Asian Journal of Chemistry Vol. 17, No. 1 (2005), 342–348

Effect of Smoking on the Composition of Erythrocyte Membrane Phospholipids

M. TOKER, N. YILMAZ* and M. AKTAŞ†
Department of Biology, Gaziosmanpaşa University, Tokat, Turkey

E-mail: necyilmaz@hotmail.com

In this study, the effect of cigarette smoking on the composition of erythrocyte membrane cholesterol and phospholipids was studied. Three groups were formed as control, working in cigaratee factory but not smoking and working in cigarette factory and smoking. The membrane phospholipids were separated from each other with thin layer chromatography (TLC). The phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and sphingomyelin (SM) amounts for each group were measured as per cent. The ratios for sphingomyelin/phosphatidyl choline and total cholesterol/total phospholipids were also determined in per cent. The results showed that the amounts of phosphatidyl serine and sphingomyelin with ratio for total cholestrol/total phospholipid, and for sphingomyelin/phosphatidyl choline (PC) were increased significantly in smokers compared to control group and non-smokers at $p < 0.05$ level. Phosphatidyl choline and phosphatidyl ethanolamine decreased significantly in smokers at the $p < 0.05$ level. Increases in the ratios for total cholesterol/total phospholipids and sphingomyelin/phosphatidyl choline in smokers may be attributed to the alteration effect of cigaratte on erythrocyte membranes of those taking place in this group.

Key Words: Smoking, Membrane phospholipids, Erythrocyte phospholipids.

INTRODUCTION

Erythrocytes have often been used in studies on the composition of plasma membranes because they are easy to obtain. The composition of erythrocyte phospholipids in the different mammalian species is different[1]. The major phospholipid classes of the human erythrocyte membrane are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and sphingomyelin (SM)[2]. The membrane phospholipids have been separated by thin-layer chromatography. But phospholipid amounts have been determined by phosphorus determination[3].

Free cholesterol, which seems to play a role in stabilizing membranes, is also an important component in erythrocyte membranes. Generally, cholesterol in erythrocyte membranes has been measured enzymatically[4, 5].

Phospholipids are distributed asymmetrically between the inner and outer leaflets of the normal cell membrane lipid bilayer. The phosphatidyl choline and

†Atatürk University, Erzincan Education Faculty, Department of Biology, Erzincan, Turkey.

sphingomiyelin predominantly in the external leaflet and the phosphatidyl serine and phosphatidyl ethanolamine located internally. This equilibrium can be disrupted by activation of the calcium-dependent scramblase enzyme[6].

The lipid composition of erythrocytes has been shown to be changed by many factors such as diet[7], environment[8] and various pathologial conditions[9]. However, there are a few reports on the effect of exercise on the erythrocyte membrane phospholipids composition. Erythrocytes have often been used to study possible membrane changes induced by oxidative stress. Since they are easier to obtain than cells from tissues. For functional and structural reasons, erythrocytes have been shown to be constantly exposed to both extracelluler and intracellular sources of free radicals[10], and to have antioxidant defense mechanism, protecting themselves against these free radicals. However, the capacities of these mechanisms would seem to be limited, because drug-induced oxidative stresses have been found to disturb the normal phospolipid distribution across the erythrocyte membrane lipid bilayer and may consequently alter the composition of phospholipids[3, 11]. Smoking causes numerous abnormalities of phospholipid metabolism and catabolism, including dose-response effects on serum lipids, including triglycerides and phospholipid dense LDL and HDL concentrations, which have been clearly documented with most of the studies[12].

In the present work, we wish to report the effect of cigarette smoking on the composition of erythrocyte membrane cholesterol and phospholipids.

EXPERIMENTAL

Blood sampling

All blood samples were supplied from each of sixty volunteers between 24–37 years of age. After a 12 h fast venous 10 mL of blood samples were drawn into tubes and anticoagulated with 0.10 mL solution containing 30 g Na_2EDTA per 100 mL. Each blood sample obtained from smokers and non-smokers was chilled immediately at 0°C and then centrifuged at 4°C. The plasma was removed, kept at 4°C and extracted within 2 h after withdrawal of the blood sample.

Preparation of erythrocyte membranes

Blood sample was collected in a glass tube with ethylenediamine tetraacetic acid (EDTA). The plasma and buffy coat layers were separated by centrifugation at $2280 \times g$ for 30 min and red cells were obtained from blood. Erythrocyte membranes were prepared as follows: Erythrocytes were washed three times by resuspension in five volumes of 0.9% isotonic saline and centrifugation. Residual buffy coats were removed after each wash. One volume of washed erythrocytes was hemolyzed by adding two volumes distilled water and red cells were counted in a Coulter T-600. The majority of packed erythrocytes were frozen for membrane cholesterol and phospholipid determination at 20°C[13].

Extraction of phospholipids from erythrocyte membranes

The extraction of phospholipids was carried out with isopropanol. 0.5 g of membrane solution was added to 10 mL of isopropanol . The mixture was filtered

and the residue in the filter was washed twice with 10 mL of chloroform/isopropanol (9 : 14 v/v). The extract was evaporated to dryness and residue consisting of membrane lipids dissolved in chloroform was made up to 5 mL and used for phospholipids analysis. The solvents used in all the experiments contained 10 mg 2,6-di-*tert*-butyl-*p*-cresol as oxidant in order to avoid oxidative degradation during extraction[14].

Thin-layer chromatographic separation of erythrocyte membrane phospholipids

To determine the concentration of the individual neutral lipids, it was chromatographed with an aliquot of lipid extract on a 0.25 mm layer of silica gel with hexane-diethyl ether-glacial acetic acid 80 : 20 : 1 containing 50 mg of BHT per 100 mL to prevent auto-oxidation during chromatography. The plate was sprayed with 2,7-dichloro-fluorescein and the cholesterol, cholesteryl ester and triglyceride spots, which were completely separated, were each scrapped off and eluted three times with 2 mL aliquots of methanol-chloroform 1 : 2. The phospholipid composition was determined in triplicate by TLC of aliquots of the lipid extract on silica gel 0.25 mm thick, in chloroform-methanol-glacial acetic acid-water 25 : 15 : 4 : 2 containing 50 mg of BHT per 100 mL. The TLC plates were prepared with water. The scrapped off spots were digested directly for phosphrorus determination as described[15].

Total membrane phosphorus was determined in triplicate by Barlett's method[16].

The cholesterol of erythrocyte membrane was measured by cholesterol oxidase method[17].

RESULTS AND DISCUSSION

The effect of smoking on erythrocyte membrane phospholipids, cholesterol, total cholesterol/total phospholipids and sphingomyelin/phosphatidyl choline ratios were compared in three groups.

To compare the results of lipid values, analysis of variance test was used and to determine the difference among the groups, Duncan test was employed.

The values for erythrocyte membrane cholesterol, total phospholipids and phospholipid fractions, belonging to smokers and non-smokers working for cigaratte factories and control group are given in Table-1.The values for the ratio total cholesterol/total phospholipids and sphingomyelin/phosphatidyl choline of control group, smokers and non-smokers are given in Table-2.

The distribution of the phospholipid fractions in smokers was determined as percentage: PC: 30.75 ± 1.43, PE: 24.59 ± 2.55, PS: 18.67 ± 2.99, SM: 26.14 ± 1.25. Membrane TC and TPL levels were found as 1.48 ± 0.10 mg per 10^{10} cells and 3.41 ± 0.31 mg per 10^{10} cells. In non-smokers and in the control group, these values were determined respectively as 33.14 ± 1.74, 29.64 ± 2.29, 14.19 ± 1.04, 22.89 ± 1.51, 33.44 ± 1.82, 29.56 ± 2.19, 14.38 ± 1.01 and

22.71 ± 1.21. In non-smokers and control group, membrane TC and TPL values were found respectively as 1.19 ± 0.98, 3.14 ± 0.16, 1.16 ± 0.09 and 3.09 ± 0.26 mg per 10^{10} cell.

It is noticed from Tables 1 and 2 that the total membrane cholestrol, total membrane phospholipid, phospholipid fraction values increased significantly for smokers ($p < 0.05$). Total cholesterol and total phospholipid levels in the erythrocytes were higher in the non-smoking group than in the conrol group but did not change significatly. On the other hand, phosphatidyl choline and phosphatidyl ethanolamine values decreased significantly at level of $p < 0.05$. No differences were detected significantly among these parameters for non-smokers and control groups. The ratio of total cholesterol to total phospholipid (TC : TPL) increased significantly in smokers. But this ratio was not changed in other groups.

TABLE-1
ERYTHROCYTE MEMBRANE CHOLESTROL AND PHOSPHOLIPID CONTENT IN SMOKERS, NON-SMOKERS AND CONTROL GROUP

Lipid	Control group		Smoking group		Non-smoking group	
	Mean	SD	Mean	SD	Mean	SD
TC	1.16	0.09	1.48	0.10*	1.19	0.09
PE	29.56	2.19	24.59	2.55*	29.64	2.29
PS	14.38	1.01	18.67	2.99*	14.19	1.04
PC	33.44	1.82	30.70	1.43*	33.14	1.74
SM	22.71	1.21	26.14	1.25*	22.89	1.51
TPL	3.09	0.26	3.41	0.31*	3.14	0.16

TC, Total cholestrol; PE, Phosphatidyl ethanolamine; PS, Phosphatidyl serine; PC, Phosphatidyl choline; SM, Sphingomyelin; TPL, Total phospholipid.
Values are percentages of mean mass: * $p < 0.05$. The total cholesterol and total phospholipid are expressed as mg 10^{10} cell.

TABLE-2
RELATIVE CHOLESTOROL AND PHOSPHOLIPID COMPOSITIONS OF ERYTHROCYTE MEMBRANES IN SMOKING, NON-SMOKING, AND CONTROL GROUP

Lipid	Control group		Smoking group		Non-smoking group	
	Mean	SD	Mean	SD	Mean	SD
TC/TPL	0.380	0.030	0.438	0.049*	0.380	0.030
SM/PC	3.680	0.060	0.850	0.050*	0.690	0.060

In this study, the effects of smoking on erythrocyte membrane cholesterol and major phospholipids was examined.

In present study, the PC and PE levels in the erythrocyte membranes were significantly decreased in smokers. PC and PE values in the erythrocyte membranes did not change significantly in the control group and non-smokers.

This change may be due to lipid peroxidation, because, cigarette smoking increases phospholipase A_2 activity. Smoking increases phospholipase A_2 activity through at least two mechanisms: (1) Free radicals from cigarette smoke peroxidize vulnerable polyunsaturated fatty acids, which are cleaved and repaired by a variety of cytosolic and membrane phospholipase A_2[18,19]. (2) Nicotine and cotinine directly activate phospholipase A_2 in retina[20] and placenta[21].

On the other hand, PC may exchange between plasma and erythrocyte membrane. Exchange of peroxidized PC in erythrocyte membranes with plasma PC may explain the absence of a difference in the fatty acid composition of the membrane PC in smokers. Possible exchange of PC between erythrocytes and plasma may be due to smoking[10]. The decrease in PC and PE values would be due to conversion of phosphatidyl choline and phosphatidyl ethanolamine into phophatidyl serine[22].

In this study, the values for sphingomyelin and phosphatidyl serine increased significantly for smokers compared to non-smokers and control groups. When the phospholipids of erythrocyte membrane were studied in the control group by different researchers, it was found that the phosphatidyl serine amount was found approximately half of the other phosholipid amounts. On the other hand, sphingomyelin was found in relatively lesser amount compared to other phospholipids, except phosphatidyl serine. In normal subjects, the PS amounts were determined respectively as 14.8, 13.1, 13.0 and 12.7% by Philips and Dodge[15], Neerhout, Rouser *et al.* and Broekhyuse. Among these researchers, only the results of Brookhyuse were lower than others. The sphingomyelin values were 25.5, 24.1, 26.9 and 25.8%, respectively[23]. In the present study, the values of phosphatidyl serine and sphingomyelin for control group were 14.38 ± 1.01 and 22.71 ± 1.21% respectively. The amounts of phosphatidyl serine and sphingomyelin occurred lower than fractions of other phospholipids.

Total cholesterol and total phospholipid amounts were found as 1.48 ± 0.10 mg/10^{10} cells, 3.41 ± 0.31 mg/10^{10} cells in smokers and these values were significantly at level $p < 0.05$. But, in other groups also, similar results were obtained. In a study, the total phospholipid values were found 148 ± 3.48 mg/dL in the serum from mice treated with nicotine and 131.0 ± 3.27 mg/dL in the serum of control group. In this study, the difference was observed significant at the level of $p < 0.01$.[24]

The length and unsaturation of the phospholipid acyl chains, structures of phospholipid classes and the ratios of cholesterol/phospholipid, sphingomyelin/phosphatidyl choline have an important role in membrane fluidity. The increase in the ratios SM/PC and TC/TPL decreases the membrane lipid fluidity[25-28]. In our study, the ratios for sphingomyelin/phosphatidyl choline and cholesterol/phospholipid for control group were found as 0.68 ± 0.06, 0.38 ± 0.03, respectively. These values were found as 0.69 ± 0.06 and 0.38 ± 0.03 for non-smokers working in cigarette factories and 0.85 ± 0.05 and 0.438 ± 0.049 for

smokers. Our results show that the ratio of TC/TPL in the erythrocyte membranes of smokers was found to increase. The ratio of SM to PC also increased in smokers. Therefore, smoking would appear to decrease membrane fluidity[29].

Conclusion

The lipid components of membranes have an important role in vital functional systems such as prostaglandin synthesis, cation transport systems and signal transfer in biological systems and the composition of membrane lipids are affected by alterations occurring in plasma lipids.

The alteration in membrane phospholipid levels causes changes in erythrocyte characteristics[30].

In intact erythrocytes, all the PS and most of the PE are confined to the inner leaflet of the membrane bilayer, whereas PC and SM have been found to be predominantly localized in the outer leaflet[31]. Both *in vitro* and *in vivo* oxidative stress in erythrocytes has been shown to change the phospholipid distribution, only PS[11] or both PS and PE[3] moving from the inner bilayer to the outer bilayer. As the morphology of erythrocytes has been found to be dependent on the transbilayer distribution of incorparated phospholipids[32]. The changes in phospho lipid distribution induced by oxidative stress during smoking may affect erythrocyte shape. According to our results we can say that the membrane fluidity of erythrocytes of smokers would be different from others.

REFERENCES

1. R.M.C. Dawson, N. Hemington and D.B. Lindsay, *Biochem. J.*, **77**, 226 (1967).
2. G.B. Philips, J.T. Dodge and C. Howe, *Lipids*, **4**, 544 (1969).
3. S.K. Jain, *Biochim. Biophys. Acta*, **937**, 205 (1988).
4. P. Baldini, S. Incerpi, S. Lambert-Gardini, A. Spinedi and P. Luli, *Diabetes*, **38**, 825 (1989).
5. M. Garnier, J.R. Attali, P. Valensi, E. Delatour-Hanss, F. Gaudey and Koutsorus, *Metabolism*, **39**, 794 (1990).
6. R.F. Azwaal and A.J. Schroit, *Blood*, **89**, 1121 (1997).
7. J.J. Agren, H. Pekkarinen, H. Litmanen and O. Hanninen, *Eur. J. Appl. Physiol.*, **63**, 393 (1991).
8. P.J.C. Kuiper, A. Livne and N. Meyerstein, *Biochim. Biophys. Acta*, **248**, 300 (1971).
9. Y. Okano, T. Yamauchi, T. Sekiya, H. Iida and I. Hasegawa, *Clin. Chim. Acta*, **88**, 237 (1978).
10. D. Chiu, F. Kuypers and B. Lubin, *Semin. Hematol.*, **26**, 257 (1989).
11. A. Arduini, A. Stern, S. Storto, M. Belfiglio, G. Mancinelli, R. Scurti and G. Federici, *Arc. Biochem. Biophys.*, **273**, 112 (1989).
12. W.Y. Craig, G.E. Palomaki and J.E. Haddow, *BMJ*, **298**, 784 (1989).
13. H.G. Rose and M. Oklander, *J. Lipid Res.*, **6**, 428 (1965).
14. E. Peuchent, R. Wolf, C. Salles and R. Jensen, *Anal. Biochem.*, **181**, 341 (1989).
15. G.B. Phillips and J.T. Dodge, *J. Lipid Res.*, **8**, 676 (1967).
16. G.R. Barlett, *J. Biol. Chem.*, **234**, 466 (1959).
17. H.K. Naito, in: L.A. Kaplan and A.J. Pasce (Eds.), Clin. Chem. Theory, Analysis and Correlation, Mosby Company, Missouri, pp. 968–1004 (1989).
18. J.K. Beckman, S.M. Borovits and I.M. Burr, *J. Biol. Chem.*, **262**, 1479 (1987).
19. S. Nigam and T. Schewe, *Biochem. Biophys.*, **488**, 167 (2000).

20. B.V. Sastry and M.E. Hemontolor, *J. Ocul. Pharmacol. Ther.*, **14**, 447 (1998).
21. B.V. Sastry, M.B. Change, M.E. Hemontolor and T.A. Goddijn-Wessel, *Pharmacogol. Ther.*, **57**, 104 (1998).
22. G. Szymanka, A. Max, I. Melzner, M.G. Sarzala and R. Rüdel, *Gen Physiol. Biophys.*, **5**, 179 (1986).
23. L.L.M. Van Denen and J. Gier, in: D.M. Surgener (Ed.), The Red Blood Cell, Academic Press, New York, pp. 148–214 (1974).
24. M.S. Latha, P.L. Vijayammaland P.A. Kurup, *Indian J. Med. Res.*, **98B**, 44 (1993).
25. T.A. Hagve, Q. Lie and M. Gronn, *Scand. J. Clin. Lab. Invest.*, **44**, 39 (1993).
26. C. Popp-Snijder, J.A. Schouten, A.P. Dejong and E.A. van der Veen, *Scand. J. Clin. Lab. Invest.*, **44**, 39 (1984).
27. E. Berlin, S.J. Bhathena, J.T. Judd, P.P. Nair, D.Y. Jones and P.R. Taylor, *Metabolism*, **38**, 790 (1989.)
28. M. Shinitzky and Y. Bareenholz, *Biochim. Biophys. Acta*, 515, 367 (1978).
29. P.J. Talmund and S.E. Humpries, *Curr. Opin. Lipidol.*, **12**, 405 (2001).
30. J.S. Owen and N. McIntyre, *Biochem. Biophys. Acta*, **510**, 168 (1978).
31. A.J. Werkleij, R.F.A. Zwaal, B. Roelofsen, P. Compturius, D. Kasteliin and L.L.M. Van Denen, *Biochem. Biophys. Acta*, **323**, 178 (1973).
32. D.L. Daleke and W.H. Huestis, *J. Cell. Bio.*, **108**, 1375 (1989).

(*Received*: 26 March 2004; *Accepted*: 25 August 2004) AJC-3534

NANOTECHNOLOGY: SCIENCE AND APPLICATION
NANOTECH INSIGHT

20–25 FEBRUARY, 2005

Contact:
Photoenergy Center
Ain Shams University
Abbassia, 11566 Cairo, Egypt
Email contact@nanoinsight.net
URL http://www.nanoinsight.net