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Comparison between in vitro lipid peroxidation in fresh sheep platelets and peroxidative processes during sheep platelet ageing under storage at 4°C

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Abstract

Incubation of sheep platelet crude membranes with xanthine oxidase (XO)/hypoxanthine/Fe^{2+}-ADP revealed: (i) a fast peroxidative response – with a maximal linear rate of 14 nmol malondialdehyde (MDA) equivalents/mg protein, as evidenced by the thiobarbituric acid test – and a decrease in the polyunsaturated fatty acid (PUFA) content of the platelet crude membranes; (ii) a decrease in the lipid fluidity in the deep lipid core of the membranes but not at the membrane surface; (iii) a dramatic inhibitory effect on glucose 6-phosphatase (Glc-6-Pase) but not on acetylcholinesterase activity. Platelets were also aged by storage at 4°C in their own plasma or in Seto additive solution. In these media, platelet aggregates were visible and the effects on platelet phospholipids, PUFA, lipid extract fluorescence, crude membrane fluidity and membrane-bound enzyme activities were assessed for comparison with those observed in in vitro lipid peroxidation. The sensitivity of membranes from stored platelets to lipid peroxidation was also assessed. Storage of platelets in plasma for 5 days was associated with different changes in their crude membranes such as decreases in arachidonic acid contents, the decrease not being avoided by the presence of phospholipase A_2 inhibitors, increases in MDA equivalents, conjugated dienes and lipid extract fluorescence, decreases in the amounts of MDA equivalents formed by platelet crude membranes treated with the oxidizing agents, changes in membrane fluidity and inhibition of Glc-6-Pase. All these alterations were less pronounced or even abolished after platelet storage in Seto. These findings suggest that platelet lipid peroxidation due to XO/hypoxanthine/Fe^{2+}-ADP and platelet membrane alterations observed after platelet ageing under storage at 4°C share common features. Also, as regards the prevention of peroxidative processes, Seto solution permits better storage of sheep platelets than plasma.

Keywords: Lipid peroxidation; Ageing; Platelet storage

Abbreviations: AChE, acetylcholinesterase; BHA, 3(2)-*tert*-butyl-4-hydroxyanisole; DPH, 1,6-diphenyl-1,3,5-hexatriene; Glc-6-Pase, glucose 6-phosphatase; l-PE, lysophosphatidylethanolamine; MDA, malondialdehyde; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid(s); TBARS, thiobarbituric acid-reactive substance(s); TMA-DPH, 1-[4-(trimethylammonium)-phenyl]-6-phenyl-1,3,5-hexatriene; XO, xanthine oxidase

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1. Introduction

Besides the requirement of platelets to maintain vascular integrity, the removal of most platelets from the circulation is believed to be age-dependent [1]. The absence of a nucleus in platelets suggests that failures in the maintenance of normal homeostasis might occur during ageing since platelets probably lack the means for synthesizing proteins for the repair of enzymes and cellular structures. It has been proposed that there is a relationship between the ageing of platelets and their metabolic rate, possibly related to changes in mitochondrial functions during platelet ageing, since platelets have no means for mitochondrial turnover. Moreover, the mitochondrial theory of ageing [2] relates cell ageing to the continuous attack of mitochondrial DNA by oxygen-derived radicals and other toxic species. It has recently been reported that in platelets, during senescence, alterations of the mitochondrial hydrophobic subunits of Complex I could occur, as indicated by decreased rotenone sensitivity in ageing [3]. Nevertheless, such changes may not necessarily be related to an accumulation of mitochondrial DNA mutations, but rather to alterations in the lipid composition and fluidity of the mitochondrial membrane [4,5], due to different processes such as oxygen-related peroxidation events.

Previous results from our laboratory have indicated that during the storage (in vitro ageing) of sheep erythrocytes at 4°C changes in the phospholipid profile and polyunsaturated fatty acid contents occur, such changes being at least partly related to peroxidation events that occur during the storage period [6,7]. Additionally, during storage platelets undergo several biochemical, structural and functional alterations – similar to those that occur during platelet activation and aggregation – that decrease platelet viability and reduce the half-life of the cells [8]. Moreover, although the life span of platelets stored in plasma at 22°C is higher than that of refrigerated platelets, storage at 4°C for 1–3 days not only permits platelets to retain some of their hemostatic functions [9] but also increases transfusion safety [10]. Among the different media used for platelet storage, synthetic media afford similar or even better preservation of platelet function after up to 5 days in comparison with storage in autologous plasma [11]. Platelet membranes contain high levels of polyunsaturated fatty acids (PUFA), the cellular components most susceptible to peroxidation. Lipid peroxidation of membranes induced by reactive oxygen species is able to alter the structure and function of membrane components involved in pathological events and could be one of the mechanisms responsible for membrane and cellular ageing [12,13]. Nevertheless, little information is available about the changes resulting in platelet senescence, death and removal from the circulation. Xanthine oxidase (XO), which has been implicated as a significant source of these reactive oxygen species, may be released into the circulation, where platelets are located, producing oxidant injury to cell membranes [14,15].

The present study compares the susceptibility of fresh and stored sheep platelets to lipid peroxidation due to XO/hypoxanthine/Fe^{2+}-ADP and the occurrence of peroxidation processes during platelet storage at cold temperature in two different media, i.e., their own plasma and a synthetic media called Seto. As will be shown, membrane alterations that were brought about in vitro displayed features in common with those observed under storage. We therefore set up in vitro peroxidation studies with a view to providing a mechanistic model to account for some of the effects occurring in platelet ageing under storage conditions.

2. Materials and methods

2.1. Materials

Acetylthiocholine chloride, adenosine diphosphate (ADP), BF_3-methanol reagent, bovine serum albumin (BSA), 3(2)-*tert*-butyl-4-hydroxyanisole (BHA), dimethyl sulfoxide (DMSO), 1,6-diphenyl-1,3,5-hexatriene (DPH), 5,5′-dithiobis(2-nitrobenzoate) (DTNB), glucose 6-phosphate (Glc-6-P, disodium salt), hypoxanthine, tetrahydrofuran, 2-thiobarbituric acid, Tris (hydroxymethyl) aminomethane, 1-[4-(trimethylammonium)-phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) and XO were from Sigma Chemical, Aldrich Chemical or Supelco (Sigma-Aldrich Química, Madrid, Spain). Thin-layer chromatography plates (Silicagel G, type 60) and isooc-

tane were from Merck (Darmstadt, Germany). The remaining products were from Panreac or Probus (Barcelona, Spain).

2.2. Platelet preparation and storage conditions

Platelets were isolated from the fresh blood of adult sheep (*Ovis aries* L.) sacrificed at a local slaughterhouse. Platelets were obtained as described earlier [16], using citrate-phosphate-dextrose-adenine as anticoagulant. For the storage of platelets in autologous plasma or in Seto additive solution, 60 ml portions of platelet-rich plasma (PRP) containing $50–60\times10^9$ cells were transferred to a 300 ml Teruflex T-300 transfer bag (Terumo, Tokyo, Japan) using a sterile connection device. The Teruflex bags used for the storage of platelets in Seto solution were centrifuged at $4300\times g$ for 10 min to obtain platelet pellets. The supernatant plasma was then separated and replaced by 60 ml of Seto solution (90 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 4.9 mM NaH_2PO_4, 20.1 mM Na_2HPO_4, 23 mM Na acetate, 17 mM Na_3 citrate, 28.8 mM maltose, 23.5 mM glucose, pH 7.4) [11] using the sterile connection device. Penicillin G (300 IU/ml) and streptomycin sulfate (300 μg/ml) were added and all bags (those with plasma or Seto) were then shaken vertically for 1 h at 22°C to resuspend the pellets. The bags were then stored at 4°C for up to 8 days with continuous shaking in an ABT 5 platelet tumbler at 6 rpm (SBS Instruments, Madrid, Spain). No bacterial growth was detected at the end of the storage period. Lactate dehydrogenase (LDH, EC 1.1.1.27) activity, a marker of platelet lysis, was determined in the media after storage by the method of Wroblewski and La Due [17].

2.3. Recovery of platelets and aggregates

PRPs or plasma-poor platelet concentrates (PCs) were first centrifuged at $600\times g$ for 10 min to remove traces of aggregates. The supernatants were then withdrawn from the bags under vacuum and centrifuged at $4300\times g$ for 10 min to obtain the platelet fraction (platelets). Platelets and aggregates were washed once in buffer A (5 mM Tris, 0.14 M NaCl, pH 7.4) and recovered by recentrifugation. For the collection of crude membranes, fresh or stored platelets and aggregates were resuspended in 5 mM Tris-HCl, pH 7.4, and kept on ice for 10 min to allow them to swell. They were then homogenized by passing them 30 times through a syringe fitted with a 22-gauge needle. The homogenates were centrifuged at $100\,000\times g$ for 1 h at 4°C and the pellets (crude membranes) were washed once in buffer A, resuspended in the same buffer, and stored at −80°C until use.

2.4. Peroxidation assays

In vitro peroxidation was carried out using XO/hypoxanthine/Fe^{2+}-ADP as indicated previously [18,19]. Samples from fresh or stored platelets (0.5–1.0 mg of platelet membranes) were incubated with 100 μl of mixture A (10 mM ADP and 1 mM $FeCl_3$ dissolved in buffer A) and 50 μl of 20 mM hypoxanthine at 1 ml final volume adjusted with buffer A. After air had been bubbled into the mixture, the reaction was started by the addition of 10 mU XO and further incubation at 37°C for a standard time of 120 min (in some experiments different periods of time were assayed as indicated in the appropriate figure legend). The reaction was stopped after cooling on ice by the addition of 100 μl 0.5 M EDTA, pH 7.4. Controls including samples without mixture A, hypoxanthine and XO, and samples in which XO had been previously inactivated by heat, were always assayed in parallel. The extent of lipid peroxidation in the in vitro assays and in stored platelet crude membranes was assessed by measurements of thiobarbituric acid-reactive substances (TBARS) [20] and expressed as malondialdehyde equivalents (MDA, nmol/mg protein). Additionally, conjugated dienes in stored platelet samples were measured by monitoring the absorbance at 233 nm of detergent-dispersed platelet crude membranes (0.05 mg protein in 10 mM phosphate buffer, pH 7.1, containing 1% Lubrol PX), and calculated using the molar absorption coefficient of 2.52×10^4 M^{-1} cm^{-1} [21].

Peroxidized platelet membranes used for fatty acid analyses and fluorescence anisotropy determinations were first centrifuged at $200\,000\times g$ for 30 min to eliminate the peroxidation mixture and then washed once with buffer A.

2.5. Lipid extraction, fractionation and identification of phospholipid classes, and fatty acid analysis by gas–liquid chromatography (GLC)

Total lipids were extracted from samples by the method of Rose and Oklander [22], slightly modified, as reported previously [23]. Phospholipid classes were fractionated by two-dimensional thin layer chromatography [24] and phospholipid contents were determined by measuring lipid phosphorus after hydrolysis of the lipid extracts with $HClO_4$ [7]. For fatty acid analysis, an aliquot of the lipid extracts from each sample (10–15 μg lipid phosphorus) was transmethylated using the BF_3–methanol reagent. The fatty acid methyl esters of total lipids were partitioned in a water/petroleum hydrocarbon system, brought to dryness under nitrogen, dissolved in 10 μl of isooctane and separated in a KNK-3000-HRGC gas chromatograph (Konik Instruments, Barcelona, Spain), as indicated previously [23].

2.6. Enzyme determinations

Glucose 6-phosphatase (Glc-6-Pase) was determined by the method of Baginski et al. [25]. Acetylcholinesterase (AChE) activity was determined by measuring the hydrolysis of acetylthiocholine chloride as indicated previously [26]. Specific activities were expressed as mU/mg protein. One unit (U) of Glc-6-Pase is equal to 1 μmol phosphorus released per minute. One unit of AChE is defined as the amount of enzyme that forms 1 μmol of thiocholine per minute, which yields 1 μmol of nitromercaptobenzoate (molar absorptivity of 13 600 M^{-1} cm^{-1} at 412 nm).

The protein contents of different samples were estimated by the method of Bradford [27], using BSA as standard.

2.7. Measurement of lipid fluorescence

Lipid fluorescence was measured in the lipid extracts from fresh or stored platelets and aggregates as indicated previously [23]. For this purpose, the lipid extracts from the different samples dissolved in chloroform/methanol (1:1, v/v) were measured using a Hitachi F-4010 spectrofluorimeter (Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 350 nm and 450 nm, respectively, and bandpass values of 5 nm for excitation and emission. The lipid fluorescence values shown in the results are relative Hitachi F-4010 units. One fluorescence unit is equivalent to 1 μg quinine sulfate/ml of water.

2.8. Measurement of fluorescence polarization

Crude membranes from control and treated platelet and aggregates were labeled with the 'fluidity probes' DPH and TMA-DPH as recently reported by us [23]. Freshly prepared 0.5 mM DPH (10 mM stock solution in tetrahydrofuran) or TMA-DPH (10 mM stock solution in DMSO) were added to each membrane sample resuspended in 4 ml PBS (10 mM sodium phosphate buffer, 150 mM NaCl), pH 7.4, at a ratio of 0.5 nmol probe per 100 μg protein (50:1 phospholipid/probe ratio). TMA-DPH remained specifically in the crude membranes after very rapid incorporation, thus making preincubation unnecessary, while DPH was incorporated after preincubation at 37°C for 30 min. Fluorescence was measured on a Hitachi F-4010 spectrofluorimeter at 10, 25 and 37°C. The excitation and emission wavelengths used with DPH and TMA-DPH were 360 and 430 nm, and 365 and 428 nm, with slit widths of 10 (excitation) and 20 nm (emission), respectively. Fluorescence anisotropy was calculated according to Shinitzky and Barenholz [28] as described previously [23].

2.9. Statistical analysis

Statistical analysis was performed using Student's *t*-test. Significance was determined at $P < 0.05$.

3. Results and discussion

Peroxidation produces changes in the structure of cells due to changes in the cell chemical composition, which can result in membrane disorganization and variations in their physical and functional properties, with concomitant inactivation of some membrane-bound enzymes, leading to defects in cell function and the decline in cell performance that characterizes senescence. In fact, uncontrolled peroxidation of biomembranes may be sufficient to cause cell death [12].

Study of lipid peroxidation in biomembranes includes the use of non-enzymic or enzyme-catalyzed systems that enhance lipid peroxidation. The XO/hypoxanthine/Fe^{2+}-ADP enzymatic system was chosen as a model peroxidation system because preliminary experiments had shown that other systems, such as Fe^{2+}/ascorbic acid (1.5 mM/10 mM), $FeSO_4/H_2O_2$ (0.2 mM/0.12 mM), H_2O_2/sodium azide/$CuSO_4$ (2 mM/1 mM/10 μM) were ineffective for the formation of MDA equivalents in the sheep platelet system used by us.

Although enzymatic lipid peroxidation is clearly observed in an $O_2^{\bullet-}$-generating system with XO [29], it is not well understood how water-soluble O_2 and iron initiate lipid peroxidation in the hydrophobic membrane core, where PUFA are located. It has been proposed that the initiation and dynamics of lipid peroxidation induced by XO/Fe^{3+}-ADP in negatively charged liposomes involve a reduction of Fe^{3+} by the O_2 generated by XO. The reduced iron, ferrous iron, or a perferryl species, then forms an alkoxyl radical via a Fenton-like reaction which penetrates the hydrophobic region of the membrane and triggers the initiation of lipid peroxidation [30].

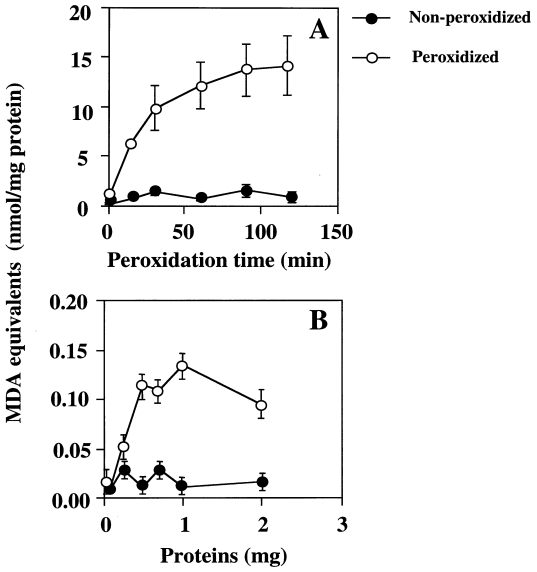

Fig. 1. Effect of peroxidation time (A) and amount of proteins (B) on TBARS formation in sheep platelet crude membranes. Samples were peroxidized as described in Section 2 for different times (A) or for 120 min (B). Data represent means ± S.E.M. of three experiments done in triplicate.

3.1. Conditions of in vitro peroxidation

The first set of experiments was designed to determine the best conditions for peroxidation. Although the amount of TBARS detected after peroxidation is not necessarily identical to the amount of MDA produced [18], incubation of the sheep platelet crude membrane system with the peroxidation mixture clearly resulted in the formation of MDA equivalents in a time-dependent manner, maximal TBARS formation occurring after 90–120 min of incubation with the oxidizing agents (Fig. 1A) and at protein

Table 1
Changes in the phospholipid composition of sheep platelets before and after storage in plasma or Seto solution, aggregates from the storage media and peroxidized platelet crude membranes

Phospholipids	Fresh platelets (control)	Peroxidized platelet membranes	Stored platelets		Aggregates from storage media	
		120 min	48 h		48 h	
			Plasma	Seto	Plasma	Seto
PE	29.5 ± 1.8	24.3 ± 0.6*	19.5 ± 0.4*	28.4 ± 1.4	17.4 ± 1.0*	32.3 ± 3.7
PC	30.9 ± 1.1	32.3 ± 1.4	43.7 ± 1.1*	31.0 ± 1.1	47.4 ± 3.2*	32.7 ± 2.3
PS+PI	12.3 ± 0.5	14.4 ± 1.2	9.3 ± 1.1	13.7 ± 0.8	8.0 ± 1.0	10.7 ± 0.3
SM	25.5 ± 0.5	23.9 ± 0.9	21.9 ± 0.6	24.4 ± 0.3	20.6 ± 0.6	22.7 ± 0.9
l-PC	0.9 ± 0.3	0.7 ± 0.5	1.5 ± 1.3	0.6 ± 0.2	5.2 ± 1.7	ND
l-PE	ND	4.3 ± 0.5*	ND	ND	ND	ND
DPG	1.3 ± 0.2	0.9 ± 0.7	1.9 ± 0.7	1.7 ± 0.8	3.3 ± 1.7	1.7 ± 1.2

Data are given in percentages of total phospholipid contents and are means ± S.E.M. of four individual experiments done in duplicate. ND, not detected; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS+PI, phosphatidylserine+phosphatidylinositol; SM, sphingomyelin; l-PC, lysophosphatidylcholine; l-PE, lysophosphatidylethanolamine; DPG, diphosphatidylglycerol.
By rows, *$P < 0.05$ vs. fresh platelets (control).

Table 2
Changes in the fatty acid composition of total lipids from sheep platelets before and after storage in plasma or Seto solution, aggregates from the storage media and peroxidized platelet crude membranes

Fatty acid composition	Fresh platelets (control)	Peroxidized platelet membranes	Stored platelets		Aggregates from storage media	
		120 min	48 h		48 h	
			Plasma	Seto	Plasma	Seto
14:0	1.1 ± 0.1	1.8 ± 0.3*	1.9 ± 0.2*	1.8 ± 0.3*	2.3 ± 0.3*	1.6 ± 0.3
15:0	0.6 ± 0.1	0.7 ± 0.2	0.7 ± 0.3	0.9 ± 0.2	1.3 ± 0.3	0.8 ± 0.1
16:0	17.6 ± 1.0	23.6 ± 0.7*	22.6 ± 0.6*	21.1 ± 1.0*	23.2 ± 1.7*	21.1 ± 1.1*
16:1(*n*–7)	1.9 ± 0.1	3.0 ± 0.3*	2.4 ± 0.5	3.0 ± 0.4*	4.1 ± 0.5*	3.1 ± 0.5*
17:0	0.8 ± 0.1	1.0 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.9 ± 0.4	0.8 ± 0.4
18:0	15.1 ± 1.0	17.2 ± 0.9	17.5 ± 1.1	15.6 ± 0.7	16.9 ± 1.4	15.7 ± 0.6
18:1(*n*–9)	27.8 ± 0.7	35.9 ± 2.7	28.7 ± 0.6	30.7 ± 1.6	25.4 ± 2.2	29.2 ± 1.0
18:2(*n*–6)	13.3 ± 0.6	12.1 ± 0.5	13.1 ± 1.3	12.8 ± 0.9	13.2 ± 1.8	13.6 ± 0.4
18:3(*n*–3)	6.4 ± 1.6	2.3 ± 1.6*	6.4 ± 1.3	3.5 ± 0.9	7.9 ± 2.6	6.3 ± 0.8
18:4(*n*–3)	3.8 ± 0.5	ND	0.8 ± 0.1	2.4 ± 0.6	ND	ND
20:4(*n*–6)	11.0 ± 0.5	2.7 ± 0.2*	6.0 ± 0.5*	9.3 ± 0.8	3.6 ± 0.6*	10.1 ± 1.2
20:5(*n*–3)	0.9 ± 0.3	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	ND	ND
SFA	34.9 ± 1.6	43.3 ± 0.6*	43.9 ± 0.8*	39.8 ± 0.3	43.1 ± 0.6*	39.9 ± 0.8
MUFA	30.0 ± 2.3	40.1 ± 0.2*	31.4 ± 0.3	33.7 ± 1.3	28.8 ± 1.6	32.3 ± 0.6
PUFA	35.0 ± 2.3	16.6 ± 0.5*	24.8 ± 0.8	27.7 ± 0.8	25.4 ± 1.6*	30.3 ± 2.1
Total UFA	65.1 ± 1.0	56.7 ± 0.3*	56.1 ± 0.8*	61.4 ± 0.9	54.1 ± 0.7*	62.6 ± 2.7
SFA/UFA	0.54 ± 0.03	0.76 ± 0.01*	0.78 ± 0.02*	0.65 ± 0.02	0.80 ± 0.03*	0.64 ± 0.04
UI	137.9 ± 4.8	82.0 ± 1.0*	99.2 ± 0.5*	116.3 ± 1.4	96.1 ± 5.0*	118.8 ± 7.6

Data are given in percentages of total fatty acid contents and are means ± S.E.M. of four experiments with GLC determinations done in triplicate. ND, not detected; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; UFA, unsaturated fatty acids. The unsaturation index (UI) was calculated as the sum of the percentage by weight of each fatty acid times the number of olefinic bonds.
By rows, $*P < 0.05$ vs. fresh platelets (control).

concentrations of 1 mg in the assay medium (Fig. 1B). It is well known that at the concentration used in our peroxidation system ADP can activate platelets and trigger the enzyme-catalyzed arachidonic acid cascade sometimes known to produce MDA [31]. Therefore, although it could be argued that the TBARS detected after peroxidation reactions represent a mixture of oxidant-induced TBARS and enzymically formed TBARS, the second possibility seems to be unlikely because to our knowledge platelet activation by ADP has only been described in non-activated intact cells and not in platelet crude membrane preparations. Also, the addition of the antioxidant BHA (0.1–1 mM) to the reaction mixture always prevented TBARS formation (data not shown).

We next tested how our model peroxidation system might affect the structure and function of sheep platelet membranes. For this purpose, the phospholipid and fatty acid composition, lipid extract fluorescence, fluorescence anisotropy, as well as the activity of two membrane-bound enzymes, Glc-6-Pase and AChE, were studied. The effects observed with the model system were compared to those occurring with ageing sheep platelets in storage with their own plasma or with Seto synthetic medium. For comparison, we also include the data obtained with the platelet aggregates formed during storage in both media, since alterations during storage that decrease platelet viability seem to be similar to those that occur during platelet activation and aggregation [32].

3.2. Platelet ageing under storage conditions

Different studies have shown that pH values below 6.1 produce irreversible platelet damage, with loss of function and viability. Nevertheless, during platelet ageing under our storage conditions, pH values re-

mained stable (7.53 ± 0.01 and 7.46 ± 0.03 or 7.60 ± 0.02 and 7.48 ± 0.04 after 0 or 5 days of storage in plasma and Seto solutions, respectively). Additionally, LDH activities remained at low levels in the supernatants from storage media after 5 days of storage (about 0.1 and 0.7% of the original contents for Seto solution and plasma, respectively), indicating a weak degree of platelet lysis during the in vitro ageing period. Aggregate formation was also visible during storage. Therefore, the changes described so far cannot be attributed either to acidification of the storage media or platelet death due to cell lysis.

3.3. *Effects on phospholipid composition, fatty acid profiles and lipid extract fluorescence*

Table 1 shows the phospholipid composition of total lipids from platelet crude membranes after 120 min of peroxidation as well as those of stored platelets and aggregates formed during platelet storage for 2 days. A significant decrease in the content of PE with a parallel increase in the l-PE percentage ($P < 0.05$) was observed after lipid peroxidation treatments. No changes were observed during cell ageing under storage in Seto, but platelets stored in plasma showed significant differences in the phospholipid profile, with a lower content of PE (as occurred in external peroxidized samples) and a parallel increase in PC percentages (about 1.5-fold in both cases as compared to parent cells, $P < 0.05$). The phospholipid composition of aggregates was similar to that found in platelets, changes in the PE and PC percentages only being visible in the aggregates formed in plasma.

Table 2 shows the fatty acid composition of total lipids from samples equivalent to those described in Table 1. With respect to in vitro peroxidation, the most significant change was the decrease in the PUFA content of the platelet crude membranes – especially the arachidonic acid species, which decreased by more than 70% – which was also indicated by the increase in the saturation/unsaturation ratio (1.3-fold) and the decrease in the unsaturation index (1.4-fold). Again, enzymically catalyzed arachidonic acid degradation due to platelet enzyme activation by ADP used in the peroxidation mixture is unlikely because we were using platelet crude membranes rather than intact cells. Additionally, PUFA

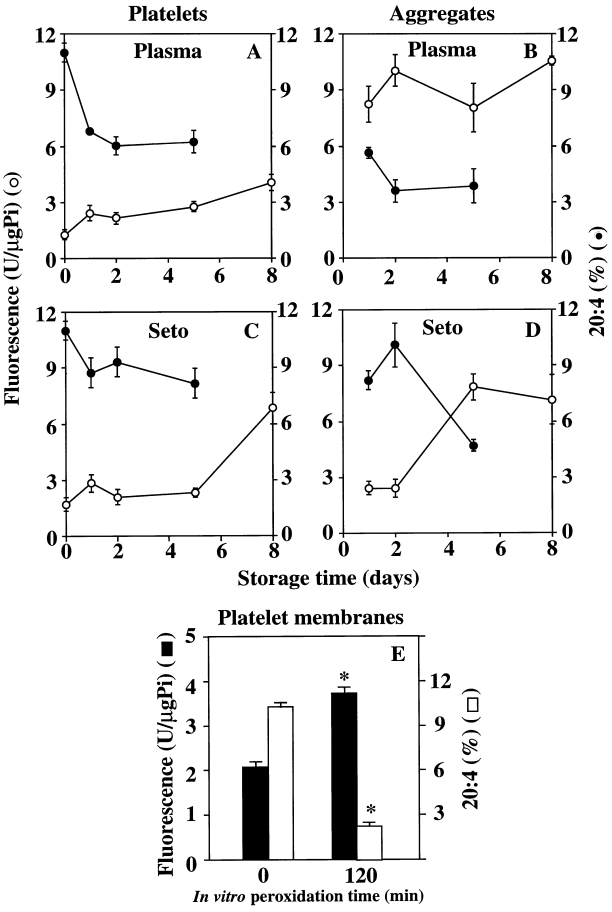

Fig. 2. Relationships between increases in lipid extract fluorescences and decreases in arachidonic acid contents. Lipid extract fluorescence and arachidonic acid (20:4) percentages in platelets and aggregates after platelet storage in plasma (A,B) or Seto (C,D) media, and in peroxidized platelet crude membranes (E) were determined as indicated in Section 2. Amounts of 20:4 are expressed as percentages of total fatty acids. Data represent means ± S.E.M. of five experiments done in triplicate. In E, $^{*}P < 0.05$ as compared to non-peroxidized membranes (0 time).

degradation could be almost completely prevented by adding BHA (0.1–1 mM) to the reaction mixture (data not shown). With respect to platelet ageing under storage, the changes in the PUFA profile were mainly observed in the samples from plasma: the percentages of 20:4 were reduced ($P < 0.05$) 1.8-fold and 3-fold in aggregates and platelets, respectively, only trace amounts of 18:4 fatty acid being detected. The unsaturation indices only revealed a significant decrease (1.4-fold) in all materials stored in plasma.

Fig. 2A and C show that the arachidonic acid content of platelets decreased as from the first day

of storage, this decrease being more pronounced in the case of platelets stored in plasma. These decreases ran in parallel with increases in the lipid extract fluorescence of stored platelets. Fig. 2B shows that the 20:4 fatty acid content of aggregates formed after 1 day of platelet storage in plasma was 50% lower than that of fresh platelets. This fatty acid content decreased 40% more with longer periods of platelet storage in plasma. Fig. 2D shows that the arachidonic acid contents of aggregates formed after 1–2 days of platelet storage in Seto solution were 2–3-fold higher than those of aggregates in plasma, showing a 50% reduction only after 5 days of storage. These decreases also ran in parallel with increases in the lipid extract fluorescence of aggregates, especially in the case of storage in Seto solution. Aggregates formed in plasma already showed the highest fluorescence after 1 day of storage from all materials studied. The higher losses of arachidonic acid seen to occur in aggregates as compared to platelets could indicate that in aggregates the fatty acid could be degraded by both peroxidation-induced and enzymically catalyzed systems. This possibility is quite likely considering that aggregates represent activated platelets in an aggregation state. Fig. 2E shows an increase in the lipid extract fluorescence and a decrease in the 20:4 fatty acid content after 120 min of crude membrane peroxidation.

All the lipid data reported so far for aging platelets under storage conditions indicate a lipid depletion that was mainly detected in platelets stored in plasma. These lipid changes could be due to at least three different phenomena: (i) membrane fragmentation by microvesiculation during storage; (ii) the contribution of phospholipase A_2; (iii) membrane lipid peroxidation, a phenomenon that finally produces fluorescent chromolipids. We have already demonstrated that microvesiculation is not the main cause of lipid depletion under the aging conditions used in this work [23]. A contribution by phospholipase A_2 can also be ruled out because the presence of phospholipase A_2 inhibitors (aristolochic acid, 5 μmol/50×10^9 platelets; EDTA/EGTA/NaF mixture, 15 mM final concentration each) did not prevent the decreases in arachidonic acid (10.1 ± 0.3, 2.9 ± 0.2, 1.5 ± 0.5 and 4.0 ± 0.7% of total fatty acid contents for non-stored platelets; platelets stored in plasma for 5 days without phospholipase A_2 inhibitors, and platelets stored in plasma for 5 days in the presence of aristolochic acid or the EDTA/EGTA/NaF mixture, respectively). Accordingly, the involvement of other platelet enzymes of the arachidonic acid cascade acting downstream from phospholipase A_2 is very unlikely. Also, phospholipase A_2 has been shown to be inactivated after cell storage at 4°C [33]. By contrast, lipid peroxidation correlates well with the decrease in PE and the accumulation of fluorescent lipid with a concomitant decrease in PUFA in the platelets stored in plasma described by us here, as well as with other data shown below on the amount of MDA equivalents and conjugated dienes and the sensitivity of crude membranes from stored platelets to in vitro peroxidation (see Section 3.6). Significantly, a decrease in the content of PE was also detected in the platelet membranes peroxidized in vitro as described above. Other evidence also supports the idea that lipid peroxidation occurs during specific storage conditions in unfractionated erythrocytes [6,7] and human platelets [34].

3.4. Effects on membrane fluidity

The changes in the lipid composition of sheep platelet membranes after lipid peroxidation were accompanied by alterations in the physical state of the membranes, as indicated by the fluorescence probes used in this work.

The data reported here for the fluorescence anisotropy of DPH or TMA-DPH molecules embedded in platelet membranes represent the average fluidity of different domains in the lipid bilayers from crude membranes. DPH is known to be located within the hydrophobic core of membranes. TMA-DPH is anchored in close proximity to the bilayer surface, therefore yielding information about the bilayer lipid environment quite close to the surface, where negative components are located [35].

The microviscosity of the crude membranes from platelets or aggregates, expressed as anisotropy obtained with DPH and TMA-DPH, is shown in Fig. 3. The higher absolute values of TMA-DPH fluorescence anisotropy as compared to DPH suggest a more rigid nature of the surface crude membrane domain probed by TMA-DPH. With respect to lipid peroxidation (Fig. 3A,D), changes were only detected with DPH, whose fluorescence anisotropy increased

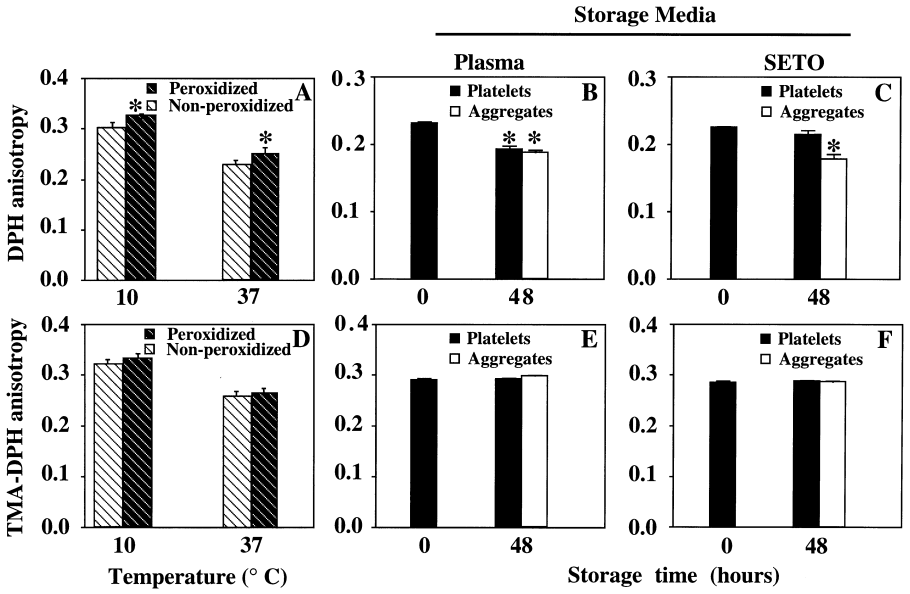

Fig. 3. Changes in the fluorescence anisotropy values of DPH and TMA-DPH. The probes were embedded in crude membranes from sheep platelets before and after 120 min of peroxidation (A,D), or in crude membranes from platelets (closed bars) and aggregates (open bars) after platelet storage in plasma (B,E) or in Seto (C,F) medium. Fluorescence anisotropy values were determined at 10 or 37°C for the peroxidation studies, and 37°C for in vitro ageing under storage conditions. Data represent means ± S.E.M. of three experiments done in triplicate. A,D: $*P < 0.05$ as compared to non-peroxidized membranes (open bars) at each temperature. Rest of panels: $*P < 0.05$ as compared to non-stored platelets.

significantly. Accordingly, the membrane surface does not seem to be affected by the oxidizing agents. This kind of behavior is similar to that observed in the case of erythrocytes peroxidized with the same enzymatic system [36], and to that of other types of cell membranes after peroxidation with different systems [37,38]. In platelets, anisotropy changes were only visible with DPH for storage in plasma (about 13% decrease, $P < 0.05$) (Fig. 3B). In aggregates, decreases in DPH anisotropy were observed in both storage media (Fig. 3B,C). Changes in fluidity have also been found in stored and aged human erythrocytes [39]. As a whole, the data show that both in vitro peroxidation and ageing under storage in plasma are associated with changes in fluidity. Ageing under storage in Seto solution was associated with less pronounced changes in fluidity, visible only after longer storage periods (5 days, data not shown), which is in accordance with the lower decreases in the PUFA contents in this case.

3.5. Effect on Glc-6-Pase and AChE activities

Membrane-bound enzymes can be affected by free radicals. The oxidation of amino acids essential for enzyme activity or protein conformation, the interaction of proteins with particular products of peroxidation and/or changes in membrane composition, organization and fluidity can cause inactivation or activation of some membrane-bound proteins.

Fig. 4 shows that AChE activity was not strongly affected by the oxidizing agents. By contrast, Glc-6-Pase was more sensitive to lipid peroxidation, 40 and

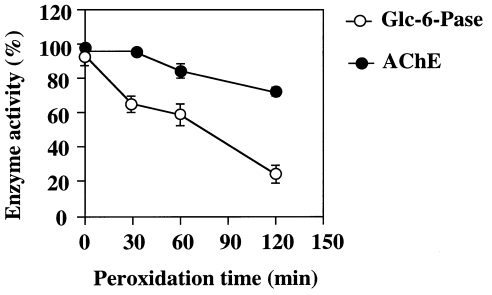

Fig. 4. Effect of peroxidation time on AChE and Glc-6-Pase activities of platelet membranes. Fresh sheep platelet crude membranes were peroxidized as described in Section 2. Data are expressed considering the value obtained at 0 min as 100% (Glc-6-Pase, 0.61 ± 0.04 μmol P_i/minute × mg protein), and represent means ± S.E.M. of three experiments done in triplicate.

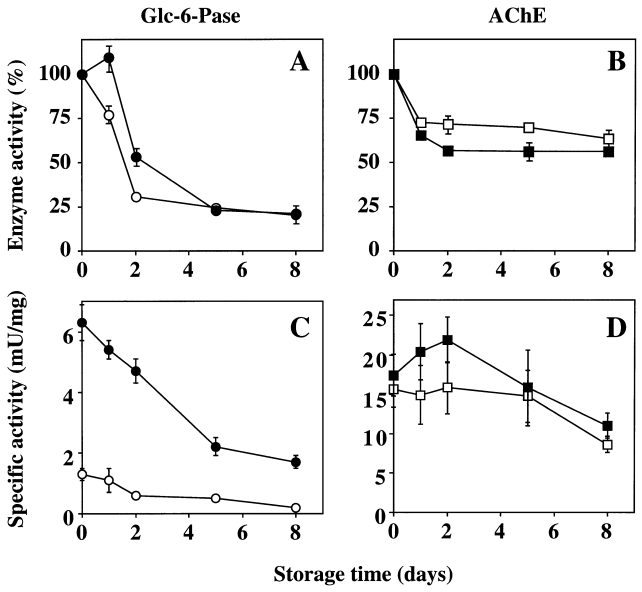

Fig. 5. Effect of storage on AChE and Glc-6-Pase activities. Sheep platelets were stored in plasma (open symbols) or Seto (closed symbols) medium for up to 8 days. At different storage times, platelets from appropriate Teruflex bags were separated and enzyme activities determined as indicated in Section 2. Data are expressed considering the value obtained on day 0 as 100% and represent means ± S.E.M. of three experiments done in triplicate.

70% of the enzyme activity being lost after 60 or 120 min of treatment, respectively. Additionally, the addition of BHA (0.1–1 mM) to the reaction mixture effectively prevented Glc-6-Pase inactivation (data not shown). In other sources, inactivation of Glc-6-Pase by peroxidation has been related to changes in the composition and/or structure of the lipid bilayer [40] and modification of thiol [41] and/or amino groups [42]. In our case, preliminary studies have shown that the amount of protein thiols in sheep platelet membranes decreases significantly after 45 min of treatment with XO-hypoxanthine/Fe^{2+}-ADP (data not shown).

Fig. 5A and C show that total or specific Glc-6-Pase activities decreased significantly in platelet membranes after storage in both media. Additionally, changes in total or specific AChE activities (Fig. 5B,D) were less pronounced or basically undetectable, respectively, in both storage media. Although inactivation of the endoplasmic reticulum-bound Glc-6-Pase could be related to the effects of peroxidation processes occurring during storage, interpretation of the AChE data is difficult since it is known that during platelet storage, the shedding of AChE-enriched vesicles from the platelet plasma membrane, where AChE is located through a GPI anchor, does occur [23,43]. Therefore, the data shown in Fig. 5B are in accordance with the notion of losses of AChE due to vesiculation, especially since AChE does not seem to be sensitive to lipid peroxidation, as shown in Fig. 4. The data on AChE specific activities shown in Fig. 5D are also consistent with this interpretation.

3.6. *Sensitivity of crude membranes from platelets and aggregates to in vitro peroxidation after storage*

Initial levels of TBARS in platelets (prior to storage) were always lower than 0.02 nmol MDA/mg protein. After platelet ageing under storage in plasma they ranged from 0.6 to 1.0 nmol/mg protein, which is quite low when compared with the TBARS produced after in vitro peroxidation (Fig. 1). Nevertheless, it is possible that TBARS could be masked by their reaction with free amino groups from platelets or even that they were mainly released to the storage medium, therefore rendering them undetectable. Fig. 6 depicts the sensitivity of the membranes from stored platelets and the aggregates formed to XO/hypoxanthine/Fe^{2+}-ADP treatment. Thus, if platelets are already peroxidized during the storage time, their susceptibility to in vitro peroxidation should be lower. Actually, storage of platelets for 5 days in plasma was accompanied by a 3-fold decrease in the amount

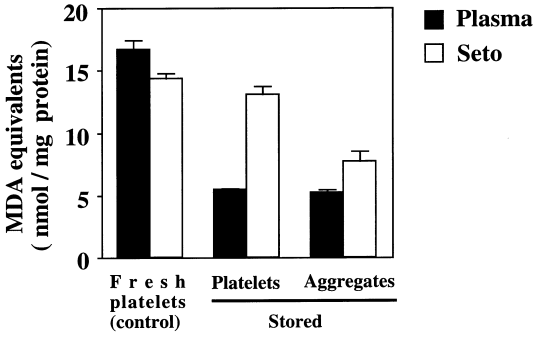

Fig. 6. Sensitivity to lipid peroxidation of platelet membranes and aggregates after sheep platelet storage in plasma or Seto medium. The sensitivity of crude membranes from samples stored for 5 days to XO-hypoxanthine/Fe^{2+}-ADP treatment was determined. Data are expressed as the formation of MDA equivalents (nmol/mg protein) and represent means ± S.E.M. of five experiments done in triplicate.

of TBARS formed by their membranes after treatment with the oxidizing agents. By contrast, membranes from platelets stored in Seto solution for 5 days formed more or less the same amount of TBARS as membranes from unstored platelets. Membranes from aggregates formed in plasma or Seto solution produced the same amounts or 40% less TBARS, respectively, as those produced by membranes from platelets stored in the same media. Membrane peroxidation in platelets stored in plasma was also confirmed by the increase in conjugated dienes. After 5 days of storage, the concentration of the dienes was about 6 times the initial value (8 ± 2, 14 ± 3 and 55 ± 7 nmol/mg protein for crude membranes from non-stored platelets or platelets stored in Seto or plasma, respectively). All these data indicate (i) that storage in Seto somehow protects platelets against peroxidative processes and (ii) that in this medium the aggregates formed are more peroxidized than the platelets. The presence of acetate and phosphate in the Seto medium provides a critical buffering effect, and acetate oxidation suppresses lactate production [44]. Phosphate has also been purported to inhibit lipid peroxidation [45], which would be in accordance with the minimal degree of peroxidation found by us in platelets stored in Seto solution.

In summary, lipid peroxidation of platelet crude membranes due to XO/hypoxanthine/Fe^{2+}-ADP and the occurrence of peroxidation processes during platelet ageing under storage at cold temperature share features in common with the changes observed in PUFA degradation and lipid extract fluorescence, crude membrane fluidity and membrane-bound enzyme activities. Moreover, Seto solution permits better storage of sheep platelets than plasma as regards the prevention of peroxidative processes. Therefore, the in vitro peroxidation model might be useful for carrying out studies on some of the effects occurring during platelet ageing in storage.

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