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Exposure to Malondialdehyde Induces an Early Redox Unbalance Preceding Membrane Toxicity in Human Erythrocytes

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This work investigated the oxidative injury to human red blood cells (RBCs) by the exposure to exogenous malondialdehyde (MDA), in a physiological environment. When a 10% RBC suspension was incubated in autologous plasma, in the presence of 50 μM MDA, 30% of MDA entered into the cells. A time-course study showed that MDA caused early (30–120 min) and delayed (3–18 h) effects. MDA caused a fast depletion of reduced glutathione, and loss of the glucose-6-phosphate dehydrogenase activity, followed by a decrease of HbO_2. Accumulation of methemoglobin, and formation of small amounts of hemichrome were later evident. Also, an HbO_2-derived fluorescent product was measured in the membrane. The redox unbalance was followed by structural and functional damage to the membrane, evident as the formation of conjugated diene lipid hydroperoxides, concurrent with a sharp accumulation of MDA, consumption of membrane vitamin E, and egress of K^+ ions. SDS--PAGE of membrane proteins showed formation of high molecular weight aggregates. In spite of the marked oxidative alterations, the incubation plasma prevented a substantial hemolysis, even after a 18 h incubation. On the contrary, the exposure of RBCs to 50 μM MDA in glucose-containing phosphate saline buffer, resulted in a 16% hemolysis within 6 h. These results indicate that the exposure to MDA causes a rapid intracellular oxidative stress and potentiates oxidative cascades on RBCs, resulting in their dysfunction.

Keywords: MDA; Oxidative stress; Antioxidant defenses; Red blood cells; Hemoglobin

INTRODUCTION

Malondialdehyde (MDA) and a number of alkanals and alkenals are major end-products of the oxidation of polyunsaturated fatty acids.[1] These molecules may react with a variety of nucleophilic sites in DNA, proteins, and phospholipids, generating various types of adducts.[1–4] This leads to the assumption that the reactive aldehydes can ingenerate specific cellular stress responses, thereby acting as cytotoxic second messengers for the primary free radicals that initiated lipid peroxidation. In contrast to the compounds which they have been formed from, the lipid-derived aldehydes are water-soluble, which allows them to diffuse from the place of generation to other targets within the cell, or even propagate the injury outside to intact cells. It has recently been shown that the exposure to reactive aldehydes such as hydroxynonenal or MDA causes an increase in the intracellular peroxide levels in cultured rat hepatocytes[5] and aortic smooth muscle cells.[6]

Circulating erythrocytes are extremely oxidation-susceptible, in that their membranes are rich in polyunsaturated fatty acids, and they are continuously exposed to a high concentration of oxygen and hemoglobin. The latter can act as a powerful catalyst for the initiation of free radical chain oxidation under

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a number of circumstances.[7–9] Nevertheless, red blood cells (RBCs) possess a variety of mechanisms to preserve their integrity and function, primarily to maintain their hemoglobin in a reduced state. They include antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glucose-6-P dehydrogenase, and reducing molecules such as reduced glutathione.[10–13] A few reports described the alterations to the membrane structure[14,15] and function,[15,16] by exposure of isolated erythrocytes to exogenous MDA. In the light that the exposure to reactive aldehydes may trigger the formation of peroxides in cells,[5,6] it is possible that the generation of an intracellular redox unbalance precedes the injury to the RBC membrane. Also, because three principal MDA forms can exist in aqueous solutions, whose reactivity is influenced by pH and presence of proteins in the medium,[1] it is not clear if and to what extent the physiological environment could affect the MDA toxicity to RBCs. With this in mind we investigated the effect of exogenous MDA on the intracellular redox status, and researched the oxidant events leading to membrane damage and eventual lysis in a model of human erythrocyte exposed to MDA in diluted plasma, in order to approach the conditions closest to its physiological status.

MATERIALS AND METHODS

Malondialdehyde (MDA) was prepared by acid hydrolysis of 1,1,3,3-tetraethoxypropane (Sigma, St. Louis, Mo) according Suttnar et al.[17] Ethylenediaminetetraacetic acid (EDTA), DTNB, α-tocopherol, G-6P dehydrogenase kit, TRIS, bovine methemoglobin, catalase from human erythrocytes, Na-dithionite, acrylamide, bis-acrylamide, tetramethylenediamine (TEMED), Na-dodecyl sulfate, Coomassie blue, and molecular weight markers were from Sigma. Sephadex G-25 was from Pharmacia. All other solvent and chemicals were of the highest purity grade.

Red Blood Cell Treatment

Blood samples were obtained from apparently healthy individuals by venipuncture, with informed consent. EDTA (1 mg/ml blood) was used as an anticoagulant. RBCs were sedimented at 1000 g for 10 min and washed three times with phosphate-buffered saline (PBS), pH 7.4. Supernatant and buffy coat were carefully removed by aspiration after each wash. RBCs were diluted with suitable amounts of autologous plasma, diluted 4-fold with PBS containing 100 U penicillin/ml, to obtain a final 10% hematocrit (HT). This suspension was then incubated at 37°C in the absence (control RBCs) or in the presence of MDA. The extent of hemolysis was determined as follows. A volume of the incubation mixture was diluted with 40 vol of PBS and centrifuged at 1000 g for 10 min to precipitate the cells. The absorbance of the supernatant was then evaluated at 540 nm. Similarly, a volume of the incubation mixture was treated with 40 vol of 5 mM sodium phosphate buffer, pH 7.4 (hypotonic PBS), to yield complete hemolysis, and supernatant after centrifugation was evaluated spectrophotometrically at 540 nm. The percentage of hemolysis was calculated from the ratio of the absorbances.

Analytical Determinations

MDA into the erythrocytes was determined by precipiting the cells after incubation, and treating them with hypotonic PBS. This suspension (5 μl) was mixed with 0.2% (w/w) TBA in 0.1 M Na-acetate buffer, pH 3.5. After heating at 95°C for 60 min, the reaction solution was centrifuged, and an aliquot of 20 μl supernatant was analyzed by HPLC following Fukunaga.[18]

GSH in the RBCs was determined by titration with DTNB as previously reported.[19]

The oxidation status of hemoglobin was assayed as follows. The RBCs from a 1 ml incubation mixture were lysed in 40 vol of hypotonic phosphate buffer and precipitated. Aliquots of the supernatant in a final volume of 1.0 ml of PBS were scanned at 500–700 nm, and the concentration of the oxidation products of hemoglobin in mixtures of HbO_2, methemoglobin (metHb), and hemichrome calculated according to Winterbourn.[20]

To test whether MDA can react with HbO_2, oxyhemoglobin was freshly prepared from met-Hb by reduction with excess Na-dithionite in deareated 0.01 M K^+ phosphate buffer pH 7.8. Reduced hemoglobin was chromatographically purified on a Sephadex G-25 column (1.2 × 20 cm), using the same phosphate buffer as eluent. A small amount (2–3%) of methemoglobin was always present in the preparation. A 1.6 mM HbO_2 solution was incubated with 15 μM MDA, at 37°C. Spectra were monitored between 500 and 700 nm at 30 min intervals, for 6 h.

Erythrocyte vitamin E (α-tocopherol) was determined by reverse phase HPLC with fluorescence detection (ex 290 nm; em 335 nm). Samples (5 ml) of the incubation mixtures were centrifuged at 1000g for 10 min, after addition of 25 μl of 40 mM BHT. The RBC pellet was re-suspended with 1 ml of PBS containing 0.5% pyrogallol, and vitamin E was extracted by mixing with two volumes of absolute ethanol, followed by two successive extractions with six and two volumes of petroleum ether. The organic extracts were gathered, dried under nitrogen, re-suspended in several microliters of suitable solvent

and injected on top of a Supelco Supelcosil™ (Bellefonte, PA) LC-18 HPLC column (0.46 × 25 cm).

K^+ ions were measured in aliquots of incubation plasma with a SpotLyte Analyzer (Menarini Diagnostic, Italy) using a flow-through potassium electrode.

Ghosts were prepared by three washing-centrifugation cycles (30 min at 20,000 g, 4°C) with 20 vol of hypotonic PBS, and suspended in PBS. Conjugated diene (CD) lipid hydroperoxides were extracted from 500 μl of the suspension with 3 ml of a mixture $CHCl_3$:CH_3OH (2:1). The organic extract was evaporated under a nitrogen stream, re-suspended in cyclohexane and quantified spectrophotometrically at 234 nm, using a molar extinction coefficient of 27,000.[21]

The activity of the glucose-6-phosphate dehydrogenase was assayed on the cells precipitated from the incubation mixture, and re-suspended in PBS to obtain a 40% HT. Subsequent treatment of the cells and the measurement of the NADPH absorbance was according to the instructions of the manufacturer of the diagnostic kit. All assays were carried out in duplicate.

The effect of MDA on catalase was studied by incubating purified catalase (0.2 μM) in PBS, at 37°C, in the absence and in the presence of either 15 or 50 μM MDA. Then aliquots of the incubation mixture were taken and the catalase activity was measured as described,[22] at 30 min intervals, for 120 min.

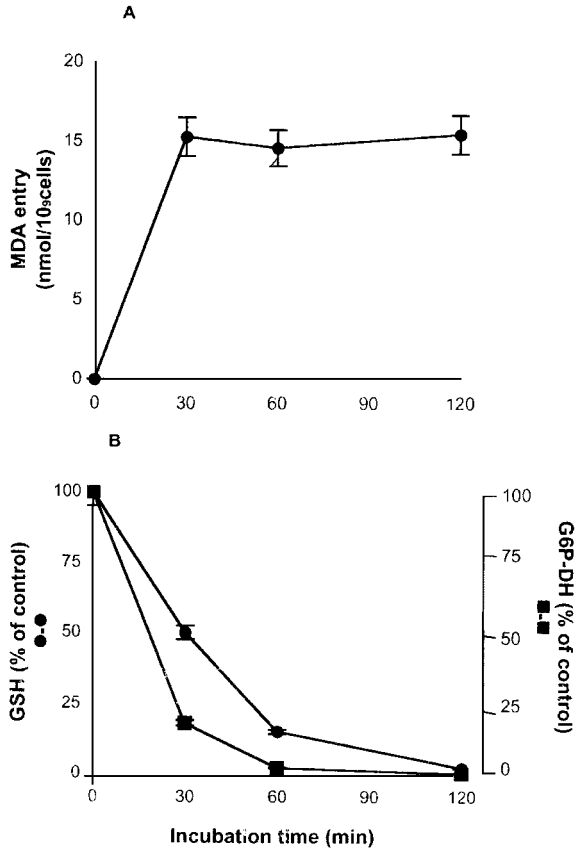

FIGURE 1 Time course of MDA entry (A) and GSH depletion and G6P-DH inhibition (B) in human RBCs (HT 10%) incubated in diluted autologous plasma, in the presence of 50 μM MDA. Values are the mean ± SD of eight separate experiments.

Analysis of Membrane Proteins

Ghosts, prepared as above, were suspended in PBS, and proteins determined by the BioRad colorimetric method, according to Bradford.[23] RBC membranes were mixed with 50 μl of an SDS-Tris buffer solution [2% SDS, 10% (v/v) glycerol, 0.03% bromophenol blue, 63 mM Tris–HCl, pH 6.8] and put in a boiling water bath for 10 min. SDS-PAGE was carried out on a 0.75 mm thick slab gel with 4.4 and 10% gels for condensation and separation, respectively, according to Laemmli.[24] The amount of protein layered was 30 μg. Protein bands were stained by Coomassie Brillant Blue R-250. The gel system was calibrated for molecular weight determination by measuring the migration of standard proteins (range 16–205 kDa).

Fluorescent Products

Fluorescent products were measured in RBC ghosts (100 μg protein/ml PBS) at 460 nm excitation and 525 nm emission,[25] with a F-2000 Hitachi spectrofluorimeter.

Statistical Analysis

Conventional methods were used for calculation of means and standard deviations. Comparison between control-RBCs and RBCs exposed to MDA was performed by unpaired Student's t-test.

RESULTS

MDA Entry and Redox Status of RBC

The exposure of human erythrocytes to 50 μM MDA, in diluted plasma, caused a rapid accumulation of 15 ± 1.2 nmol MDA/10^9 cells within 30 min. This amount remained substantially constant for the following 90 min (Fig. 1A). Variations of the redox status of erythrocytes were first explored by measuring the intracellular GSH, and the activity of the G6P-DH (Fig. 1B). GSH showed a 50% fall within 30 min, then it further decreased until 60 min, and a total loss was observed at 120 min. The activity of G6P-DH dramatically decreased at 30 min, when it was 20% of the control, and it was completely lost at 60 min (Fig. 1B).

The hemoglobin status is also a sensitive index of the oxidative stress in RBCs.[26] We then measured the amount of HbO_2, methemoglobin and hemichrome, at time intervals during the incubation of

erythrocytes with MDA. HbO_2 underwent a progressive decrease. Small amounts of metHb were formed within 3 h, with a further increase during the following 3 h. A sharp accumulation was observed after 6 h of incubation. Methemoglobin represented 42% of the total Hb concentration in the cells after 18 h (Fig. 2). Small amounts of hemichrome, representing the product of a more extensive hemoglobin oxidation, was observed at longer incubation times (12–18 h), concurrent with the accumulation of metHb. This suggests that, at least partially, hemichrome could be generated from methemoglobin.

The formation of hemoglobin oxidized products did not appear the consequence of a direct MDA-HbO_2 interaction. To investigate about this point the absorbance spectrum of hemoglobin was monitored during the incubation of 1.6 mM HbO_2 in the presence of 15 μM MDA. No spectral variation was observed, indicating that reaction of MDA with Hb did not occur under conditions similar to the experiments with the RBCs (not shown).

No changes in the GSH content, G6P-DH activity, and Hb oxidation status were observed in the RBCs incubated in diluted plasma, in the absence of MDA.

Effect of MDA on the Catalase Activity

The possibility was checked that MDA can interact with and inhibit catalase, a major antioxidant enzyme in the RBCs. To this purpose purified catalase at a concentration similar to that of human erythrocytes (10% HT)[27] was incubated in PBS at 37°C, in the presence or in the absence of either 15 or 50 μM MDA, and the enzyme activity was assayed on aliquots of the incubation mixtures at time intervals. The enzyme incubated in the absence of MDA (control) did not undergo any loss of activity for 0–120 min (not shown). On the contrary, the enzyme exposed to MDA was inhibited (Fig. 3). MDA caused a 25 and 38% inactivation, at 15 μM and at 50 μM, respectively, after a 30 min incubation. A further 15% loss of activity was observed by the exposure to 15 μM MDA for 90–120 min, however the inhibition by 50 μM MDA was not significantly modified after an incubation longer than 30 min (Fig. 3).

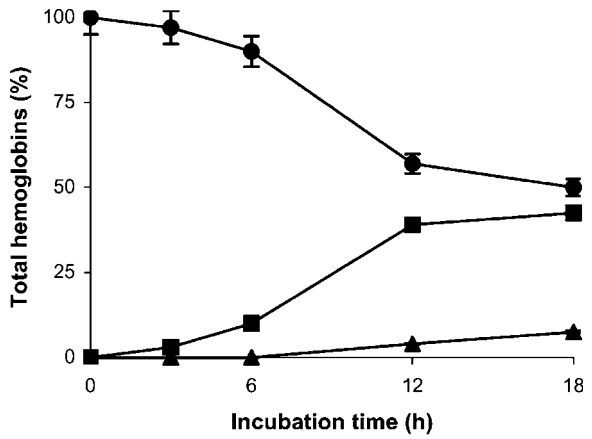

FIGURE 2 Oxyhemoglobin (●), methaemoglobin (■) and hemichrome (▲) in human RBCs (HT 10%) incubated in diluted autologous plasma, in the presence of 50 μM MDA. The values are expressed as the percent of total hemoglobins, and are the mean ± SD of five separate experiments.

Generation of Fluorescent Products

Fluorescent products with characteristic excitation and emission spectra are formed by the reaction of HbO_2 with H_2O_2,[25,28,29] and precipitate onto the membrane. Ghosts from the erythrocytes incubated in the presence of 50 μM MDA, for 60 min, exhibited fluorescence with excitation at 460 nm and emission at 525 nm, the extent of which increased along with the incubation (Table I). This fluorescence, considered as due to the fluorescent hemoglobin product, was distinct from that generated by the formation of MDA adducts with membrane components (not shown).

Membrane Damage by Exposure of Human Erythrocytes to MDA

A prolonged incubation of RBCs in plasma, in the presence of 50 μM MDA, caused accumulation of malondialdehyde in the cells (Fig. 4A), suggesting oxidation of membrane lipids. In addition, the conjugated diene lipid hydroperoxides markedly increased after a 6 h incubation, and were two-fold (410 ± 23 nmol/10^{10} cells) with respect to the conjugated dienes of control RBCs (200 ±

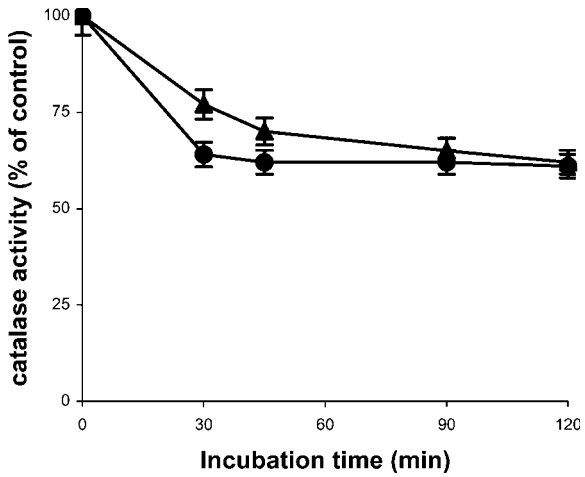

FIGURE 3 Effect of the exposure to MDA on the activity of catalase. The enzyme at 0.2 μM was incubated in the presence of either 15 (●) or 50 μM (▲) MDA for the time indicated, then the activity was measured spectrophotometrically as reported in "Materials and Methods". The results are expressed as the percent of the activity measured in the RBCs incubated in the absence of MDA (control, control), and are the mean ± SD of three separate experiments.

TABLE I Fluorescent oxidation products from hemoglobin in RBC membrane (λ ex 460 nm; λ em 525 nm). For experimental details see "Materials and Methods". Values are the means ± SD of n separate experiments. (*with respect to control, $P < 0.001$; **with respect to 1 h incubation, $P < 0.01$; ***with respect to 6 h incubation, $P < 0.05$ (Student's t-test)

Treatment	Incubation time (h)	F.U./mg membrane protein	n
RBC control	18	0.050 ± 0.010	3
50 μMDA	1	0.550 ± 0.068*	6
	6	0.698 ± 0.088**	6
	18	0.912 ± 0.150***	6

14 nmol/10^{10} cells) after 18 h (Fig. 4B). Concurrent with the oxidation of membrane lipids, a decrease of vitamin E was observed. The amount of vitamin E was not significantly varied in RBCs after a 3 h incubation in the presence of MDA, then it progressively decreased. Vitamin E was 50% of that in control erythrocytes after 18 h (Fig. 4C).

Membrane proteins were modified after exposure of RBCs to MDA. The SDS gel electrophoresis of RBC membrane proteins provided evidence of proteinaceous high molecular weight adducts, which did not penetrate the gel, after a 30 min incubation (not shown). The intensity of this band, which was accounted for by the reaction of the MDA in the medium with membrane components, did not change after 60 min of incubation, but increased after 18 h (Fig. 5, lanes 2, 3), as the result of the adducts formed by the MDA generated in the cells.

K^+ Efflux

Damage to the erythrocyte membrane can affect the ion exchange and movement between the inner milieu and the environment. The K^+ concentration in the incubation plasma was 1.25 ± 0.13 meq/l, a value which increased slightly to 1.65 ± 0.16 meq/l after the incubation of RBCs for 18 h, in the absence of MDA. MDA at 50 μM caused a K^+ efflux which led to a marked rise of the $[K^+]$ to 26.14 ± 5.6 meq/l of incubation plasma (Fig. 6).

Effect of MDA on the Hemolysis of RBCs

Damage to the membrane lipids and proteins of RBCs may be accompained by hemolysis. RBCs incubated with diluted plasma alone, for 0–18 h at 37°C, were stable and did not show hemolysis (not shown). RBCs incubated in diluted plasma in the presence of 50–500 μM MDA, underwent a modest hemolysis, depending on the MDA concentration. MDA concentrations below 100 μM did not markedly affect the cell integrity. In the presence of 500 μM MDA the hemolysis was 15% after 18 h (Fig. 7). When the same experiments were carried out by incubating the erythrocytes in glucose-containing PBS, the hemolytic effect of MDA dramatically increased. A 16% hemolysis was observed after a 6 h incubation in the presence of 50 μM MDA (Fig. 7).

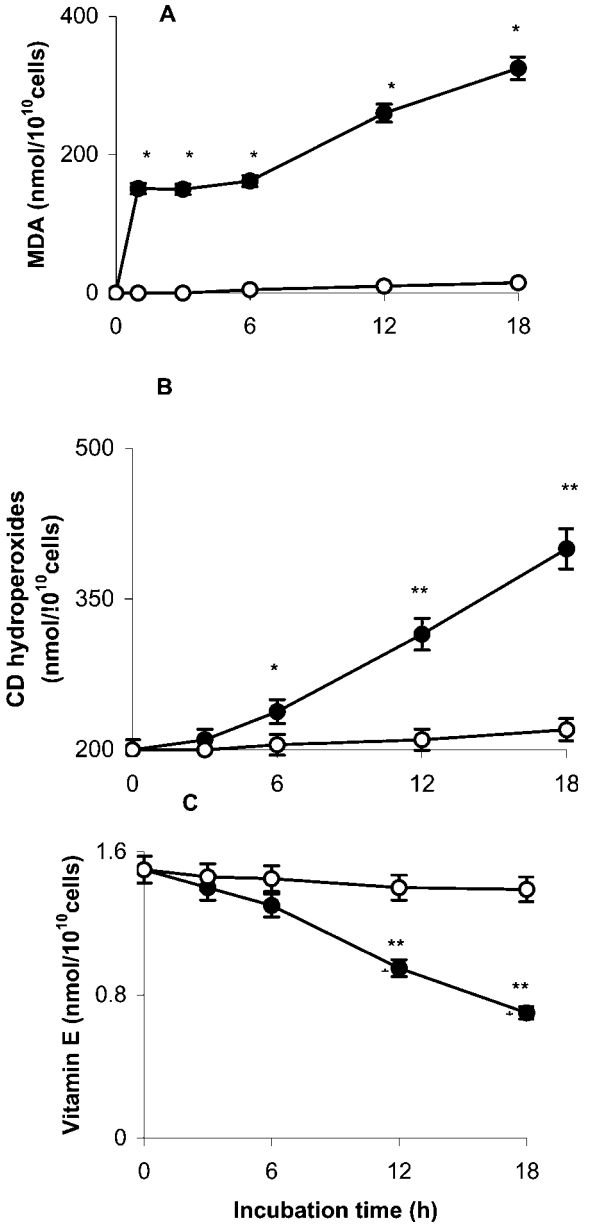

FIGURE 4 Time course of MDA accumulation (A), CD hydroperoxides formation (B) and vitamin E depletion (C) in human RBCs (HT 10%) incubated in diluted autologous plasma, in the absence (open symbols, control) or in the presence (closed symbols) of 50 μM MDA. Values are the mean ± SD of five separate experiments. With respect to the relevant control, values were significant with $*P = 0.05$ and $**P < 0.001$ (Student's t-test).

DISCUSSION

Formation of adducts with various cell components has appeared as a major cause of cytotoxicity from the lipid oxidation-produced aldehydes.[1] However, recent evidence has been provided that such

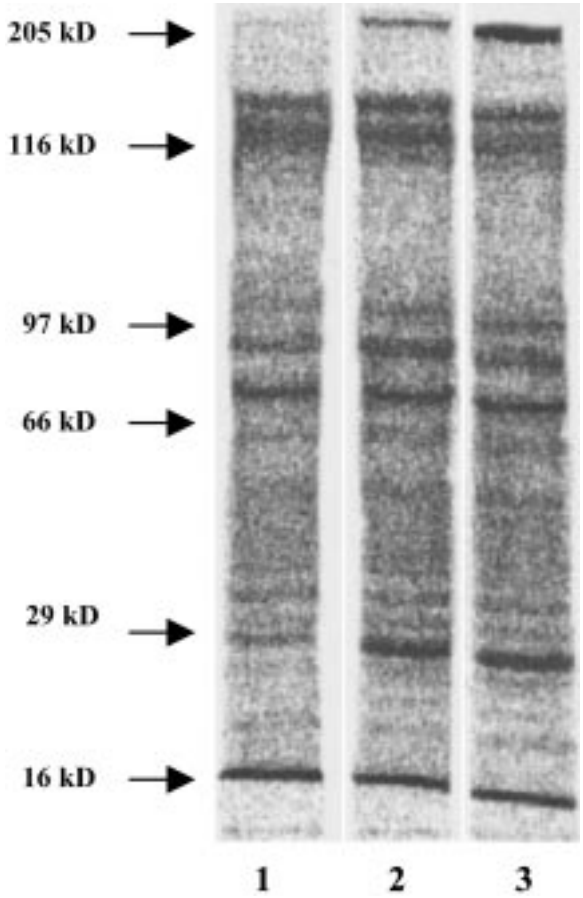

FIGURE 5 SDS-PAGE pattern of membrane proteins from human RBCs (HT 10%) incubated in diluted autologous plasma, in the absence (lane 1, 18 h incubation), or in the presence (lane 2, 60 min incubation; lane 3, 18 h incubation) of 50 μM MDA. The arrows indicate standard proteins and their molecular weights. The amount of protein layered was 30 μg in each case.

compounds may themselves be inducers of oxidative stress.[5,6] Malondialdehyde, a highly reactive bifunctional molecule, has been shown able to cross-link erythrocyte phospholipids and proteins, to affect membrane fluidity and to impair various

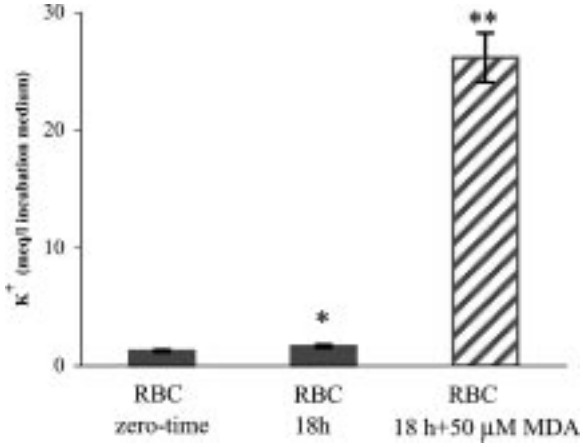

FIGURE 6 Efflux of K^+ from RBCs (HT 10%) incubated for 18 h in the absence (full bar) or in the presence (dashed bar) of 50 μM MDA. Values are the means ± SD of three separate experiments. With respect to RBCs zero-time values were significant with $*P < 0.05$ and $**P < 0.001$ (Student's t-test).

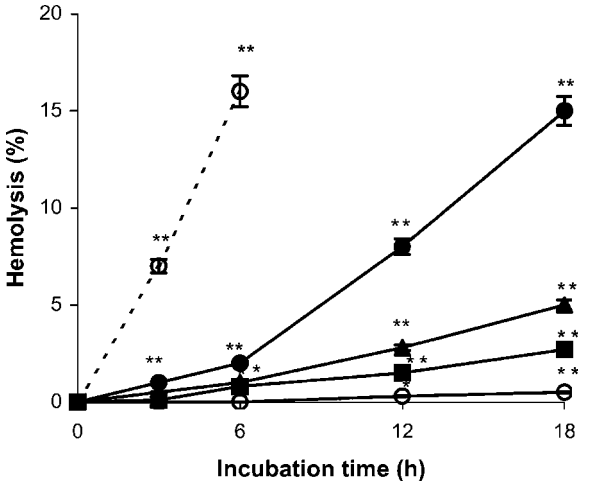

FIGURE 7 MDA-induced hemolysis of human RBCs (HT 10%) incubated in diluted autologous plasma (—) or in PBS (- - -). MDA concentration was 50 (○), 100 (■), 250 (▲) and 500 μM (●). Values are the mean ± SD of 3 separate experiments. With respect to the relevant control, values were significant with $*P < 0.01$ and $**P < 0.001$ (Student's t-test).

membrane functions, which causes a decreased RBC survival.[3,15,30–34] Our time-course study showed that the exposure of intact human RBCs to 50 μM exogenous MDA, in autologous plasma for 0–18 h, causes early (30–120 min) and delayed (3–18 h) effects. While observing the rapid formation of adducts of MDA with membrane components, we found that MDA first causes a cellular redox unbalance, which ensues in oxidation of membrane lipids, and finally in impairment of the membrane protein function. A substantial hemolysis was not observed, unless the erythrocytes were exposed to 500 μM MDA for 18 h.

The exposure to MDA induced changes in the redox status of the intracellular compartment of RBCs. Under the conditions applied, 15 μM MDA rapidly entered into the cells, followed by the rapid consumption of reduced glutathione, a major component to regulate the erythrocyte redox environment,[35,36] and oxidation of hemoglobin. The loss of GSH paralleled the entry of MDA into the cells, which cannot be ascribed to reaction with MDA. Indeed, although capable of forming products with cysteine, MDA does not react with GSH.[1,37,38] On the other hand, our finding that purified HbO_2 did not react with MDA suggested that the hemoglobin oxidation status in the RBCs was another expression of the intracellular oxidative stress.

Malondialdehyde has been shown capable of inactivating the purified glucose-6-P dehydrogenase.[39] This suggests that, either formed in tissues or if it enters the tissue, MDA will not simply modify structural proteins, but can modify functional proteins also. In accordance, we observed that the entry of MDA into the erythrocyte was rapidly followed by a sharp decrease of the G6P-dehydro-

genase activity, suggesting that inactivation of this enzyme by MDA can also occur under cell conditions. An increase of the erythrocyte G6P-DH activity has been reported under oxidative stress *in vivo*.[40–42] This may be interpreted as an adaptive mechanism leading to enhanced synthesis of antioxidant enzymes in nucleated erythrocyte precursors, following the production of reactive oxygen species,[43,44] and the occurrence of GSH unbalance.[45,46]

Under physiological conditions, the hemoglobin autoxidation continuously exposes the erythrocyte to the oxidant attack of H_2O_2,[47] a process counteracted by the combined activity of catalase, GSH-peroxidase, and GSH.[10–12] The impairment of the shunt activity, and the consequent loss of NADPH, will affect the activity of the enzyme systems involved in the recycling of glutathione, and in maintaining the oxidation status of hemoglobin, in the RBCs. The catalytic activity of catalase has been shown to depend on the presence of NADPH.[48] Oxidized glutathione (GSSG) is generated via the action of glutathione peroxidase in the detoxification of H_2O_2, and is recycled back to GSH by the NADPH-dependent glutathione reductase. Thus, the loss of NADPH will affect the activity of the system GSH-peroxidase/GSH-reductase, which can account for the loss of GSH. Moreover, because of the unarrested hemoglobin autoxidation, the defective activity of these enzymes, entails that H_2O_2 will accumulate in the MDA-treated RBCs. It is known that the genetic deficiency of G6P-DH will result in impairment of disposing H_2O_2.[49] Our findings that MDA inhibits catalase purified from human erythrocytes also suggest an additional way to impair the antioxidant defense in the RBCs, and would further support that hydrogen peroxide may accumulate.

After depletion of the RBC antioxidant defenses, the coexistence of H_2O_2, HbO_2 and met-Hb may lead to a number of consequences. Characteristic fluorescent hemoglobin degradation products are formed during the reaction of hydrogen peroxide with RBCs,[25] and accumulate in the membrane. In our experiments, fluorescent products with characteristic excitation and emission spectra were formed and accumulated in the RBC membrane following exposure to MDA, indicating reaction of hemoglobin with H_2O_2.[28] On the other hand, precipitation of oxidized products, formed by the action of H_2O_2 on methemoglobin, would cause the oxidation of membrane lipids,[7–9] which was also observed. Indeed, after the initial elevation of MDA in the RBC, due to the MDA entry, a steep increase was observed lately. Measurements of the CD hydroperoxides and vitamin E in the membrane supported that the accumulation of MDA was to be related to the membrane lipid oxidation.

Finally, a progressive K^+ efflux was observed after the exposure of RBCs to MDA. Since no significant hemolysis occurred under the conditions of our experiments, this was accounted for by a dysfunction of membrane proteins. The K^+ leak appeared related to the time-dependent accumulation of MDA, that is with the extent of the oxidative stress and lipid peroxidation in the erythrocyte, a finding in accordance with previous reports.[16] A direct link between the onset of lipid peroxidation and the eventual inactivation of certain membrane-bound proteins, going through adduct formation and protein cross-linking by aldehydes such as MDA, has been demonstrated.[50] In addition, H_2O_2-activated hemoglobin can cross-link and damage erythrocyte membrane proteins.[51] Although a number of ion transporters can be involved, it may be interesting to mention that the activity of the K^+ Cl^- exchanger of the erythrocyte membrane is enhanced by both the depletion of GSH and accumulation of metHb,[52] thereby causing efflux of K^+ under oxidative stress.

It has recently been suggested that membrane protein modification is the relevant factor leading to the RBC lysis.[53] In spite of the marked oxidative alterations inside the cell and impairment of membrane ion transport systems, 50 μM MDA did not cause hemolysis under the conditions of our experiments. To this end ten times higher MDA concentrations had to be used, or RBCs had to be incubated in saline buffer. While suggesting that the ultimate hemolytic effects of MDA are somewhat prevented by the plasma defenses, this points out that a severe MDA-induced oxidative stress occurs in RBCs well before hemolysis is evident. In other terms, a redox unbalance is caused to RBC by amounts of MDA much lower than those necessary to modify and inactivate structural proteins.

Conclusively, in accordance with recent reports that exposure to reactive aldehydes such as 4-HNE causes the intracellular production of peroxide,[5,6] this paper provides evidence that MDA itself can elicit an oxidative stress in RBCs, possibly due to the unhalted generation of H_2O_2. In our experimental model, the inhibition of G-6P dehydrogenase would lead to the observed depletion of GSH, as the result of a decrease of NADPH and impairment of the glutathione reductase activity. In addition, the induction of methemoglobin and reaction with H_2O_2 will eventually cause precipitation of hemoglobin degradation products, with oxidation of membrane lipids[7,8] and damage to proteins.[51] The plasma antioxidant defense may somewhat prevent the MDA-stimulated hemolysis, but it cannot prevent the oxidative stress of the cells.

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