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α- Tocopherol Binding Activity of Red Blood Cells in Smokers

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Despite high plasma levels of vitamin E, red blood cell membranes contain relatively low levels of vitamin E. This suggests the existence of a selective vitamin E uptake/regeneration system in human red blood cell membranes. α-Tocopherol binding sites on human red blood cells are thought to be involved in the uptake of α-tocopherol from the plasma. To understand the role of the uptake system we have compared the α-tocopherol content and binding activity of red blood cells from smokers and non-smokers. The specific binding of [^{3}H]α-tocopherol to pure red blood cell preparations from smokers (n = 7, 28.4 ± 2.8 years) was 30.6 ± 3.2 fmoles per 3×10^8 red blood cells and for non-smokers (n = 17, 27.9 ± 1.3 years) was 41.7 ± 3.7 fmoles per 3×10^8 red blood cells. Thus α-tocopherol uptake activity was significantly lower in smokers ($P = 0.05$). Red blood cells from smokers contained less (1.8 ± 0.4 μg/gHb) α-tocopherol than non-smokers (2.8 ± 0.3 μg/gHb), ($P < 0.05$), despite plasma levels of α-tocopherol being similar: 12.9 ± 0.8 μM in non-smokers vs. 12.7 ± 0.5 μM in smokers. However, adjusting plasma α-tocopherol for total plasma cholesterol plus triacylglycerols showed α-tocopherol levels were higher ($P < 0.01$) in non-smokers (2.84 ± 0.10 μmol α-tocopherol/mmol [cholesterol+triacylglycerol]) than in smokers (2.36 ± 0.11 μmol α-tocopherol/mmol [cholesterol+triacylglycerol]). The reduced α-tocopherol levels in red blood cells from smokers may be due to impairment of α-tocopherol uptake activity. The reduced levels of α-tocopherol in smokers red blood cells was not associated with any changes in cell membrane fluidity. At present it is not known whether supplementation of smokers with vitamin E would normalise the α-tocopherol uptake activity of red blood cells.

Keywords: Smoking, α-tocopherol uptake, red blood cells, α-tocopherol, membrane fluidity

Abbreviations used: BHT, butylated hydroxytoluene; BMR, Basal metabolic rate; Hb, Haemoglobin; HPLC, High pressure liquid chromatography; HDL, High density lipoproteins; LDL, Low density lipoproteins

INTRODUCTION

Vitamin E (RRR-tocopherol) is the major lipid-soluble antioxidant in human red blood cell membranes, where it plays an important role in the suppression of free radical-induced lipid peroxidation.[1,2] Hence, the regulation of α-tocopherol concentrations in membranes is critically important in maintaining red blood cell membrane structure and function. During their relatively short life, in which no protein synthesis occurs, human red blood cells come in contact

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with free radicals produced from many sources, e.g. oxidised haemoglobin, drugs, etc., which ultimately can produce very reactive superoxides.[3] Efficient defence systems in human red blood cells are located in both the cytosol and membrane. The membrane itself contains vitamin E as the major, if not only, lipid soluble chain breaking antioxidant.[4] Although the level of vitamin E in the human red blood cell membrane is relatively low compared with plasma levels,[4] the normal red blood cell membranes from healthy volunteers do not contain any significant amounts of lipid peroxides when freshly isolated. This suggests the existence of a selective system for vitamin E uptake and/or regeneration in red blood cell membranes.[5] Vitamin E deficiency leads to increased membrane fragility and decreased cell survival time under conditions of oxidative stress—as happens in premature babies. The control of cellular uptake of vitamin E is therefore a potentially important step in the regulation of vitamin E metabolism. In fact, the existence of the α-tocopherol uptake system in several cell types, such as human red blood cell, adrenocortical cells, endothelial cells and liver membranes has been reported.[6-10] However, no information is available on the uptake activity in oxidative stress conditions such as smoking. Cigarette smoking can induce various deleterious effects on cellular systems by perturbing both plasma and cellular antioxidant systems.[11,12] The increased tendency of erythrocytes of smokers to peroxidise was reported despite smokers having similar plasma vitamin E levels as in non-smokers.[12-14] The increased tendency of erythrocytes of smokers to peroxidise can be abolished by supplementation with huge amounts of α-tocopherol[13] indicating that smokers may have a greater requirement for vitamin E than non-smokers. It is possible that impairment of the vitamin E uptake system in red blood cells may be partly responsible for enhanced susceptibility to peroxidation in smokers as these cells would accumulate less than adequate amounts of vitamin E despite having similar plasma levels of vitamin E as non-smokers. However no information is available regarding the effects of cigarette smoking on vitamin E uptake by red blood cells of smokers. To address this issue we are currently investigating the α-tocopherol uptake activity of human red blood cells in smokers.

In this paper, our data demonstrate that red blood cells have lower α-tocopherol uptake activity in smokers compared with the non-smokers, and that the reduced uptake activity was associated with the lower levels of α-tocopherol accumulation in membranes which may give rise to an increased susceptibility to lipid peroxidation.

MATERIALS AND METHODS

Materials

D-α-[^{3}H]Tocopherol (specific activity 55Ci/mmol) was purchased from Amersham International (Amersham, U.K). Unlabelled D-α-tocopherol was kindly supplied by Henkel Corporation, USA. Dibutyl phthalate was obtained from Sigma, Poole, UK. Hionic fluor and soluene-350 were purchased from Packard, USA. All other reagents used were of analytical grade quality. Purity of α -[^{3}H]Tocopherol was monitored routinely by using high performance liquid chromatography (HPLC) throughout the study.

Preparation of Pure Red Blood Cells

Blood collected from age and sex matched smokers (n = 7, 28.4 ± 2.4 years) and non-smokers (n = 17, 27.9 ± 1.3 years) after an over-night fast was used in this study to investigate the α-tocopherol uptake activity of red blood cells. Blood was collected in sodium citrate (final concentration, 13 mM) as an anti-coagulant. Immediately after collection, blood was centrifuged at 180 × g for 15 min. and the plasma was removed by aspiration. The remaining red blood cell concentrate was made up to the original volume with phosphate buffered saline (PBS), pH 7.4, and spun at 3000 × g for 10 min. at 4°C. The red blood cell concentrate was then passed through a cellulose: α-cellulose (1:1,w/w) column to

remove residual platelets and white blood cells.[15] The red blood cell preparation was then spun again at 3000 × g at 4°C and the upper layer was removed. The concentrate was washed once again with PBS buffer. Finally, PBS was added to red blood cell concentrate to produce an haematocrit of about 30%. The purity of the red blood cell preparation was routinely checked for the presence of other blood cells using a Serono-Baker automated cell counter (Serono-Baker diagnostic Inc., USA).

[^{3}H]α-Tocopherol Binding Assay

[^{3}H] α-tocopherol binding activity of the pure red blood cell preparation was measured according to a previously published method[6] but with extensive modifications.[16] Binding characteristics of α-tocopherol with these cells were described before by us.[16] Typically, 300×10^6 cells were incubated with 20 nM [^{3}H]α-tocopherol in 500μl at 4°C for 2h with regular shaking. Incubations were performed in triplicate in 25 mM Tris-HCl, pH 7.4 containing 5 mM glucose, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 1 mM EDTA and 1% bovine serum albumin in a total volume of 500μl. [^{3}H]α-Tocopherol in alcohol was added with the final concentration of the latter in all assays at 0.4%. After the incubation, the mixture was carefully placed on a layer of dibutyl phthalate and assay buffer and was centrifuged at 12,000 × g for 3 min. at 4°C. The red cell pellet was then solubilised in soluene-350: propan-2-ol (1:1,v/v) and oxidised with 30% H_2O_2. The scintillant hionic fluor was added and the radioactivity measured. Since only about 1% of the added [^{3}H]α-tocopherol bound to red blood cells, the concentration of the free ligand was essentially constant throughout the incubation. Similar phenomenon was also observed with the binding of prostaglandin E_1 to human erythrocyte membranes.[17] Although this phenomenon is common to most of the biological receptors, the physiological significance is not clearly understood.

Parallel experiments were run using a 600-fold excess unlabelled α-tocopherol in the above incubation mixture to determine the non-specific binding. The value was subtracted from the total α-tocopherol binding to calculate the specific binding.

Determination of Tocopherols, Carotenoids and Retinol in Red Blood Cells and Plasma

Tocopherols (α and γ), β-carotene and retinol in red blood cells and plasma were determined according to Hess et al.[18] Briefly, 1 ml of pure red blood cells from smokers or non-smokers were mixed with 1 ml of 25% ascorbic acid and 2 ml of ethanol and the mixture was incubated at 70°C for 10 min. Then 2 ml of 10 M KOH was added to the mixture and further incubated for 30 min. at 70°C. 1.2 ml hexane containing butylated hydroxytoluene (BHT) was then added, the solutions shaken thoroughly and the upper hexane layer transferred to a microfuge tube. Retinyl palmitate as an internal standard was added to the hexane together with 400 μl of distilled water. The solutions were mixed thoroughly and centrifuged at 6000 × g for 5 minutes. The hexane layer was dried down in a speed vac for about 10 min. without being allowed to dry completely. The sample was reconstituted in 200 μl DEA [20% (v/v) 1,4 dioxan, 20% (v/v) ethanol, 60% (v/v) acetonitrile] and shaken for 10 min. before application to the HPLC column. Beckman Ultrasphere ODS 5 μm (25 cm × 4.6 mm I.D.) in a column oven set at 28°C was used. The flow rate was 1.5 ml/min and the injection volume was 100 μl. The run time was 18 minutes. The mobile phase for the column for a total volume of 1 L was: acetonitrile (684 ml), tetra-hydrofuran (220 ml), methanol (68 ml), 1% ammonium acetate (28 ml) and 2% BHT (25 ml). α and γ-tocopherols were detected by fluorescence emission at 295 nm whereas β-carotene, retinol and retinyl palmitate were detected using a UV spectrophotometer.

For the determination of plasma tocopherols, carotenoids and retinol, 200 μl of plasma was mixed with 200 μl of water and 400 μl of ethanol containing retinyl palmitate as internal standard

and vortexed for 10 seconds. 800 µl of hexane (containing 0.05% BHT) was added, the microtube shaken for 10 min. and then centrifuged for 5 min. at 6000 × g. The hexane layer was removed and dried. The sample was then reconstituted in 200 µl of DEA and shaken for 5–10 min. before application to the HPLC column which was used to measure the tocopherols, β-carotene and retinol levels in plasma as described above for red cells.

Plasma levels of vitamin C were determined according to the method as described before.[19] Briefly, the plasma samples were centrifuged at 10,000 rpm for 10 min. at 4°C and the supernatant was used for vitamin C determination by HPLC using a Nucleosil (ODS 5 µm 25 cm × 4.6 mm I.D.) (A. Jones Chromatography, U.K.) column. The mobile phase was made up of sodium acetate buffer, pH 5.0 containing EDTA, homocysteine, acetonitrile with a flow rate of 0.55 ml/min. Ascorbic acid was detected at 263 nm.

Determination of Plasma Lipids and Lipoprotein Fractions

It is generally agreed that lipid-standardization is a prerequisite for interpretation of plasma vitamin E levels.[20] α-Tocopherol standardized for cholesterol and triacylglycerols is known to reflect the plasma vitamin E status almost as powerfully as the previously suggested α-tocopherol/total lipid ratio and slightly better than the α tocopherol/cholesterol ratio.[20] In this study individual plasma tocopherol levels were standardized for cholesterol and cholesterol plus triacylglycerols. The cholesterol content (mmol/l) of all lipoprotein fractions and plasma triacylglycerol was determined as described before.[21] LDL cholesterol was then calculated from plasma cholesterol, triacylglycerol and HDL cholesterol by using the Friedwald formula.[22]

Determination of Red Cell Membrane Microviscosity

Erythrocyte membrane fluorescence polarisation was determined according to Dutta-Roy *et al.*.[23] Typically, the haemoglobin free white ghosts (50 µg protein) in 50 mM Tris-HCl buffer, pH 7.4 containing 5 mM $MgCl_2$ were labelled with a fluorescence probe by incubating an equal volume of 2 µM 1,6-diphenyl-1,3,5-hexatriene (DPH) dispersion in the same buffer for 1 hour at 23°C. The steady-state fluorescence polarisation was measured in a Perkin-Elmer Luminescence spectrophotometer (LS-5B) fitted with a polariser accessory. Excitation and emission wavelengths were 358 and 430 nm, respectively. Excitation and emission slits were 5 nm. The steady-state fluorescence polarisation, P, was calculated from the equation (i):

$$P = \frac{I_{VV} - G.I_{VH}}{I_{VV} + G.I_{VH}} \quad \text{(i)}, \quad G = \frac{I_{HV}}{I_{HH}}$$

where I_{VV}, and I_{VH} are the fluorescence intensities recorded with the analysing polariser oriented, respectively, parallel and normal to the vertically oriented of the polarised excitation beam. G is the grating correction factor. I_{HV} and I_{HH} are the fluorescence intensities determined with the emission polariser vertically and horizontally when the excitation polariser was set in the horizontal position. Light scattering errors were minimised by assuring that measured anisotropies were concentration-independent. The steady-state fluorescence anisotropy (r_s) was obtained from the equation (ii).

$$r_s = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2G.I_{VH}} = \frac{2P}{3 - P} \qquad \text{(ii)}$$

The fluorescence background of a blank sample (unlabelled membranes) never exceeded 15% of the fluorescence signal of membranes labelled with DPH.

Statistical Analysis

Differences between the smokers and non-smokers were assessed by means of Students *t* test. Differences were considered significant when the P value ≤ 0.05. Results are expressed as means ± SEM.

TABLE I Baseline characteristics of smokers and non-smokers

Characteristics	Non-smokers n = 17	Smokers n = 7	P value
Age (y)	27.9 ± 1.3	28.4 ± 2.83	ns
Weight/height^2 (Kg/m^2)	25.2 ± 0.8	24.9 ± 0.75	ns
Cigarettes smoked/day	—	~20	
BMR (kcal/day)	1716.5 ± 29.9	1828.8 ± 47.3	0.05
Cholesterol (mmol/l)	3.84 ± 0.24	4.21 ± 0.22	ns
Triacylglycerols (mmol/l)	0.76 ± 0.11	1.21 ± 0.22	0.02
HDL cholesterol (mmol/l)	0.81 ± 0.06	0.63 ± 0.08	0.04
LDLcholesterol (mmol/l)	2.69 ± 0.19	3.03 ± 0.21	ns

Experimental conditions were as described in the Methods section. Values given as mean ± SEM. Differences between the groups tested by Student's t-test.

RESULTS

Table I shows the base line characteristics of smokers (n = 7) and non-smokers (n = 17) used in this study. No significant differences in plasma total cholesterol and LDL cholesterol between these two groups were observed. However, differences in BMR, plasma levels of triacylglycerol and HDL-cholesterol between smokers and non-smokers reached statistical significance. Plasma HDL-cholesterol was lower in smokers (0.63 ± 0.08 mmol/l) compared with non-smokers (0.81 ± 0.06 mmol/l, P < 0.04) whereas the reverse was true for plasma triacylglycerol levels (1.21 ± 0.22 mmol/l for smokers vs. 0.76 ± 0.11 mmol/l for non-smokers, P < 0.02).

Cigarette smoking was associated with alterations of antioxidants status both in plasma and in red blood cells.[11,12] Table-II shows the various antioxidants concentrations in smokers and non-smokers in this study. The plasma levels of α-tocopherol were similar both in smokers (12.65 ± 0.46 μM) and non-smokers (12.88 ± 0.81 μM). After adjusting for total plasma cholesterol plus triacylglycerols these values were significantly different (P < 0.01) with non-smokers having higher levels (2.84 ± 0.10 μmol α-tocopherol/mmol [cholesterol + triacylglycerol]) than

TABLE II Plasma antioxidant concentrations in smokers and non-smokers

Antioxidant	Non-smokers n = 17	Smokers n = 7	P value
α-tocopherol (μmol/l)	12.88 ± 0.81	12.65 ± 0.46	ns
α-tocopherol/cholesterol (μmol/mmol)	3.38 ± 0.11	3.05 ± 0.16	0.055
α-tocopherol/cholesterol+ triacylglycerols (μmol/mmol)	2.84 ± 0.10	2.36 ± 0.11	0.006
γ-tocopherol (μmol/l)	0.87 ± 0.09	1.07 ± 0.21	ns
γ-tocopherol/cholesterol (μmol/mmol)	0.23 ± 0.02	0.27 ± 0.06	ns
γ-tocopherol/cholesterol+ Triacylglycerols (μmol/mmol)	0.19 ± 0.02	0.21 ± 0.05	ns
β-carotene (μmol/l)	0.36 ± 0.04	0.19 ± 0.01	0.005
retinol (μmol/l)	1.29 ± 0.06	1.38 ± 0.08	ns
retinol/cholesterol (μmol/mmol)	0.36 ± 0.02	0.34 ± 0.03	ns
vitamin C (μmol/l)	48.57 ± 3.84	23.97 ± 5.770	.001

Experimental conditions were as described in Methods section. Values given as mean ± SEM, Differences between the groups tested by Student's one-tailed t-test.

smokers (2.36 ± 0.11 μmol α-tocopherol/mmol [cholesterol + triacylglycerol]). Plasma levels of β-carotene were significantly lower ($P < 0.005$) in smokers (0.19 μmol/l) than in non-smokers (0.36 ± 0.04 μmol/l). Similarly, plasma concentrations of vitamin C were also significantly lower in smokers (23.97 ± 5.97 μmol/l) compared with non-smokers (48.57 ± 3.84 μmol/l) $P < 0.001$. The range of plasma concentrations of vitamin C in smokers was 3.7–39.8 μmol/l whereas 28.1–62.5 μmol/l in non-smokers.

In order to examine the effect of smoking on the α-tocopherol uptake activity of red blood cells, binding of [^{3}H]α-tocopherol to pure red blood cells preparations from smokers and non-smokers was determined. Red blood cells prepared by the above method were found to be devoid of any white blood cells and platelets. The specific binding of α-tocopherol to red blood cells was 30.6 ± 3.2 fmoles per 3×10^8 red blood cells for smokers, and 41.2 ± 3.7 fmoles per 3×10^8 red blood cells for non-smokers, ($P = 0.05$).

To determine whether reduced α-tocopherol uptake activity of red blood cells resulted in less accumulation of α-tocopherol, concentrations of α-tocopherol in these cells from smokers and non-smokers were measured. The pure red blood cells of smokers contained lower concentrations (1.80 ± 0.28 μg/gHb) of α-tocopherol compared with non-smokers (2.79 ± 0.30 μg/gHb), $P < 0.05$, which may reflect the results of reduced α-tocopherol uptake activity in these cells. However, because of large variability in the binding of α-tocopherol and its content in red blood cells both in smokers and non-smokers, no significant correlation was observed between these two measures ($r = 0.4$ for smokers and non-smokers).

However despite the reduced amount of α-tocopherol in red blood cells, fluorescence polarisation of the cell membranes showed no significant differences were observed in the values for membrane fluorescence anisotropy (r_s) viz. smokers 0.292 ± 0.003, $n = 14$ for smokers and smokers 0.290 ± 0.004, $n = 18$ for non-smokers.

DISCUSSION

An important area of vitamin E research concerns the quantitative assessment of the need for this antioxidant system to protect the body from free radical-induced damage. However, it is impossible to develop biologically relevant estimates of vitamin E requirements until we have a better understanding of the mechanisms responsible for its extracellular and intracellular transport both in physiological conditions and in oxidative stress.[10,24] Several studies have shown that there were no differences in plasma α-tocopherol levels between smokers and non-smokers and yet red blood cells of smokers are more susceptible to peroxidation.[13,14] Therefore, the aim of this study was to investigate whether cellular uptake and metabolism of α-tocopherol was impaired in red blood cells of smokers.

Data presented in this paper clearly demonstrate that α-tocopherol levels of red blood cells were lower in smokers despite total circulating levels of α-tocopherol being similar to those of non-smokers. However, total α-tocopherol adjusted for plasma cholesterol plus triacylglycerol was lower in smokers ($P < 0.05$); no such difference was observed for γ-tocopherol. As expected from other studies both plasma β-carotene and vitamin C levels were also significantly lower in smokers.[13,25,26] However, in this study, the range of plasma concentrations of both α-tocopherol and vitamin C was smaller than those observed in some studies.[25] This may have been due to the fact that volunteers selected in this study were from the Rowett Institute and they were closely matched for age, sex, and body mass index and had similar socio-economic background.

It is not known whether the reduced binding activity in smokers' red blood cells is due to a reduction in the number of binding sites and/or their affinity for α-tocopherol but the lower uptake of α-tocopherol in smokers' red blood cells may partly explain the lower concentrations

of α-tocopherol. Smokers had about a third less α-tocopherol in their red blood cells despite their having similar α-tocopherol plasma levels in absolute term to non-smokers. The higher plasma concentrations of cholesterol and triacylglycerols of smokers which led to lower α-tocopherol level adjusted for total plasma cholesterol and triacylglycerols, may also be linked if α-tocopherol access to red blood cell membrane is mediated through exchange with the lipoproteins.

Gas phase cigarette smoke contains approximately 1×10^{15} radicals per puff.[11,12] The relatively high amounts of these free radicals and other reactive oxygen species in cigarette smoke can deplete antioxidants and initiate the peroxidation of lipids and of modifying cellular and membrane proteins.[11,12] The α-tocopherol uptake sites in red blood cell membranes is reported to be protein in nature[7] so damage to binding sites of either the protein itself or of the surrounding lipids by smoking may also explain the observed reduced α-tocopherol uptake activity. The membrane fluidity depends in part on lipid constituents such as plasma cholesterol, cholesterol/phospholipid ratios, and the number of unsaturated double bonds in the fatty acids in phospholipids.[27] The role of membrane fluidity with reference to the activity of membrane-associated functions such as enzymes, receptors and ion channels has been reported.[28] Membrane fluidity of cells has also been shown to be altered by membrane lipid peroxidation.[28,29] Erythrocyte membrane fluidity has been inconsistently shown to be affected in smokers.[30] In this study we did not observe any change in average membrane fluidity so it seems unlikely that the reduced uptake activity was due to the changes in membrane fluidity. It may be possible that smoking may have direct damaging effect on the protein. Erythrocyte membrane proteins are susceptible to free radical attack, a phenomenon suggested to play a role in irreversible damage during cell aging,[31,32] seems therefore the more likely explanation. High concentrations of glucose was also shown to reduce the binding of α-tocopherol to cultured endothelial cells.[9] The glucose-mediated oxidative damage of cells is suggested to be one of the mechanisms responsible for the reduction of α-tocopherol uptake activity.

Several studies have shown that smokers have lower plasma levels of vitamin C and β-carotene due to both lower intake and increased metabolic use.[25] The decreased plasma concentration of vitamin C and β-carotene in smokers may limit regeneration of oxidised α-tocopherol and demonstration that large doses of oral α-tocopherol abolish the enhanced susceptibility of red blood cells to peroxidation[13] may mean that extra vitamin E could normalise the α-tocopherol uptake activity of red blood cells. If this occurs, however, the α-tocopherol may need to be provided over several weeks to allow its full protective effect to be seen in newly produced red blood cells.

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References

[1] A. T. Diplock (1983). The role of vitamin E in biological membranes. *Ciba Foundation Symposia*, **101**, 45–55.
[2] G. Traber and L. Packer (1995). Vitamin E: Beyond antioxidant function. *American Journal of Clinical Nutrition*, **62S**, 1501S–1509.
[3] B. Halliwell and J. M. C. Gutteridge (1985). *Free Radicals in Biology and Medicine*, Claredon Press, Oxford,
[4] G. W. Burton, A. Webb and K. U. Ingold (1985). A mild, rapid, and efficient method of lipid extraction for use in vitamin E-lipid ratios. *Lipids*, **20**, 29–39.
[5] A. Constantinescu, D. Han and L. Packer (1993). Vitamin E recycling in human erythrocyte membranes. *Journal of Biological Chemistry*, **268**, 10906–10913.
[6] A. E. Kitabchi and J. Wimalasena (1982). Specific binding sites for d—-tocopherol on human erythrocytes. *Biochimica et Biophysica Acta*, **684**, 200–206.
[7] J. Wimalasena, M. Davis and A. E. Kitabchi (1982). Characterization and solubilization of the specific binding sites for d-—tocopherol from human erythrocyte membrane. *Biochemical Pharmacology*, **31**, 3455–3461.
[8] A. E. Kitabchi, J. Wimalasena and J. Barker (1980). Specific sites for α-tocopherol in purified isolated adrenocortical cell membranes, *Biochemical Biophysical Research Communications*, **96**, 1739–1746.

[9] M. Kunisaki, F. Umeda, T. Yamauchi, M. Masakado, H. Natawat (1993). High glucose reduces specific binding for d-alpha tocopherol in cultured aortic endothelial cells. *Diabetes*, **42**, 1138–1146.

[10] A. K. Dutta-Roy, M. J. Gordon, F. M. Campbell, G. G. Duthie and W. P. T. James (1994). Vitamin E requirements, Transport and Metabolism: Role of α-tocopherol-binding proteins. *Journal of Nutritional Biochemistry*, **5**, 562–570.

[11] D. F. Church and W. A. Pryor (1985). Free radical chemistry and cigarette and its toxicological implications. *Environmental Health Perspectives*, **64**, 111–126.

[12] G. G. Duthie, J. R. Arthur and W. P. T. James (1991). Effects of smoking and vitamin E on blood antioxidant status. *American Journal of Clinical Nutrition*, **53**, 1061S –1063S.

[13] G. G. Duthie, J. R. Arthur, W. P. T. James and H. M. Vint (1989). Antioxidant status of smokers and non-smokers: effects of vitamin E supplementation. *Annals of New York Academy of Sciences*, **570**, 435–438.

[14] W. S. Stryker, L. A. Kaplan, E. A. Stein, M. J. Stampfer, A. Sober and W. C. Willet (1988). The relation of diet, cigarette smoking, and alcohol consumption to plasma beta carotene and alpha-tocopherol levels. *American Journal of Epidemiology*, **127**, 283–289.

[15] E. Beutler, C. West and K. G. Blume (1976). The removal of leukocytes and platelets from whole blood. *Journal of Laboratory of Clinical Medicine*, **88**, 328–333.

[16] M. C. Bellizzi, A. K. Dutta-Roy and W. P. T. James (1997). High D-glucose does not affect binding of α-tocopherol to human erythrocytes. *Molecular and Cellular Biochemistry*, (in the press).

[17] A. K. Dutta-Roy and A. K. Sinha (1985). Binding of Prostaglandin E_1 to human erythrocyte membrane. *Biochimica et Biophysica Acta*, **812**, 671–678.

[18] D. Hess, H. E. Keller, B. Oberlin, R. Bonfanti and W. Schuep (1991). Simultaneous determination of retinol, tocopherols, carotenes and lycopene in plasma by means of high performance liquid chromatography on reversed phase. *International Journal for Vitamin and Nutrition Research*, **61**, 232–238.

[19] M. Gleeson, J. D. Robertson and R. J. Maughan (1987). Influence of exercise on ascorbic acid status in man. *Clinical Science*, **73**, 501–505.

[20] D. I. Thurnham, J. A. Davies, B. J. Crump, R. D. Situnayake and M. Davis (1986). The use of different lipids to express serum tocopherol-lipid ratios for the measurements of vitamin E status. *Annals of Clinical Biochemistry*, **23**, 514–520, 1986.

[21] C. C. Allain, L. S. Poon, C. S. G. Clan, W. Richmond and P. D. Fu (1974). Enzymatic determination of total serum cholesterol *Clinical Chemistry*, **20**, 470–475.

[22] Atherosclerosis Society, Prevention of coronary heart disease: scientific background and new clinical guidelines. *Nutrition, Metabolism and Cardiovascular Diseases*, **2**, 113–156, 1992.

[23] A. K. Dutta-Roy, L. Hoque and B. J. Paterson (1993). Prostaglandin E_1 binding sites in rabbit erythrocyte membranes. *European Journal of Biochemistry*, **213**, 1167–1173.

[24] A. K. Dutta-Roy (1997). α-Tocopherol-binding proteins: Purification and Characterization. *Methods in Enzymology*, (in the press)

[25] C. Bolton-Smith, C. E. Casey, K. F. Gey, W. C. S. Smith and H. Tunstall-Pedoe (1991). Antioxidant vitamin intakes assessed using a food-frequency questionnaire—correlation with biochemical status in smokers and non-smokers. *British Journal of Nutrition*, **65**, 337–346.

[26] R. A. Riemersma, M. Oliver, R. A. Elton and K. F. Gey (1990). Plasma antioxidant and coronary heart disease; vitamin C and E, and selenium. European Journal of Clinical Nutrition **44**, 143–150.

[27] C. D. Stubbs and A. D. Smith (1990). Essential fatty acids in membrane-Physical properties and function. *Biochemical Society Transactions*, **18**, 779–780.

[28] A. K. Dutta-Roy (1994). Insulin mediated processes in platelets, monocytes/macrophages and erythrocytes: Effects of essential fatty acid metabolism. *Prostaglandins Leukotrienes and Essential Fatty Acids*, **51**, 385–399.

[29] M. Choe, C. Jackson and B. P. Yu (1995). Lipid peroxidation contributes to age-related membrane rigidity. *Free Readical Biology & Medicine*, **18**, 977–984.

[30] M. Boynard, F. Ribier, R. Guillet, F. Driss, P. Perrotin, F. Gaudey, C. Pautou and B. Nalpas (1994). Assessment of red blood cell deformability in healthy adult subjects volunteers—Influence of sex, age, cigarette smoking, menstrual cycle and oral-contraceptives. *Clinical Hemorheology*, **14**, 255–262.

[31] C. Signorini, M. Ferrali, L. Ciccoli, L. Sugherini, A. Magnani and M. Comporti (1995). Iron release, membrane protein oxidation and erythrocyte aging. *FEBS Letters*, **362**, 165–170.

[32] P. Hochstein and S. K. Jain (1981). Association of lipid peroxidation and polymerization of membrane-proteins with erythrocyte aging. *Federation Proceedings*, **40**, 183–188.