Free Rad. Res., Vol. 28, pp. 205–214
Reprints available directly from the publisher
Photocopying permitted by license only

Chain-Breaking Antioxidants and Ferriheme-Bound Drugs are Synergistic Inhibitors of Erythrocyte Membrane Peroxidation

ERIC DAILLY, SAÏK URIEN*,† and JEAN PAUL TILLEMENT

Laboratoire de Pharmacologie, Faculté de Médecine, F-94010 Créteil Université Paris XII, France

Accepted by Prof. S. Orrenius

(Received 15 June 1997; In revised form 20 October 1997)

Induced erythrocyte membrane peroxidation (EMP) is considered as an accurate model of reperfusion injuries and as such was used to investigate protective effects of various drugs. EMP was induced by an azo initiator and monitored by oxygen uptake. Both hydrophilic (ascorbic acid) and lipophilic (α-tocopherol, probucol, nicanartine) chain-breaking antioxidants as well as ferriheme-bound drugs (deferoxamine, chloroquine) inhibited EMP. When *antioxidants and ferriheme-bound drugs were* combined, synergistic effects were observed. It is proposed that ferriheme compounds which catalyse peroxide induced lipid peroxidation were blocked by deferoxamine and/or chloroquine. So these drugs inhibited at least partly the membrane peroxidation process and added their effects to the ones of chain-breaking antioxidants.

Keywords: Antioxidant, peroxidation, erythrocyte, chloroquine, deferoxamine, ferriheme

INTRODUCTION

Numerous physiopathological states such as inflammation, hypoxia-reperfusion and radiation[1] generate excess of reactive oxygen species in blood. One of the consequences of this overproduction is circulating lipids peroxidation including low density lipoproteins peroxidation which are involved in the atherosclerosis process.[2] Erythrocyte membrane peroxidation (EMP) has been observed in diseases such as sickle cell anemia,[3–5] malaria[6] and hemolytic anemia[7] where simultaneously plasma antioxidants levels were found decreased,[7–11] suggesting that the plasma antioxidant capacity would contribute to the erythrocyte membrane antioxidant system and thus would be connected with oxidative stress suffered by erythrocytes.

To study this phenomenon, we now propose a method to measure the plasma capacity of inhibition of erythrocyte membrane peroxidation (IEMP). This method is derived from the initial technique of Wayner *et al.* based on lipid peroxidation induced by an azo initiator and monitored by oxygen consumption with a Clark-type electrode.[12] In this preliminary study, we apply this

* Corresponding author. Tel.: (+33) 1 49 81 36 71. Fax: (+33) 1 49 81 35 94.
† Present address: Laboratoire de Pharmacologie, Faculté de Médecine, 8 rue du Général Sarrail, 94010 Créteil, France.

technique to plasma of healthy volunteers, then to plasma loaded with several drugs.

MATERIALS AND METHODS

Chemicals

2,2'—azobis (2—amidinopropane)—dihydrochloride (AAPH) was purchased from Wako Pure Chemical Industries. Nicanartine was obtained from Merz (Frankfurt am Main, Germany), chloroquine sulfate was from Specia Rhône-Poulenc Rorer (Paris, France), probucol was from Marion Merrel (Levallois—Perret, France). Others chemicals were obtained from Sigma.

Preparation of Erythrocytes

Venous blood was obtained from healthy subjects (7 males and 9 females) 23–29 years old and collected into K_3—EDTA vacutainers™. Blood was centrifuged (3000 rpm, 5 min) and plasma was stored at –80°C. Erythrocytes were stored in autologous plasma at +4°C. Assays were done in triplicate as soon as possible within 24 hours. Erythrocytes were washed three times with NaCl 0.9% just before assay.

IEMP Assay with Erythrocytes (Figure 1)

According to Wayner et al. (12), lipid peroxidation was initiated by AAPH decomposition at 37°C to yield peroxyl radicals at a known and constant rate Ri. When lipid EMP was established, plasma was added and decreased the lipid peroxidation rate. The inhibition period of EMP, T plasma, was defined as the duration of the plasma decrease of the peroxidation rate. Then, the lipid peroxidation rate returned to its initial value. IEMP was defined as

$$IEMP_{plasma} = (Ri \,.\, T\ plasma)/f \qquad (1)$$

where f is the plasma dilution in buffer. Ri was estimated by adding trolox (a chain-breaking antioxidant) when the plasma inhibition period was over. The second inhibition period of EMP induced by trolox, **T** trolox, was defined as

$$IEMP_{trolox} = (Ri \,.\, T\ trolox)/[trolox] \qquad (2)$$

where [trolox] is trolox concentration. Ri was calculated as follows

$$Ri = (2 \,.\, [trolox])/T\ trolox \qquad (3)$$

on the basis that trolox is trapping 2 peroxyl radicals per molecule.

$IEMP_{plasma}$ was calculated according to

$$IEMP_{plasma} = (2 \,.\, [trolox] \,.\, T\ plasma)/(T\ trolox \,.\, f) \qquad (4)$$

The slope ratio P1/P2 (Figure 1) is another useful parameter to measure the inhibition of lipid peroxidation by plasma. Before plasma was added, the rate of oxygen consumption due to lipid peroxidation was described by a straight line with slope P1; during plasma inhibition period of EMP a new straight line was obtained with slope P2.

The oxygen concentration was monitored with a Clark-type Hansatech™ oxygen electrode. To increase EMP rate, linoleic acid was added in the buffer (115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM Glucose, pH = 7.4). Linoleic acid which is water insoluble was mixed with plasma to obtain an emulsion. Twenty five µl of plasma and 1 µl of linoleic acid were vortexed for 30 seconds. Twenty µl of this mixture were added to the buffer also containing 4 mM AAPH at 37°C, protected from light. The final volume in the oxygen electrode cell was 1.5 ml. One µl of washed erythrocytes were added to the cell. To obtain the plasma inhibition period, a volume of plasma loaded or not with drugs was added during EMP, followed by addition of 1 µl trolox solution (5 mM, in dimethyl formamide) when the plasma inhibition period was over.

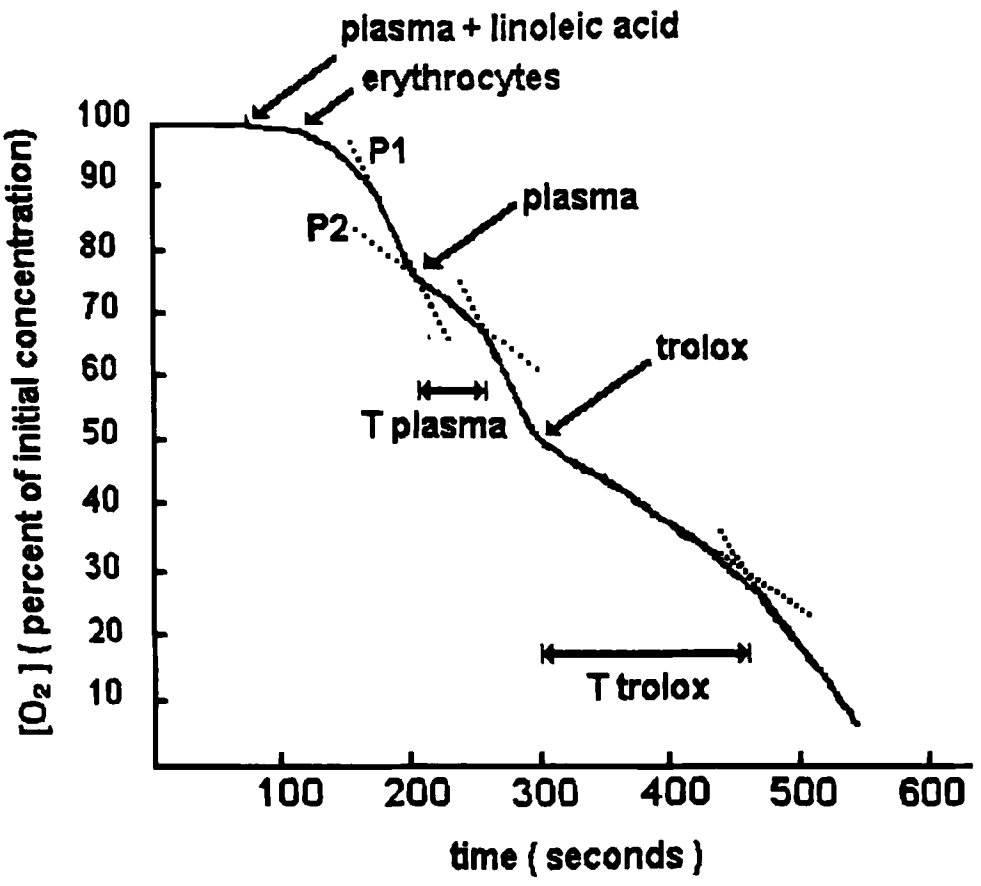

FIGURE 1 IEMP assay with the plasma and erythrocytes of a healthy volunteer. Assay is done in buffer (115 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2, 5 mM Glucose, pH=7.4) containing 4 mM AAPH at 37°C, protected from light. Oxygen concentration in the electrode cell is monitored with a Clark-type oxygen electrode. After linoleic acid-plasma mixture addition, oxygen consumption is low, indicating a slow rate of lipoprotein peroxidation. Then oxygen consumption and lipid peroxidation rates increase strongly upon erythrocyte addition. P1 is the slope of the straight line generated by oxygen consumption during lipid peroxidation. P2 is the slope of the straight line representing oxygen consumption during inhibition period of EMP induced by plasma (Tplasma). T trolox is the inhibition period of EMP due to trolox addition.

IEMP Assay with Ghosts

Hemoglobin-free erythrocytes ghosts were prepared by lysing 1 ml of cells with 15 ml of 10 mmol/l tris buffer (pH = 7.4) according to Moore et al. (25). Pellets were washed twice with tris buffer and once more with the buffer (115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM Glucose, pH = 7.4). Membrane pellets were collected after each step by centrifuging the lysed suspension at 10,000 g for 20 minutes.

The assays with ghosts were performed as described above, except that 1 µl of hemoglobin-free erythrocytes ghosts were used instead of 1 µl of washed erythrocytes.

Data Analysis

Data were analyzed by an iterative non linear regression program (MicroPharm, Inserm, 1990).

RESULTS

The IEMP assay is depicted on Figure 1. A significant inhibition of EMP required at least 20 µl of plasma for healthy volunteers. The plasma IEMP distribution is presented in Figure 2. The mean value is 1.53 ± 0.30 µM.

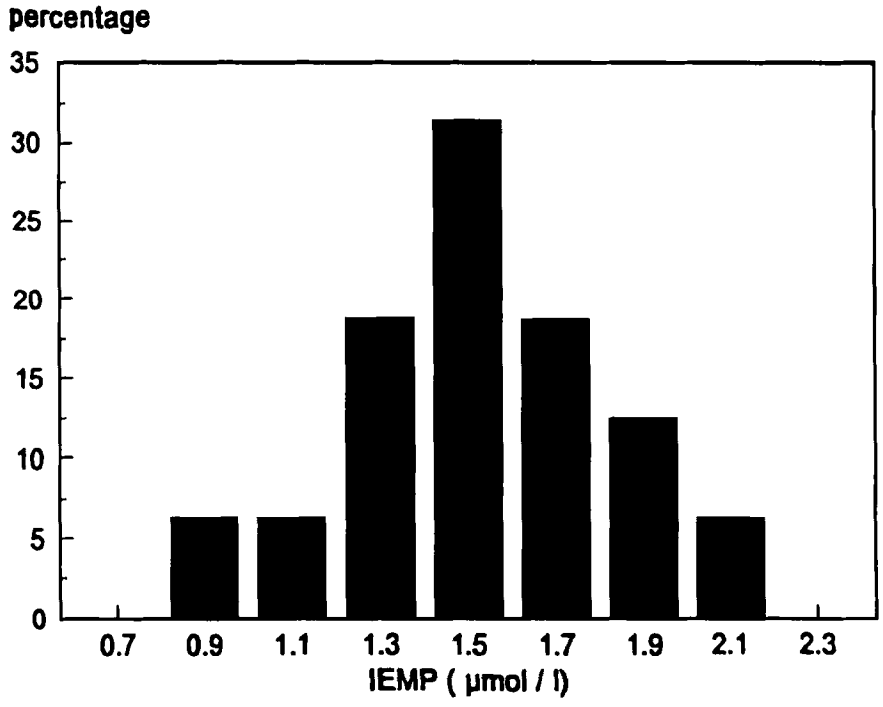

FIGURE 2 Plasma IEMP distribution in a healthy volunteer population. Plasma samples from 16 volunteers were investigated (7 males, 9 females). Results are expressed as percent of the population.

T plasma and T trolox are the classical parameters used to estimate plasma antioxidant activity.[12] The ratios T plasma/T trolox and P1/P2 were obtained from the plasma of a healthy volunteer. The following plasma volumes were added (15 µl, 20 µl, 25 µl, 30 µl, 40 µl, 60 µl). As shown in Figure 3, there was a strong correlation between the time and slope ratios ($r = 0.992$). When plasma was loaded with antioxidants, the determination of P1/P2 ratio was prefered to T plasma/T trolox as the first one is quicker. Moreover, during the inhibition period, the rate of oxygen consumption was decreased but a residual oxygen consumption level was observed. Then, at the end of the inhibition period, the oxygen concentration in electrod cell is no more sufficient to evaluate T trolox.

Antioxidant tested drugs were (i) lipophilic chain-breaking antioxidants: α-tocopherol, probucol,[14] nicanartine[13] (ii) hydrophilic chain-breaking antioxidant: ascorbic acid (iii) ferriheme- bound drug deferoxamine mesylate,[15] chloroquine sulfate.[16] The plasma of a healthy volunteer was loaded with increasing concentrations of these drugs. The relationship between P1/P2 and drug plasma concentration is depicted in Figure 4 and EC 50 values for each drug are summarized in Table I. EC 50 values presented in Table I were underestimated for α-tocopherol and ascorbic acid since these vitamins are already present in plasma. The most powerful antioxidant was probucol. Combi-nations of probucol with deferoxamine and chloroquine were also investigated. The ferriheme-bound drug concentrations were set to their EC 50 values. At these concentrations, ferriheme-bound drugs produced a slight inhibition of EMP. The combination of deferoxamine or chloroquine with probucol produced a significant increase in the antioxidant activity, i.e., a decrease of the probucol EC 50, 0.270 mM probucol alone versus 0.064 mM probucol + deferoxamine ($p < 0.01$) and 0.079 mM probucol+ chloroquine ($p < 0.01$).

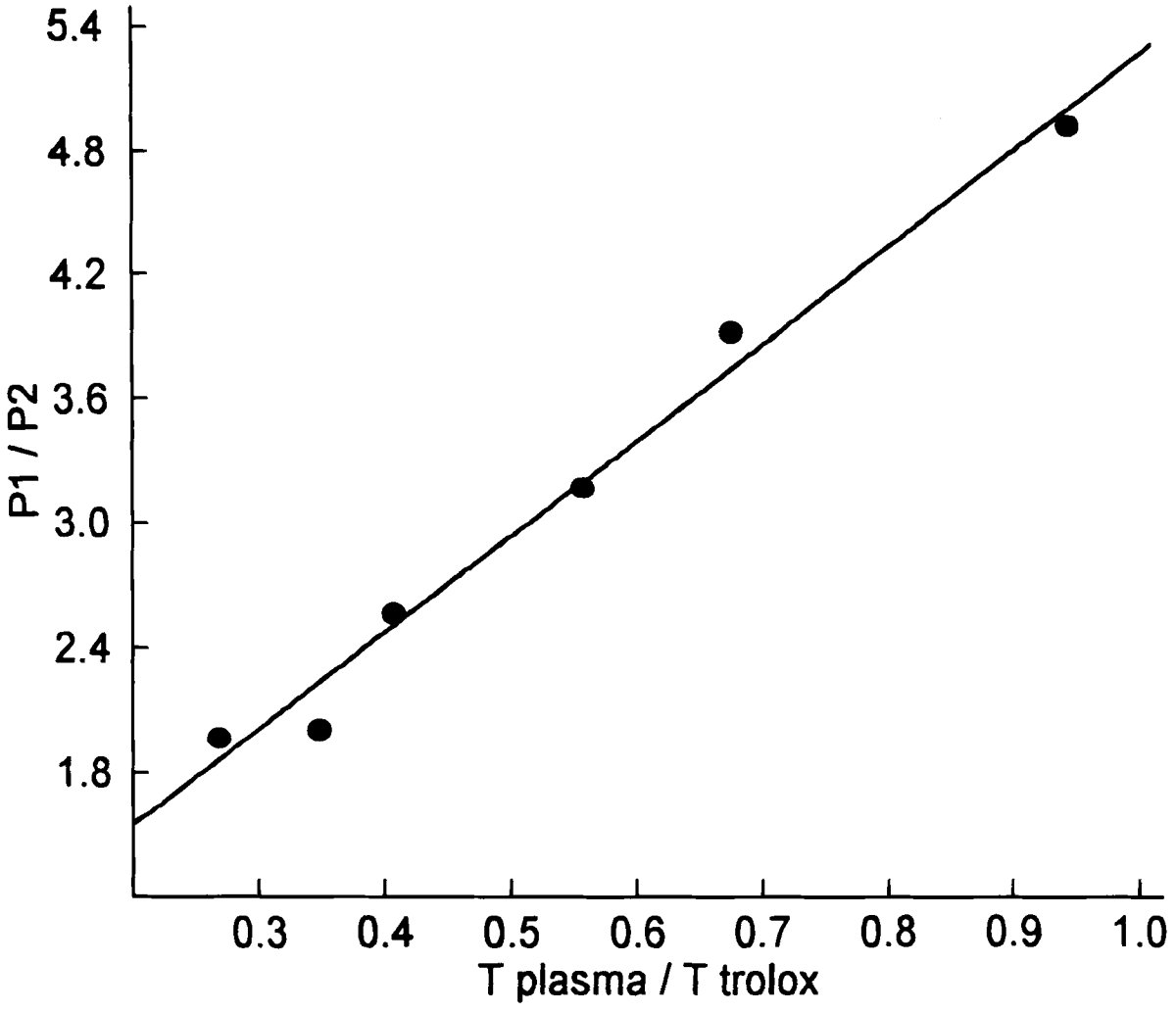

FIGURE 3 Relationship between the P1/P2 and Tplasma/Ttrolox ratios. Experiments were done with the plasma of an healthy volunteer. For each assay, the slopes and inhibition periods were estimated. The linear regression is P1/P2 = 4.575 (T plasma/T trolox) + 0.633 ($r = 0.992$).

The IEMP assay with ghosts was made to investigate the influence of oxidized hemoglobin on EMP and the lack of peroxidation inhibition by deferoxamine and chloroquine without erythrocytes. The lag time between ghosts addition and the increase of lipid peroxidation rate was in the order of thousand seconds whereas it was in the order of ten seconds with erythrocytes. In the ghosts assay, the IEMP values of plasma containing deferoxamine mesylate (10 mM) or chloroquine sulfate (12 mM) were respectively 3.10 ± 0.14 mM and 2.83 ± 0.83 mM. These IEMP values were not statistically different from those observed on plasma without drugs (2.91 ± 0.36 mM).

DISCUSSION

An interpretation of the IEMP mechanism is proposed in Figure 5. Oxygen consumption corresponds to fatty acid peroxide formation. The use of linoleic acid which is a readily oxidizable lipid increases the rate of lipoproteins peroxidation.[12]

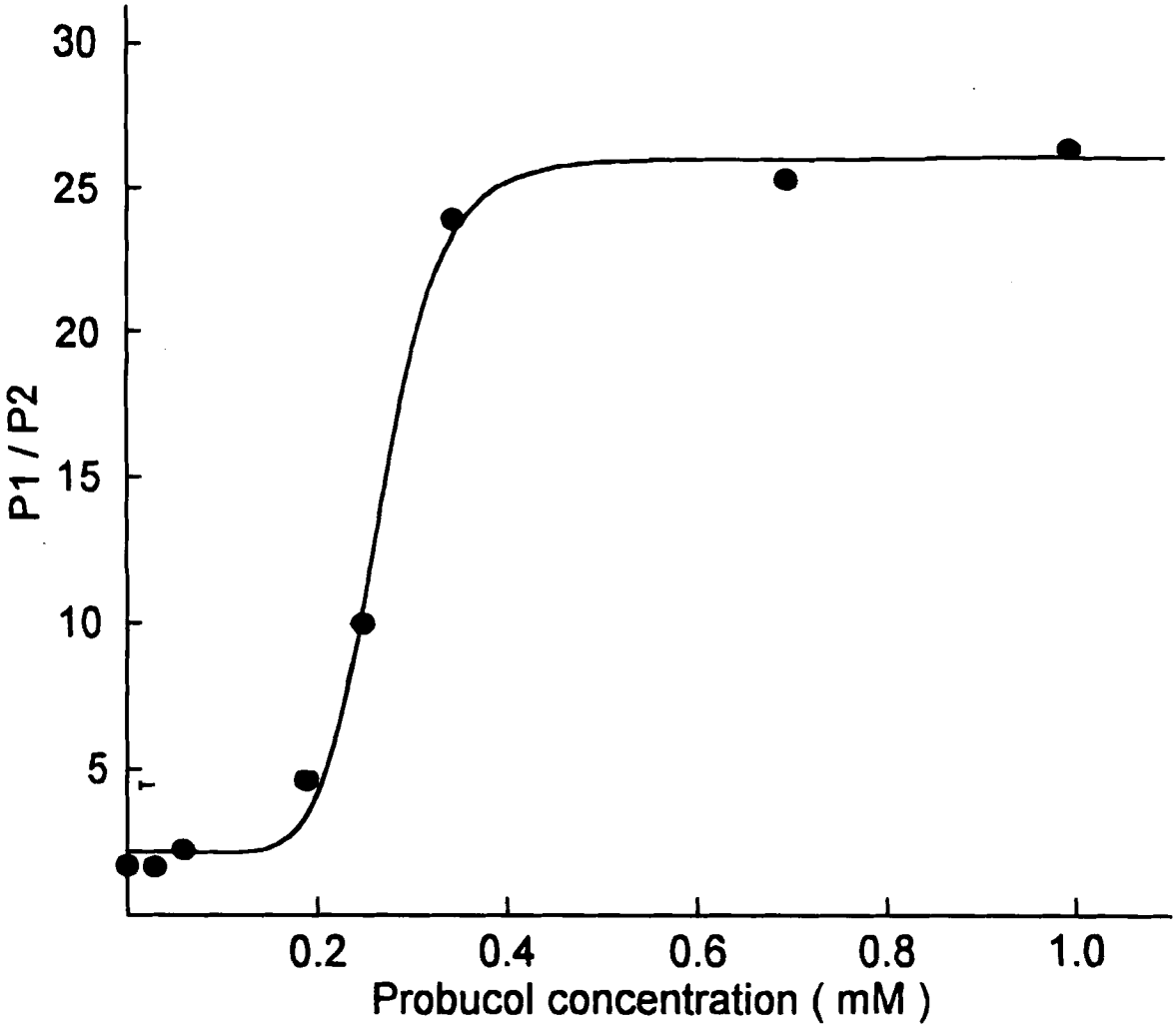

FIGURE 4 Inhibition of lipid peroxidation by probucol in the plasma of a healthy volunteer. Twenty ml of plasma containing increasing concentrations (C) of probucol were added in the electrode cell to induce an inhibition period. The ratio P1/P2 (= E) was determined in each assay. The data were fitted to the equation EE = (Emax . CH)/(EC50H+CH) where H is the Hill coefficient, EC50 is the probucol plasma concentration that produces 50% of maximum effect (Emax).

Its selection was based on the fact that Guo *et al.* showed that hydroperoxides formed in erythrocytes membrane during AAPH-induced peroxidation were mainly derived from linoleic acid.[17] The plasma peroxyl radical trapping antioxidant activity was already evidenced by Wayner *et al.* by its ability to delay lipoproteins peroxidation induced by AAPH.[12] Similarly, we showed that plasma could decrease EMP rate during a limited time interval. Our method has the advantage of being faster than the technique of Wayner *et al.* and could be used as a routine test to investigate the plasma capacity to inhibit EMP.

Chain-breaking antioxidants are able to trap free radicals directly. Lipophilic drugs such as α-tocopherol, probucol, nicanartine inhibited EMP as did ascorbic acid, a hydrophilic antioxidant (Table I). This inhibition of peroxidation is usually explained by a cooperative process between α-tocopherol and ascorbic acid.[18] In our assay, a

TABLE I Inhibition of EMP (Erythrocyte Membrane Peroxidation) by various drugs and their combinations

Drugs	EC 50 (mmol/l)
Lipophilic chain-breaking antioxidants	
probucol	0.270 ± 0.006
nicanartine	0.528 ± 0.015
alpha tocopherol	3.456 ± 0.508
Hydrophilic chain-breaking antioxidant	
ascorbic acid	0.952 ± 0.126
Ferriheme-bound drugs	
deferoxamine mesylate	5.060 ± 0.275
chloroquine sulfate	5.869 ± 0.011
Associations	
probucol with deferoxamine mesylate 5 mM	0.604 ± 0.006
probucol with chlorine sulfate 5.9 mM	0.079 ± 0.007

EC 50 is established according to equation $E = (Emax \cdot C^H)/(EC\ 50^H + C^H)$ where C is the drug concentration, H is the Hill coefficient, E is the observed effect (P1/P2), EC 50 is the drug concentration that produces 50% os maximum effect (Emax). Values are mean ± standard deviation of 2 experiments.

direct activity of ascorbic acid on free radicals generation by AAPH decomposition is also likeky to occur. But surprisingly, drugs which are not chain-breaking antioxidants like chloroquine inhibited also EMP. An explanation of this phenomenon can be deduced from the ferriheme role in EMP. Ferriheme which is both hemin (iron III photophorphyrin IX chloride) and hematin (iron III protoporphyrin IX hydroxide) is a potent catalyst of "peroxide-induced" lipid peroxidation. *This absence of catalysing activity in ghost assays explains the lag time increase before the increase of the lipid peroxidation rate.* Ferriheme released by oxidized hemoglobin promote lipid peroxidation by its catalytic ability in a Fenton reaction.[19] Deferoxamine which is an iron chelator binds ferriheme and thus inhibits hemin-induced hemolysis. This inhibition is explained by preventing ferriheme from binding to sites where it initiates hemolytic damage or by the lack of intrinsic hemolytic activity of deferoxamine or chloroquine-ferriheme complex when it binds to membrane.[20] Chloroquine also binds ferriheme with a high affinity[16] and probably shares the same inhibition mechanism. These observations suggest that (i) lipid EMP could induce hemoglobin oxidation generating oxidized hemoglobin like ferriheme derivatives[21] and that (ii) chloroquine and deferoxamine could inhibit EMP by binding these derivatives. According to our data (Table I), there is a synergy between ferriheme-bound drugs and chain-breaking antioxidants. *An interest of the synergistic combination is the decrease of the effective plasma concentration of probucol. In our assays, probucol EC50 value reduced from 0.270 ± 0.006 mM to 0.064 ± 0.006 mM, value close to the in vivo plasma concentration observed (Cmax = 0.190 mM).*[22]

EMP is a chain reaction which is developed from an initial oxidation process and contributes to hemolysis.[23] Our interpretation of EMP agrees with observations in hemolytic diseases. Administration of chain-breaking antioxidant results in rapid correction of hemolysis as described in infants with cystic fibrosis and severe hemolytic anemia.[7] The hemolysis in adult and neonatal erythrocytes caused by oxidation of lipid emulsion can be inhibited by deferoxamine.[24] In sickle cell disease the addition of both ascorbate and deferoxamine is necessary to offer a significant protection of membrane.[25] An explanation of this phenomenon could be that in high levels, ascorbate can become a pro-oxidant by reacting with iron components to catalyze the formation of hydroxyl radicals.[26] The combination of ascorbate and deferoxamine could prevent this interaction and this potential pro-oxidant activity. The high levels of hemin in the membranes of sickle cells[27] could be also involved in this phenomenon. Deferoxamine is able to prevent hemin-induced peroxidation by binding with hemin. The side effects of deferoxamine can limit its use. Our results suggest that chloroquine could provide an alternative to deferoxamine in this indication.

Oxidative processes of erythrocyte membrane but also lipoproteins peroxidation may be induced by hemin. The release of hemin due to the hemolysis would involve a lipoprotein peroxidation catalysis.[28] This strongly supports the hypothesis that EMP inhibition would be a way to prevent lipoprotein peroxidation which seems

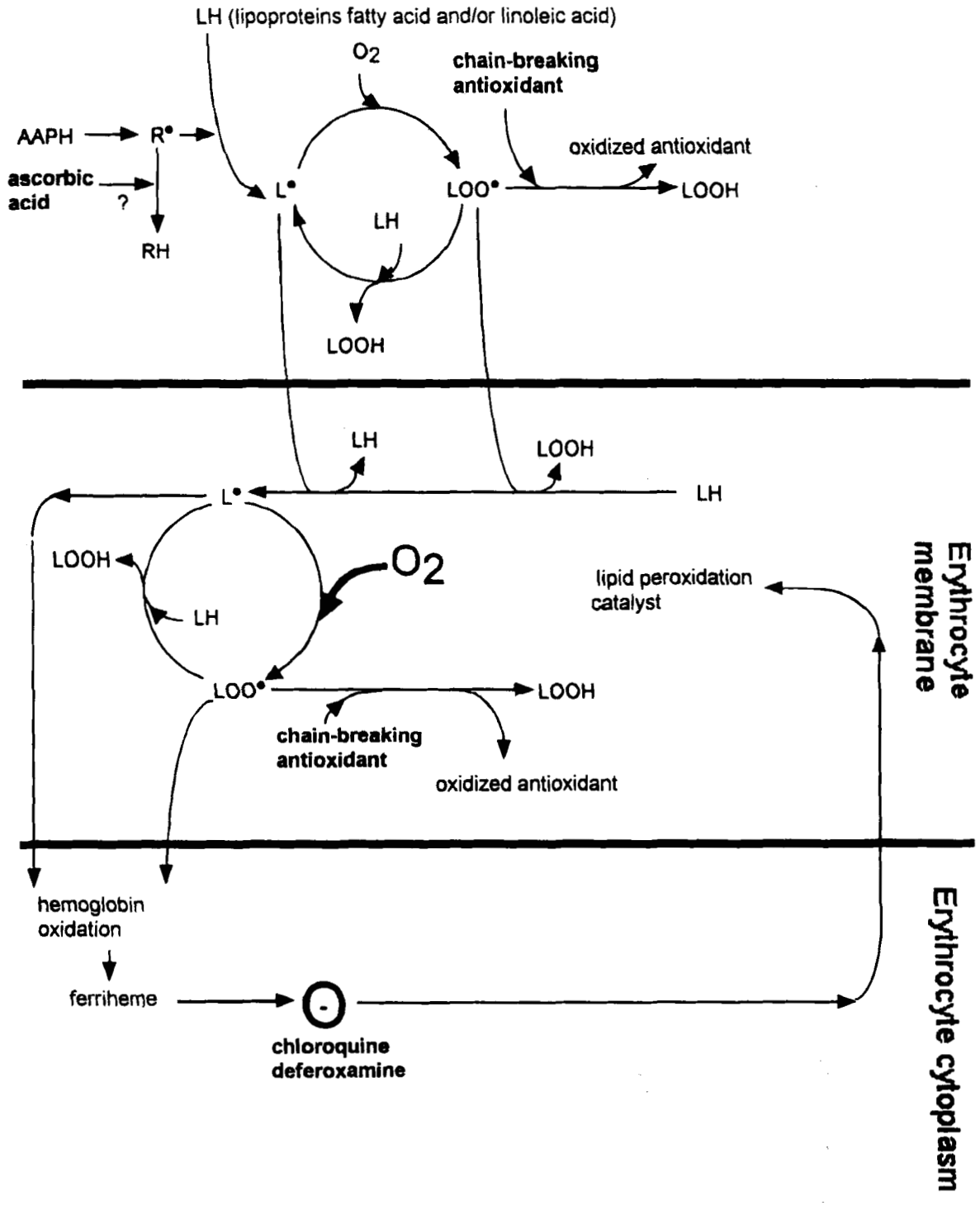

FIGURE 5 An interpretation of the erythrocyte membrane peroxidation induced by AAPH. R• is a peroxyl radical obtained from AAPH decomposition. LH is the polyunsaturated fatty acid (linoleic acid or fatty acid of lipoproteins or erythrocyte membrane) and L• is the corresponding radical. LOOH is a hydroperoxide formed from fatty acid peroxidation and LOO• is the corresponding peroxyl radical. Lipoproteins + linoleic acid generates L• and LOO• during peroxidation. These radicals can react with fatty acids in erythrocyte membranae inducing EMP. L• and LOO• formed by EMP can induce hemoglobin oxidation producing ferriheme. Ferriheme increases EMP and oxygen consumption by catalysing lipid peroxidation in erythrocyte membrane. Adapted from a scheme of LDL peroxidation (30).

to be implied in atherosclerosis.[29] Our results show that a chain-breaking antioxidant plus ferriheme-bound drug combination would be an efficient therapy in different disease but further investigations should be performed to evaluate the accuracy of this combination.

Acknowledgements

We wish to thank Merz, Specia Rhône-Poulenc Rorer and Marion Merrel laboratories for their gift of drugs. This research was supported by DRED (EA 427) and "Le Reseau de Pharmacologie Clinique".

References

[1] Freeman, B. A. and Crapo, J. D. (1982). Biology of disease: free radicals and tissue injury. *Laboratory investigation*, **47**, 412–426.

[2] Steinbrecher, U. P., Loughneed, M., Kwan, W. C. and Dirks, M. (1989). Recognition of oxidized low density lipoprotein by the scavenger receptor of macrophages results from derivatization of apolipoprotein B by products of fatty acid peroxidation. *Journal of Biological Chemistry*, **264**, 15216–15223.

[3] Das, S. K. and Nair, R. C. (1980). Superoxide dismutase, glutathione peroxidase, catalase and lipid peroxidation of normal and sickle erythrocytes. *British Journal of Haematology*, **44**, 87–92.

[4] Jain, S. K. and Shoket, S. B. (1984). A novel phospholipid in irreversibly sickled cells: Evidence for in vivo peroxidative membrane damage in sickle cell disease. *Blood*, **63**, 362–367.

[5] Hebbel, R. P. and Miller, W. J. (1984). Phagocytosis of sickle erythrocytes: Immunologic and oxidative determinants of hemolytic anemia. *Blood*, **64**, 733–741.

[6] Simoes, A. P., Van Den Berg, J., Roelofsen, B. and Op Den Kampf, J. (1992). Lipid peroxidation in Plasmodium falciparum—parasitized human erythrocytes. *Archives of Biochemistry and Biophysics*, **298**, 651–657.

[7] Wilfond, B. S., Farrell, P. M., Laxova, A. and Mischler, E. (1994). Severe hemolytic anemia associated with vitamin E deficiency in infants with cystic fibrosis. Implications for neonatal screening. *Clinical Pediatrics*, **33(1)**, 2–7.

[8] Stocker, R., Hunt, N. H. and Weidman, M. J. (1986). Antioxidants in plasma from mice infected with Plasmodium Vinckei. *Biochemical and Biophysical Research Communications*, **134**, 152–1258.

[9] Jain, S. K. and Williams, D. M. (1985). Reduced levels of plasma ascorbic acid (vitamin C) in sickle cell disease patients: its possible role in the oxidant damage to sickle cells in vivo. *Clinica Chimica Acta*, **149**, 257–261.

[10] Natta, C., Stacewicz-Sapuntazakis, M., Bhagavan, H. and Bowen, P. (1988). Low serum levels of carotenoids in sickle cell anemia. *European Journal of Haematology*, **41**, 131–135.

[11] Adelekan, D. A., Thurnham, D. I., Adelike, A. D. (1989). Reduced antioxidant capacity in pediatric patients with homozygous sickle cell disease. *European Journal of Clinical Nutrition*, **43**, 609–614.

[12] Wayner, D. D. M., Burton, G. W., Ingold, K. U., Barclay, L. R. C. and Locke, S. J. (1987). The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. *Biochimica et Biophysica Acta*, **924**, 408–419.

[13] Dailly, E., Urien, S., Wulfroth, P. and Tillement, J. P. (1996). Nicanartine improves in vitro resistance of lipoproteins to oxidation. *Pharmaceutical research*, **13**, 457–461.

[14] Mac Lean, L. R. and Hagaman, K. A. (1989). Effect of probucol on the physical properties of low density lipoproteins oxidized by copper. *Biochemistry*, **28**, 321–327.

[15] Baysal, E., Monteiro, H. P., Sullivan, S. G. and Stern, A. (1990). Desferrioxamine protects human red blood cells from hemin-induced hemolysis. *Free Radical Biology and Medicine*, **9**, 5–10.

[16] Chou, A. C., Chevli, R. and Fitch, C. D. (1980). Ferriprotoporphyrin IX fulfills the criteria for identification as the chloroquine receptor of malaria parasites. *Biochemistry*, **19**, 1543–1549.

[17] Guo, L., Ogamo, A., Ou, Z., Shinozuka, T. and Nakagawa, Y. (1995). Preferential formation of the hydroperoxide of linoleic acid in choline glycerophospholipids in human erythrocytes membrane during peroxidation with an azo initiator. *Free Radical Biology and Medicine*, **18**, 1003–1012.

[18] Sato, K., Niki, E. and Shimasaki, H. (1990). Free radical-mediated chain oxidation of low density lipoprotein and its synergistic inhibition by vitamin E and vitamin C. *Archives of Biochemistry and Biophysics*, **279**, 402–405.

[19] Tsun-Yee Chiu, D., Van Den Berg, J., Kuypers, F. A., Hung, I. J., Wei, J. S. and Liu, T. Z. (1996). Correlation of membrane lipid peroxidation with oxidation of hemoglobin variants: possibly related to rhe rates of hemin release. *Free Radical Biology and Medicine*, **21**, 89–95.

[20] Sullivan, S. G., Baysal, E. and Stern, A. (1992). Inhibition of hemin-induced hemolysis by desferrioxamine: binding of hemin to red cell membranes and the effects of alteration of membrane sulfhydryl groups. *Biochimica et Biophysica Acta*, **1104**, 38–44.

[21] Bunn, H. F. and Jandl, J. H. (1966). Exchange of heme among hemoglobin molecules. *Proceedings of the National Academy of Sciences of the USA*, **56**, 974–978.

[22] Satonin, D. K., Coutant, J. E. (1986). Comparison of gas chromatography and high-performance liquid chromatography for the analysis of probucol in plasma. *Journal of chromatography*, **380**, 401–406.

[23] Sato, Y., Kamo, S., Takahashi, T. and Suzuki, Y. (1995). Mechanism of free radical-induced hemolysis of human erythrocytes: hemolysis by water-soluble radical initiator. *Biochemistry*, **34**, 8940–8945.

[24] Kljuchnikov, S., Pitkänen, O., Raivio, K. O. and Andersson, S. (1993). Haemolysis in adult and neonatal erythrocytes caused by autoxidation of lipid emulsion (Intralipid). *Acta Paeditrica*, **82**, 348–351.

[25] Moore, R. B., Hulgan, T. M., Green, J. W. and Jenkins, L. D. (1992). Increased susceptibility of the sickle cell membrane Ca^{2+} + Mg^{2+} – ATPase to t—butylhydroperoxide: Protective effects of Ascorbate and Desferal. *Blood*, **79**, 1334–1341.

[26] Miller, D. M. and Aust, S. D. (1989). Studies of ascorbate-dependent iron-catalyzed lipid peroxidation. *Archives of Biochemistry and Biophysics*, **271**, 113–119.

[27] Kuross, S. A. and Hebbel, R. P. (1988). Nonheme iron in sickle erythrocyte membranes: associations with phospholipids and potential role in lipid peroxidation. *Blood*, **72**, 1278–1285.

[28] Miller, Y. I., Felikman, Y. and Shaklai, N. (1995). The involvement of low-density lipoprotein in hemin transport potentiates peroxidative damage. *Biochimica et Biophysica Acta*, **1272**, 119–127.

[29] Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L. and Steinberg, D. (1984). Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proceedings of the National Academy of Sciences of the USA*, **81**, 3883–3887.

[30] Esterbauer, H., Rotheneder, M., Striegl, G., Waeg, G., Ashy, A., Sattler, W. and Jürgens, G. (1989). Vitamin E and other lipophilic antioxidants protect LDL against oxidation. *Fat Science and Technology*, **8**, 316–324.