÖNÜL (2001) "Relationship Between Aging and Susceptibility to Oxidative Damage: An Assessment by Erythrocyte Membrane Proteins and Lipids," *Turkish Journal of Medical Sciences*: Vol. 31: No. 2, Article 1. Available at: https://journals.tubitak.gov.tr/medical/vol31/iss2/1

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Turk J Med Sci
31 (2001) 95-101
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Relationship Between Aging and Susceptibility to Oxidative Damage: An Assessment by Erythrocyte Membrane Proteins and Lipids

Received: May 29, 2000

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Abstract: Contradictory results have been reported from various studies regarding the effect of age on susceptibility to oxidative damage. Using different parameters, we carried out a comparative study on the erythrocytes and erythrocyte membranes of healthy elderly and young adults to determine how resistance to oxidative damage is affected by aging. Thin layer chromatography and polyacrylamide gel electrophoresis under denaturing conditions were used to estimate changes in membrane phospholipids and cytoskeletal proteins, respectively. Our results showed that these components of intact erythrocytes incubated with cumene hydroperoxide (CumOOH), a powerful oxidising agent, remained unchanged in both age groups. However, exposure of erythrocyte membranes to CumOOH induced a reduction of protein bands 1, 2, 3, and 4.1, with the appearance of high-molecular-weight aggregates. Furthermore, our data indicate that membranes from aged subjects are more susceptible to oxidative stress than those from young subjects, since these changes are higher in the aged group. Therefore, our results suggest that the intact erythrocytes of elderly individuals are equally capable of withstanding the oxidative stress induced by CumOOH, although there is a decrease in membrane defence with aging.

Key Words: Aging, oxidative stress, erythrocyte, membrane proteins and lipids, susceptibility

Introduction

Although numerous age-related studies on the level of individual antioxidants have been carried out, because of factors such as the multiplicity of antioxidants, overlap in their functions and inconsistency of changes, there is no consensus on whether the antioxidative potential of organisms is affected by the aging process (1). In order to overcome this difficulty, another strategy was employed, in which the susceptibility of tissues to experimentally induced oxidative damage was used to determine the antioxidative potential of the individual in relation to aging (2-8). Since the cell membrane is an especially important target for radical damage, and blood can reflect the liability of the whole animal to oxidative conditions, erythrocytes and erythrocyte membranes have been used extensively for determining the effects of aging in studies concerning the possible involvement of lipid peroxidation in the aging process (3-8). However, in the evaluation of biochemical parameters related to lipid peroxidation stimulated by oxidative stress, contradictory results have been reported. Some studies suggest that erythrocytes from elderly individuals and aging animals are highly susceptible to oxidative stress (3-5), while others suggest otherwise (6-8).

On the other hand, no gross changes in any of membrane components have been found in erythrocytes with advancing age (3), although the prime targets of the more reactive free radicals include unsaturated lipids in the cell membrane, amino acids in proteins, and nucleotides in DNA. There are no gross changes either in the lipid composition, or in the protein content or composition (9). Under normal conditions, the amounts

of oxy-repair enzymes or antioxidants in older organisms would possibly be sufficient to cope with the amount of damage produced. However, when an oxidant challenge exceeds the capacity of the cell's defence system, membrane damage may occur; this would make it possible to evaluate the status and interrelationships related to oxidative damage. Several investigators have reported the formation of structural changes in both proteins and lipids of the erythrocyte membrane when erythrocytes are incubated with different compounds known to induce oxidation of lipids (10-13). It has been suggested that proteins exposed to oxidised lipids undergo modifications such as protein-protein cross-linking and fragmentation, and protein-lipid adducts (10-12). The characterisation of these changes associated with membrane damage induced by various oxidative agents has been used extensively to investigate the role of oxidative membrane damage in various pathological conditions (13). Such an in vitro experimental system might also be useful in testing the antioxidant potential of tissues.

The above observations prompted us to determine the comparative liability to oxidative damage to erythrocytes in aging and young individuals by evaluation of changes in membrane protein and phospholipids to in vitro lipid peroxidation. In this study, we used the induction of oxidative stress by cumene hydroperoxide (CumOOH) in erythrocytes and erythrocyte membranes to study how the aging process affects resistance to oxidative damage. CumOOH, which mimics natural compounds, is an attractive model oxidant because its cellular actions and reactions are well documented (11). It is easily taken up by cells and is not metabolised by catalase (10, 11). The lipophilicity of CumOOH allows its use as an initiator of lipid peroxidation (8), which in turn gives rise to diffusible products. Therefore, by testing the oxidative liability of erythrocytes to CumOOH, it may be possible to visualise some of the effects of prooxidant conditions in aging.

Materials and Methods

Subjects

Subjects were young (6 males and 6 females, aged 18 to 30) or elderly (6 males and 6 females, aged 69 to 84) normal volunteers. All subjects were non-smokers and had normal blood counts for urea, glucose, creatinine, albumin, alkaline phosphatase, lactate dehydrogenase and bilirubin. None had a history of haematological abnormality, recent infectious disease or significant medical illness. The participants were instructed not to take aspirin-like drugs 2 weeks prior to blood sampling.

Preparation of Erythrocytes and Their Membranes

Fresh heparinised fasting blood samples were centrifuged at 1000 g for 10 min. Erythrocytes were separated from plasma and buffy coat and then washed three times with 10 vol of phosphate-buffered saline (PBS, pH 7.4). Erythrocytes containing a chronologically young population rich in reticulocytes were removed by discontinuous Percoll density gradient as described by Rennie (14). The gradient was built up in two layers containing Percoll with specific density values between 1.100 and 1.124 g/ml. At the end of centrifugation, the cells were collected at the interface of the Percoll above 1.124 g/ml, while the enriched fraction of reticulocytes above low density was removed. The erythrocytes were then washed two times with PBS.

White erythrocyte membranes were prepared by hypotonic haemolysis using standard procedures (15) with Tris buffer (pH 7.4) containing 0.1 mM phenylmethylsulfonylfluoride and 0.01% butylated hyroxytoluene, and membranes were finally suspended in iso-osmotic Tris-HCl. The efficiency of the method in removing the Hb from erythrocytes was checked by Drabkin's reagent. No Hb was found in the membranes. Protein concentrations in the membranes were determined by the method of Lowry et al. (16), using bovine serum albumin (Sigma Chemical Co.) as standard.

Reaction Systems

Samples used for the induction of lipid peroxidation in intact erythrocytes and erythrocyte membranes were either the erythrocyte suspension in PBS (haematocrit of 5%) or membrane (2 mg protein/ml) in 5 mM sodium phosphate, pH 7.4.

Lipid peroxidation was induced by addition of CumOOH (Sigma Chemical Co.) (final concentration, 0.2 mM). Immediately after CumOOH addition, the tubes were sealed and incubated at 37ºC with continuous shaking at 120 cycles/min. In parallel, control samples were incubated under the same conditions but without CumOOH. After 60 min, cell suspensions for intact erythrocytes were centrifuged at 7000 g for 1 min to sediment the erythrocytes. The pellet was washed with PBS and white membranes (ghosts) were prepared. The hydroperoxide reaction in membrane suspensions was stopped by placing the tubes containing the membrane suspension in ice water followed by centrifugation at 15000 g for 10 min. The membrane pellets were washed twice with 15 vol of 10 mM Tris buffer (pH 7.4). The pellets were resuspended in 1.0 ml with the same buffer. All membrane suspensions were used for protein analysis.

SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The ghosts were dissolved to a concentration of 1 mg protein /ml in SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromphenol blue, 63 mM Tris-HCl, pH 6,8) and incubated for 2 min at 95°C. SDS-PAGE was performed according to Laemmli with slight modifications, using a stacking gel of 4% and a separating gel of 7.5% (17). Staining of protein bands with Coomassie blue was carried out. The stained gel was photographed and the photographic negative used for scanning by a laser densitometer. Individual peaks from the densitometry scan were cut and weighed to determine the relative amounts of a given peak within each electrophoretic lane.

The Quantitation of Phospolipids on Thin-Layer Chromatography

Lipid extractions from erythrocyte ghosts were carried out using isopropanol and chloroform by the method of Rose and Oklander (18). Lipid extracts were dried with nitrogen and then redissolved in 50 ml of chloroform. Phospholipid classes in the lipid extract were separated by thin-layer chromatography (TLC) on silica gel H plates (Riedel-de Haen, Germany) using the chloroform-methanol-acetic acid-water (50:25:7:3, v/v) solvent system. Lipid spots on the TLC plates were visualised using iodine vapours. The lipids were identified with reference standards. The spots were scraped from the plate using a sharp razor blade, digested in a sand bath at 180°C with perchloric acid and a few drops of sulphuric acid and analysed for phosphate content individually. The phospholipid-phosphorus in the silica gel spots was quantitated by method of Fiske and Subbarow (19).Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) were quantitated in this manner for each erythrocyte membrane sample. The results for each phospholipid are expressed as a percent age.

All studies were performed in duplicate or triplicate samples and were analysed statistically by Student's test. $P<0.05$ was considered significant.

Results

Erythrocytes from young and old individuals were separated into two age classes by density gradient centrifugation. The number of reticulocytes in the high-density cell fraction studies did not show any significant differences between the elderly and young control groups.

The pospholipid composition of intact erythrocytes, which were incubated with or without CumOOH from young and old individuals, were compared (Table). In the absence of CumOOH, there was no significant difference between the phospholipid contents of the erythrocyte membrane in both the control and aged groups. In intact erythrocytes incubated with 0.2 mM CumOOH, no differences in the percentage compositions of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin phospholipids were observed between the two age groups ($p>0.05$).

Table. Membrane phospholipid composition following the oxidative stress induced by cumene hydroperoxide in intact erythrocytes and erythrocyte membranes obtained from young and elderly subjects

Phospholipids (%)	Young (n=12)		Aged (n=12)	
	CumOOH (-)	CumOOH (+)	CumOOH (-)	CumOOH (+)
Intact erythrocyte				
Sphingomyelin	25.52±1.69	25.97±081	25.42±0.79	26.55±0.33
Phosphatidylcholine	30.18±2.39	31.75±1.64	29.39±1.51	30.70±1.79
Phosphatidylserine	13.70±0.99	13.78±1.57	14.78±1.22	14.92±1.28
Phosphatidylethanolamine	30.53±1.35	28.51±2.58	30.69±0.97	27.82±1.53
Erythrocyte membrane				
Sphingomyelin	25.75±4.65	28.26±4.64	28.65±2.08	27.00±2.3
Phosphatidylcholine	24.38±2.35	26.42±3.01	27.00±3.13	28.49±3.54
Phosphatidylserine	19.10±4.12*	19.16±3.73*	19.23±3.28	21.28±3.72*
Phosphatidylethanolamine	24.46±3.66*	26.85±4.06	25.08±4.65*	23.27±5.02

Results are expressed as percentage of each phospholipid and as tandard deviation of the mean. Those marked with (*) indicate $p<0.05$ when compared to intact erythrocyte

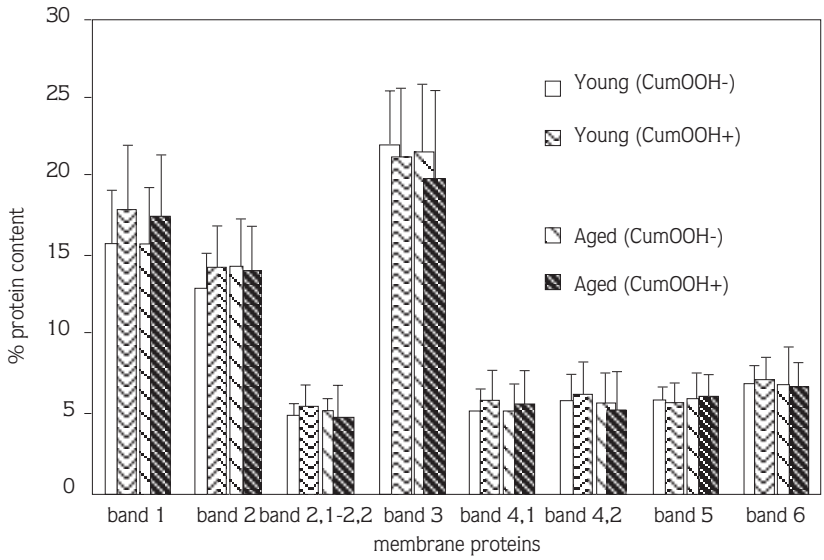


Figure 1.

In addition, after treatment with oxidative stress (0.2 mM CumOOH), qualitative and quantitative distribution of membrane proteins in erythrocytes from aged and healthy controls were analysed by SDS-PAGE. Figure 1 shows that the percentage compositions of major membrane proteins remained unchanged within each group when compared to basal conditions, i.e., the incubation absence of the CumOOH. Following oxidative challenge, no differences were also observed in any protein bands between the two age groups.

In order to assess the contribution of cytoplasmic factors to the antioxidant potential of the erythrocytes, white membranes, which have no detectable Hb, were incubated with CumOOH for one hour. Under the same conditions, despite the difference in PS and PE compositions between ghost and intact erythrocytes ($p<0.05$), CumOOH did not cause any significant change in phospholipid levels within the two age groups (Table). However, analysis of the ghost SDS-PAGE band profiles showed that some protein bands had significant changes in both age groups. Treatment with CumOOH resulted in the partial degradation of bands 1, 2, 3 and 4.1 accompanied by a formation of significant levels of high molecular weight polymers (HMWPs). We detected no differences at the other membrane protein bands. Furthermore, at this concentration, the increase of HMWP and the decreases in bands 1, 2, 3 and 4.1 were notably greater in the aged group than in the controls (Figure 2).

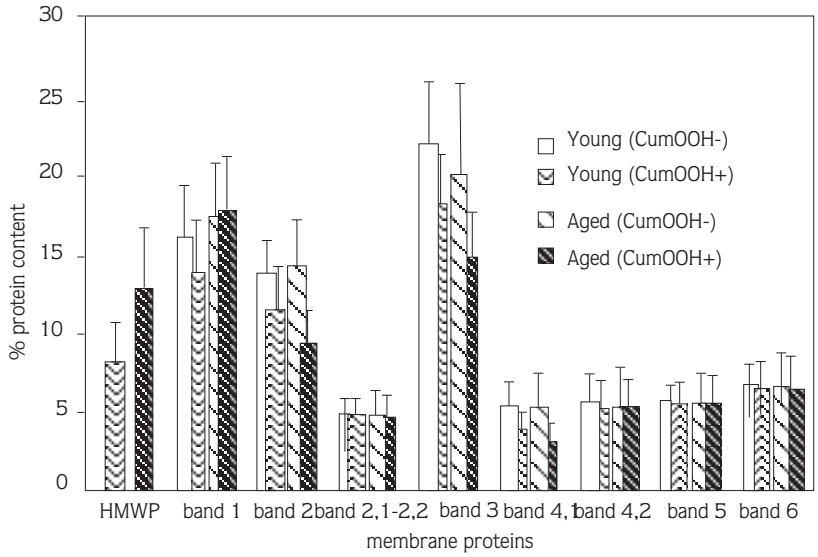


Figure 2.

Discussion

In the past several years, oxidative damage to erythrocytes has become a key word in the explanation of destructive events in many haematological disorders and in aging. In studies in this field, contradictory results showing decreased or unchanged erythrocyte susceptibility to oxidative stress with donor age have been reported (3-8).

The source of the discrepancy between these studies is not clear. In these investigations, the effect of age on the susceptibility of erythrocytes and animal tissues was estimated by different parameters such as the levels of malondialdehyde and carbonyl compounds formed during lipid peroxidation, and the activities of some enzymes which are sensitive to oxidative damage. Therefore, by a different approach, our aim was to determine whether the susceptibility of erythrocytes to oxidative damage is altered during the aging process. We attempted to characterise the changes associated with CumOOH-induced erythrocyte membrane damage, since membrane protein and phospholipids are sensitive to oxidative stress. Under normal conditions, no gross changes in any of the membrane components have been shown in erythrocytes with advancing age. Several subtle changes have been found, however, which may be of importance in determining the survival of erythrocytes in old individuals (9). It has long been known that CumOOH causes oxidative stress in erythrocytes by peroxidative cleavage of membrane lipids and that both proteins and lipids the of membrane undergo extensive structural changes in CumOOH-treated erythrocytes (10,11). It has been found that CumOOH is a suitable substrate for use in examining the consequences of oxidative stress. In our previous studies we observed that 0.2mM CumOOH was the maximum concentration which did not induce haemolysis in the erythrocytes of elderly and young adults (8). In addition, a dose curve showed no differences between the groups. Therefore, this concentration was used as the standard CumOOH concentration for comparative experiments. The reasoning behind the usage of erythrocytes other than the chronologically young population rich in reticulocytes is explained elsewhere (8).

The defence system against oxidants in the cell membrane is less effective since radicals in the membrane would be relatively sequestered from the cell's antioxidant mechanisms, which, with the exception of vitamin E, are largely cytoplasmic. In order to assess the effect of cytosolic components and the potential susceptibility to oxidative damage of erythrocyte membranes from aged individuals, we also used white membranes with no detectable Hb. In our study the extent of membrane lipid injuries was evaluated by thin-layer chromatography, while membrane protein alterations were assessed by SDS-PAGE.

The present study reports that, under conditions of low in vitro oxidant challenge and basal conditions, membrane protein patterns and phospholipid composition in erythrocytes from aged subjects remained unchanged. These data suggest that the protection mechanism against employing peroxidative stress is maintained in the intact erythrocytes of aged subjects. However, in comparison with intact erythrocytes, white erythrocyte membranes showed a complex pattern of response to CumOOH treatment. Despite the resistance to lipid peroxidation of the intact erythrocytes, the membranes prepared from erythrocytes of the elderly and young subjects were more vulnerable to CumOOH-induced oxidative stress than intact erythrocytes kept under the same conditions. Treatment of erythrocyte membranes with 0.2 mM CumOOH resulted essentially in the partial degradation of spectrin (bands 1 and 2), anion exchange (band 3), and 4.1 bands, accompanied by a significant level of HMWP formation. In addition, membranes from aged subjects were more susceptible to CumOOH than those from young subjects, because these changes were higher in the aged group. The difference in the response to CumOOH-induced oxidative stress between erythrocytes and membranes of aged individuals is in accordance with that of our previous results obtained with different parametric measurements (8). The observed difference seems to be dependent on cytoplasmic factors, since the cytosol of the erythrocytes was removed in the membrane preparation procedure. Cytoplasmic enzymes, which are protective against oxidation, may be involved (20,21). However, it is extremely difficult to evaluate the action of each participating element contributing to antioxidant activity, due to factors such as the multiplicity of antioxidants and overlap in their functions. Because the intensity of some bands was diminished and the formation of HMWP was higher in the membrane samples of elderly subjects than in the membrane samples of young subjects treated with the same concentration of CumOOH, it seems that some membrane-associated component of the membrane of elderly individuals may be inducing the oxidative effects of CumOOH. The molar ratio of phospholipid to cholesterol (PL/CH) increases with advancing age in the erythrocyte membranes (22). It is known that the ratio of PL/CH is an important factor in membrane protection (23). In addition, it has been reported that cell membrane protein

oxidation increases with age in rodent ghost (24). Grune et al. also proposed that oxidatively modified proteins can undergo direct chemical fragmentation or can form large aggregates due to covalent cross-linking reactions (25). On the basis of these suggestions, it is possible that age related changes in membrane may be partly responsible for the increase in oxidative damage. However, further investigations must be carried out to understand the mechanisms of the greater vulnerability to peroxidative stress in the membranes of elderly individuals.

Although it is known that phospholipids are sensitive to oxidative damage when oxidative stress is present, in our study, no significant differences in composition were observed. At present, the reason for the lack of effect of CumOOH on phospholipids is unknown. However, several possible explanations may be considered. Several investigators have reported that the formation of HMWP protein aggregates is independent of lipid peroxidation and is the result of a direct attack of radicals on the proteins (10-12). Furthermore, in these studies, changes in phospholipids were shown by higher oxidant stress conditions than ours (CumOOH > 1mM) (10,11). Since the TLC-method employed is not sensitive or selective enough to demonstrate oxidative changes on all phospholipids, we do not know how lipid constituents other than the membrane phospholipids studied by TLC were affected by 0.2 mM CumOOH-induced oxidative stress. There was a significant difference in the compositions of PE and PS between intact erythrocytes and cell ghosts, although the difference between younger and older individuals was not significant. At present, we are not able to explain this observed effect on PE and PS compositions. A possible explanation of this behaviour could be that during the preparation of the ghost membrane, the skeletal network undergoes rearrangement with an increase in surface area and the breaking of junction points (26). Therefore, the preparation process of the ghost membrane may affect PS and PE compositions, which exist predominantly in the inner-layer lipid. In order to examine and explain this occurrence, experiments should be expanded to a larger number of subjects.

In summary, our results suggest that the physiological antioxidant defences in the intact erythrocytes of aged subjects might suffice to prevent the oxidative damage induced by CumOOH. However, the membrane status of old adults may make them more susceptible to oxidative damage than young adults.

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