Increased Proportion of Docosahexanoic Acid and High Lipid Peroxidation Capacity in Erythrocytes of Stroke Patients

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Background and Purpose Intracellular accumulation of lipid peroxides that derive from the autoxidation of membrane polyunsaturated fatty acids reduces the deformability of erythrocytes contributing to the hemorheological disturbances observed in acute cerebral ischemia. The present study deals with the biochemical background of increased lipid peroxidation capacity in the erythrocytes of stroke patients.

Methods A complete clinical and laboratory assessment was made of 24 men and 18 women (aged 50 to 78 years; 64.5±13.9 years, mean±SD) who had an ischemic hemispheric lesion of the brain. Lipid peroxide content, lipid peroxidation capacity, superoxide dismutase activity, and fatty acid composition of erythrocytes were compared in stroke patients and 22 healthy subjects matched for age. The lipid peroxide content of the erythrocytes was estimated before and after the autoxidative test; the results were expressed as nanomoles of malondialdehyde per gram of hemoglobin. The increase of the lipid peroxide content in the erythrocytes during the autoxidative test measures the lipid peroxidation capacity.

Results In comparison with healthy subjects (1.45±0.30 nmol MDA/g Hb per 24 hours), the lipid peroxidation capacity was found to be significantly higher (4.18±0.41 nmol MDA/g Hb per 24 hours) ($P<.01$) in the erythrocytes of stroke patients. The stroke patients could be divided into two groups on the basis of lipid peroxidation capacity of their erythrocytes. Twenty patients had erythrocytes with high lipid peroxidation (<4 nmol MDA/g Hb per 24 hours), and 22 patients had very high lipid peroxidation capacity (>4 nmol MDA/g Hb per 24 hours). There was no significant difference in superoxide dismutase activity in the erythrocytes of patients compared with healthy subjects. Before the autoxidative test was conducted, the fatty acid composition in the erythrocytes of stroke patients with very high lipid peroxidation capacity was measured and found to be generally normal; only the proportion of docosahexanoic acid (22:6 n-3) was markedly ($P<.01$) increased.

Conclusions The results suggest that the erythrocytes of ischemic stroke patients with very high lipid peroxidation capacity displaying an abnormal fatty acid composition are much more vulnerable to lipid peroxidation. The increased proportion of docosahexanoic acid and the high lipid peroxidation capacity of erythrocytes play a pathogenetic role and explain the hemorheological disturbances observed in the microcirculation of stroke patients. (***Stroke.*** **1994;25:2416-2420.**)

Key Words • erythrocytes • superoxide dismutase • lipid peroxidation

High values of plasma lipid peroxides and malondialdehyde (MDA)-like materials have been found in patients with several atherosclerotic diseases such as myocardial infarction and stroke.[1-4] So far neither the origin nor the eventual effect of this phenomenon are clear. Lipid peroxides and MDA that derive from enzymatic and nonenzymatic oxidation of polyunsaturated fatty acids can be found in human plasma, where the level of these substances reflects in vivo platelet activation; indeed, the administration of aspirin, which is an inhibitor of the cyclooxygenase enzyme, decreases the plasma values of MDA-like material.[5] The recent study of Violi et al,[5] however, suggests that the increase of plasma lipid peroxides and MDA may be only an epiphenomenon of altered metabolic pathways and is not attributable to platelet hyperfunction.

Lechner et al[6] first pointed out that the reduced deformability of erythrocytes is one of several major factors contributing to the hemorheological disturbances observed in acute cerebral ischemia. Intracellular accumulation of lipid peroxides that derive from the autoxidation of membrane polyunsaturated fatty acids reduces the deformability of erythrocytes in patients with transient ischemic attack (TIA).[7,8] The present article deals with the increased levels of thiobarbituric acid (TBA)–reactive substances (including mainly lipid peroxides and MDA), which are actually detectable under the conditions of autoxidative test in the erythrocytes of stroke patients.

Because polyunsaturated fatty acids in the red blood cell membrane (particularly arachidonic acid and docosahexanoic acid) are the major sites of peroxidative damage,[9,10] we studied the fatty acid composition of red blood cells obtained from healthy subjects and stroke patients who had erythrocytes with very high lipid peroxidation capacity (LPC).

The first defense against oxygen free radicals capable of initiating lipid peroxidation is the enzyme superoxide dismutase (SOD). The activities of SOD were compared in erythrocytes of healthy subjects and stroke patients.

Received April 1, 1994; final revision received September 1, 1994; accepted September 2, 1994.

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Subjects and Methods

Twenty-four men and 18 women (aged 50 to 78 years; 64.5±3.4 years, mean±SE) who had had an ischemic lesion of the brain localized to the territory of the middle cerebral artery were included. Brain tumors and hemorrhage were excluded by computed tomographic scan. Results of Duplex and transcranial Doppler ultrasonography and cerebral angiography, when required, were evaluated in all of the patients. Clinical symptoms of stroke patients were characterized by scores of 65.3±4.1 (mean±SE) of the Matthews Scale and 45.7±8.6 (mean±SE) of the Bartel Index.

A complete clinical assessment was made of such risk factors as diabetes, hypertension, smoking, and dyslipidemia. Routine investigation included electrocardiogram, chest x-ray, measurement of blood levels of cholesterol (Dri-Stat Cholesterol-ES reagent, Beckman; range, 3.6 to 5.2 mmol/L), triglycerides (Dri-Stat Triglycerides-INT reagent, Beckman; range, 0.57 to 1.8 mmol/L), and glucose (Liquid-Stat Glucose-UV reagent, Beckman; range, 3.89 to 5.83 mmol/L). Westergren values for all but 14 patients and blood reticulocytes (8.53±0.61%) for all patients were in the normal range, and the fibrinogen level of patients (4.23±0.17 g/L) did not differ significantly from that (4.28±1.0 g/L) of healthy subjects.

Seventeen patients had blood cholesterol levels between 6.0 and 10.0 mmol/L, and 9 had hypertriglyceridemia (2.0 to 4.3 mmol/L); 4 patients had diabetes mellitus, 21 had blood pressure >150/95 mm Hg, and 6 habitually smoked >5 cigarettes per day. Medical history was checked for previous manifestations of atherosclerotic disease.

Lipid peroxide content and LPC of erythrocytes were compared in all stroke patients and in 22 healthy subjects matched for age (aged 53 to 73 years; 62.9±2.3, mean±SE). In 12 patients the SOD activity and in 5 patients with very high LPC the fatty acid composition of erythrocytes were studied.

Venous blood was withdrawn from patients before the administration of any drug interfering with the LPC, SOD activity, or fatty acid composition of erythrocytes. For erythrocyte analysis, blood was mixed with sodium citrate 3.8% (wt/vol) as an anticoagulant (1:4, vol/vol), and after centrifugation the plasma and the buffy coat were removed by aspiration. Erythrocytes were collected by centrifugation at 1000*g* after repeated washes in NaCl 0.9% (wt/vol) at 0°C to 4°C.

Lipid peroxide content and LPC were measured by the TBA color reaction.[11] The TBA-reactive material correlates well with the production of chemiluminescence, oxygen consumption, the loss of unsaturated fatty acids,[12] and the concentration of peroxides determined by other methods.[13]

Assay of the TBA-reactive lipid peroxide content of erythrocytes was performed under the optimum conditions for reproducibility as defined by Stocks and Dormandy.[11] This assay was elaborated specifically for erythrocytes and can be performed by measuring the difference in absorption between 532 and 600 μm as the basis for calculating MDA concentrations. The original method has been used in this laboratory with the following modification: The autoxidation of the erythrocyte lipids was induced by in vitro incubation of the erythrocytes under air at 37°C for 24 hours in a shaking water bath, rather than by a nonenzymatic breakdown induced by hydrogen peroxide. The cells (hematocrit, 0.10) were suspended in isotonic NaCl solution containing 10 mmol/L Veronal-Na-HCl buffer (pH 7.4). This procedure resulted in the diminution of reductive processes and the oxidation of cellular constituents and could therefore be considered an autoxidative test.

The lipid peroxide content of the erythrocytes was estimated before (LP_0) and after (LP_{24}) the autoxidative test; the results were expressed as nanomoles of MDA per gram of hemoglobin. The increase of the lipid peroxide content in the erythrocytes during incubation is the measurement of the LPC: $LPC = LP_{24} - LP_0$.

TABLE 1. Lipid Peroxidation Capacity of Erythrocytes

Subjects	n	nmol MDA/g Hb per 24 hr
Healthy subjects	22	1.45±0.30
Stroke patients	42	4.18±0.41†
High LPC, <4 nmol MDA/g Hb per 24 hr	20	2.45±0.17*
Very high LPC, ≥4 nmol MDA/g Hb per 24 hr	22	5.90±0.58†

LPC indicates lipid peroxidation capacity; MDA, malondialdehyde; and Hb, hemoglobin. Values are expressed as mean±SEM. LPC = $LP_{24} - LP_0$.

*Significant difference vs healthy subjects ($P<.05$).

†Highly significant difference vs healthy subjects ($P<.01$).

Hemolysis, extraction of hemoglobin by chloroform-ethanol precipitation, and hemoglobin estimation were carried out as described by Bartosz et al.[14] Aliquots of the final supernatants of the hemolysates were used for the measurements. SOD activity was assayed by its ability to inhibit the autoxidation of L-epinephrine at alkaline pH.[15] The activity of SOD was expressed relative to the hemoglobin content of the hemolysate.

The lipids for fatty acid analysis were extracted from the erythrocytes by an isopropanol-chloroform 11:7 (vol/vol) extraction without previous hemolysis.[16] Fatty acid methyl esters obtained by transmethylation of the probes in the presence of 5% HCl in absolute methanol at 80°C for 2.5 hours were separated on 10% DESS-PS coated onto Chromosorb W/AW, 100-120 mesh (Supelco) in 2-m-long stainless steel columns (internal diameter, 3 mm).

A dual-column flame ionization gas chromatograph (Hitachi 263) connected to a data processor (Hitachi 263-80) was used for segregation of the fatty acid methyl esters. The oven temperature was programmed from 140°C to 189°C with a rate of 1°C/min. Nitrogen gas was used as the carrier, and the flow rate was 50 μL/min. The fatty acid constituents were identified using accepted fatty acid methyl ester standards.

Data are presented as mean±SEM and were analyzed by one-way ANOVA. Statistical significance was assessed by multiple range tests including Scheffé's S test, the least significant difference test, and Tukey's honestly significant difference test. Statistical significance in these post hoc tests was at least $P<.05$. The study was approved by the ethics committee of the University Medical School of Debrecen.

Results

LPCs of erythrocytes of healthy subjects and stroke patients are shown in Table 1. These results demonstrate a moderate increase in the level of MDA in the erythrocytes of healthy subjects after the autoxidative test compared with a significantly higher ($P<.01$) increase in the erythrocytes of stroke patients.

The stroke patients can be divided into two groups on the basis of LPC of erythrocytes. A significant ($P<.01$) difference was found between the patients with erythrocytes with high LPC and those with very high LPC (Table 1).

Estimates of initial MDA levels in the erythrocytes of healthy subjects and patients are shown in Table 2. Interestingly, the lipid peroxide content of erythrocytes was estimated to be significantly ($P<.05$) lower in very-high-LPC patients compared with that of high-LPC patients. The lipid peroxide content of erythrocytes in very-high-LPC patients did not differ from that of healthy subjects.

TABLE 2. Lipid Peroxide Content of Erythrocytes

Subjects	n	nmol MDA/g Hb
Healthy subjects	22	16.6±0.7
Stroke patients	42	17.1±0.7
High LPC, <4 nmol MDA/g Hb per 24 hr	20	18.7±1.1*
Very high LPC, ≥4 nmol MDA/g Hb per 24 hr	22	15.6±0.7

LPC indicates lipid peroxidation capacity; MDA, malondialdehyde; and Hb, hemoglobin. Values are expressed as mean±SEM.
*Significant difference vs healthy subjects ($P<.05$).

The results of SOD determinations are summarized in Table 3. There was no significant difference in the erythrocytes of the patients compared with healthy subjects. The fatty acid composition in the erythrocytes of very-high-LPC stroke patients was determined before conducting the autoxidative test and was found to be generally normal; only the proportion of docosahexanoic acid (22:6 n-3) was markedly ($P<.01$) increased (Table 4).

Because lipid peroxidation may lead to breakdown of polyunsaturated fatty acids, it seems that the high level of docosahexanoic acid before the autoxidative test may be responsible for the increased rate of lipid peroxidation and the high sensitivity of erythrocytes against autoxidation in very-high-LPC stroke patients.

No correlation between the clinical symptoms and the LPC was found; however, the incidence of hypertriglyceridemia was higher in stroke patients with very high LPC than in those with high LPC.

Discussion

The increase of lipid peroxides in plasma has been considered a warning sign of vascular damage in TIA patients,[1,3] but a direct causal relationship between the plasma level of lipid peroxides and the cerebrovascular accident has not been found.[5] It is well known that the rigidity and the reduced deformability and filterability of erythrocytes play a decisive role in the pathogenesis of cerebrovascular dysfunctions.[6] Recently, biochemical investigations have been carried out to study the metabolic background of reduced hemorheological activity in erythrocytes of TIA patients.[7,8]

In our laboratory, a new method, the autoxidative test, has been formulated to simulate the special environment surrounding the erythrocytes in the microcirculation of cerebrovascular patients. Under the conditions of the autoxidative test, in vitro incubation of erythrocytes in a glucose-free medium mimics the transient starvation and peroxidative damage of cells observed in cerebral ischemia.[17,18]

TABLE 3. Superoxide Dismutase Activity of Erythrocytes

	n	U/g Hb
Healthy subjects	6	2900±710
Stroke patients	12	2680±800

Hb indicates hemoglobin. Values are expressed as mean±SEM.

TABLE 4. Fatty Acid Composition of Erythrocytes Before Conducting the Autoxidative Test in Healthy Subjects and Stroke Patients With Erythrocytes With Very High Lipid Peroxidation Capacity

Fatty Acids	Healthy Subjects (n=5)	Stroke Patients With Very High LPC (n=5)	P
16:0	27.5±3.7	25.8±3.6	NS
18:0	19.4±2.7	19.9±2.5	NS
18:1 n-9	17.2±1.8	17.2±3.0	NS
18:2 n-6	11.2±2.0	10.4±2.5	NS
20:4 n-6	18.9±2.6	16.4±1.6	NS
22:6 n-3	5.7±2.2	9.4±1.3	<.01

LPC indicates lipid peroxidation capacity. Values are expressed in percentages of all fatty acids evaluated, mean±SD.

The accelerated aging and reduced hemorheological activity of affected erythrocytes could be explained by the increased production of oxygen free radicals and the accumulation of lipid peroxides and MDA. It has been demonstrated that treating human erythrocytes with even small quantities of MDA induces membrane rigidity and reduces whole cell deformability.[19]

Red blood cell deformability, which is one of the risk factors of stroke, is dependent on the cholesterol-to-phospholipid molar ratio and membrane fatty acid composition.[20] The enhancement of lipid peroxidation in red blood cells has been observed to be accompanied by the significant loss of polyunsaturated fatty acids and cell deformability (Reference 21 and S.G.I., unpublished data, 1993).

Measurement of red blood cell deformability in cerebrovascular patients who have suffered previously from TIA has been performed in this laboratory, and a negative correlation has been found between LPC and rheological adaptability.[8] The present article deals with the biochemical background of reduced deformability observed in the erythrocytes of stroke patients. In this study, the results of the autoxidative test indicate that increased LPC and high sensitivity of erythrocytes against autoxidation exist not only in TIA but also in stroke patients.

The basal MDA content of erythrocytes from patients with an LPC <4 nmol MDA/g Hb per 24 hours was significantly higher than that of erythrocytes from control subjects. Interestingly, the basal MDA content of erythrocytes with a very high LPC did not differ significantly from that in control erythrocytes. Was the relatively lower basal MDA content associated with the higher LPC and docosahexanoic acid proportion?

The data from several studies suggest that docosahexanoic acid can be considered a pro-oxidant factor.[22-24] The predominance of pro-oxidant factors generally might reinforce the basal antioxidant defense of cells.[25] The fact that the autoxidation of lipids in the erythrocytes of control subjects has been estimated to be very limited suggests the presence of an extremely efficient protective antioxidant mechanism in the erythrocytes of healthy subjects.[26] The autoxidative test reflects a net sensitivity against autoxidation and depends not only on membrane fatty acid composition but also on the state of a selection of finely regulated, counterbalancing pro-oxidant and antioxidant factors.[27]

There is no apparent significant decrease of SOD activity in erythrocytes of the patients compared with the activity in those of healthy subjects. In this context, measuring the glutathion-dependent peroxidase activity and selenium or vitamin E content would have been relevant, even more than SOD measurement.[28] Studies of this sort are in progress in our laboratory.

It has been suggested that the susceptibility of membranes to peroxidation is affected by the proportion of polyunsaturated fatty acids in the membrane.[9,10,29] A variety of experiments performed with red blood cells from patients with various diseases indicate that all the polyunsaturated fatty acids or the sum of polyunsaturated fatty acids cannot be considered reliable markers of LPC.

Previously in this laboratory, a comparative study was carried out on the LPC and fatty acid compositon of neonatal calf and adult cattle erythrocytes. The sum of polyunsaturated fatty acids was higher in adult cells; however, the LPC was observed to be increased in calf erythrocytes with a higher proportion of arachidonic acid.[21]

Mainly, the proportions of arachidonic and docosahexanoic acids that are located in the inner leaflet of the membrane (in phosphatidylethanolamine and phosphatidylserine) influence the susceptibility of red blood cells to lipid peroxidation, whereas linoleic acid, the major unsaturated fatty acid of the outer leaflet (in phosphatidylcholine), is of minor importance.[22-24] Erythrocyte-membrane lipid peroxidation is thought to be initiated by oxygen radicals produced during the oxidation of cytoplasmic heme(II) iron.[23]

This is in good agreement with the recent finding of Urano et al[24] that significantly higher amounts of unsaturated fatty acids, arachidonic acid, and docosahexanoic acid are in the erythrocyte membranes of diabetic subjects. Reconstituted liposomes prepared from aged diabetic erythrocyte lipids are highly susceptible to superoxide-induced oxidative stress. Vitamin E has been found to be highly effective and SOD less effective in suppressing the peroxidative lysis of liposomes composed of diabetic erythrocyte lipids.

As results are expressed as mol %, the increase of n-3 fatty acids or at least of docosahexanoic acid is usually balanced by a very deep decrease in arachidonic (20:4 n-6) and linoleic (18:2 n-6) acids.[30] In the present study, a slight but not significant decrease was also reported.

Clemens et al[9] suggest that the content of polyunsaturated fatty acids in the membrane is determined by plasma fatty acids and that dietary variations may influence the susceptibility of red cells to lipid peroxidation in healthy subjects. The content of arachidonic acid in membranes of erythrocytes deficient in glucose-6-phosphate dehydrogenase (G-6-PD) has been found to be generally above normal. Fatty acid analysis of plasma, however, does not reveal significant changes between healthy subjects and G-6-PD–deficient subjects, which could explain the alteration of membrane fatty acid composition of erythrocytes.[22] Abnormalities in the fatty acid composition of plasma, erythrocytes, and platelets have been observed in cerebrovascular patients.[31]

In summary, our results suggest that the erythrocytes of ischemic stroke patients displaying an abnormal fatty acid composition are much more vulnerable to lipid peroxidation. The increased proportion of docosahexanoic acid and the high LPC of erythrocytes play a pathogenetic role and contribute to the hemorheological disturbances observed in the microcirculation of stroke patients. Quite recently, fatty acid analyses of erythrocytes of 12 stroke patients with very high LPC have been carried out in this laboratory (S.G.I., unpublished data, 1994). The results were identical, supporting the clinical importance of statistically significant changes obtained previously and published here.

With respect to patients with cerebrovascular disease, it is clearly of some importance to identify the initiating process responsible for the enhanced LPC of the erythrocytes. To clarify this question in our laboratories, further investigations are in progress.

Acknowledgments

This work was supported by the A.v. Humboldt Foundation, Germany, and by grant 1490 from the Hungarian Scientific Research Foundation (OTKA). For technical assistance we thank Maria T. Egri. For helpful discussions we gratefully acknowledge Dr M.R. Clemens.

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Stroke. 1994;25:2416-2420
doi: 10.1161/01.STR.25.12.2416
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0039-2499. Online ISSN: 1524-4628